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Determination of raltitrexed in human plasma by high performance liquid chromatography–electrospray ionization-mass spectrometry

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Abstract

A sensitive high performance liquid chromatography–electrospray ionization-mass spectrometry (HPLC–ESI-MS) assay was developed to determine raltitrexed in human plasma. After addition of benazeprilat as the internal standard (IS), methanol was used to produce a protein-free extract. Chromatographic separation was achieved with a Zorbax SB-C18 column (Narrow-Bore 2.1 mm × 150 mm, 5-μm) using a mobile phase of acetonitrile–water containing 0.1% formic acid and 2% methanol (21.9:78.1, v/v). Raltitrexed was determined with electrospray ionization-mass spectrometry. HPLC–ESI-MS was performed in the selected ion monitoring (SIM) mode using target ions at $[M+H]^+$ m/z 459.1 for raltitrexed and $[M+H]^+$ m/z 397.1 for IS. Calibration curves were linear over the range of 2.0–3000 ng/ml. The lower limit of quantification was 2.0 ng/ml. The intra- and inter-batch variability values were less than 6.7% and 10.3%, respectively. The mean plasma extraction recovery of raltitrexed was in the range of 85.2–91.1%. The method was successfully applied to determine the plasma concentrations of raltitrexed in eight Chinese colorectal cancer patients.
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Keywords: Raltitrexed; HPLC–ESI-MS; Pharmacokinetics

1. Introduction

Raltitrexed (Fig. 1A) is a novel quinazoline folate analog that selectively inhibits thymidylate synthase [1]. It has activity in advanced colorectal cancer comparable with that of fluorouracil (5-fluorouracil) plus folinic acid. Its activity is enhanced by rapid cellular entry and polyglutamation, with the polyglutamated derivatives having approximately 100-fold greater inhibitory potency than the parent compound [2]. Several articles reported the pharmacokinetic profiles of raltitrexed in animals [1,3–5] and patients [2,6–9], but few articles described the determination methods of raltitrexed in human plasma. A method based on high-performance liquid chromatography with UV detection had been developed to determine the concentrations of raltitrexed in rat plasma [10], in which the lower limit of quantification (LLOQ) was 25 ng/ml. To evaluate the pharmacokinetics of raltitrexed in humans, a more sensitive method was required. In this paper, we describe a simple, sensitive HPLC–ESI-MS method for analysis of raltitrexed in human plasma, and the LLOQ of the method is 2.0 ng/ml which is sensitive enough for the determination of raltitrexed in human plasma.

2. Experimental

2.1. Materials and reagents

Raltitrexed standard (99.5% purity) was obtained from Jiangsu chia tai-Tianqing pharmaceutical Co. Ltd. (Lianyungang, China). Benazeprilat standard (IS, 99.5% purity) was a gift from Shenzhen Salubris Pharmaceuticals Co. Ltd (Shenzhen, China). The test formulation was raltitrexed injection provided by Jiangsu chia tai-Tianqing pharmaceutical Co. Ltd. (Lianyungang, China). Acetonitrile was of HPLC grade (Merck, Darmstadt, Germany). Methanol and formic acid were of analytic-grade purity and purchased from Nanjing Chemical Reagent Co. (Nanjing, China). Distilled water, prepared from demineralized water was used throughout the study.

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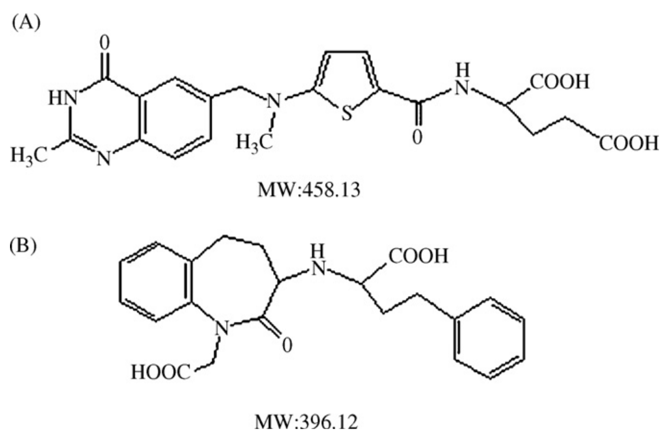

Fig. 1. Chemical structure of raltitrexed (A) and benaze prilat (B).

2.2. Instrumentation

HPLC–ESI-MS analyses were performed using an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technologies, Palo Alto, CA). And the HPLC–ESI-MS was controlled by a computer employing the HP ChemStation software (10.02 A) supplied by Agilent.

2.3. HPLC–ESI-MS conditions

The chromatographic separation was achieved on a Zorbax SB-C18 column (Narrow-Bore 2.1 mm × 150 mm, 5-μm, Agilent, Wilmington, DE, USA) with a 4 mm × 2.0 mm ID SecurityGuard C18 (5-μm) guard column (Phenomenex, Torrance, CA, USA), using a mobile phase of acetonitrile–water containing 0.1% formic acid and 2% methanol (21.9:78.1, v/v) with a flow-rate of 0.25 ml/min. The column temperature was maintained at 25 °C. HPLC–ESI-MS was carried out using nitrogen to assist nebulization. A quadrupole mass spectrometer equipped with an ESI source was set with a drying gas (N_2) flow of 10 l/min, nebulizer pressure of 40 psi, drying gas temperature of 350 °C, capillary voltage of 3.0 kV, the fragmentor voltage of 140 V and the positive ion mode. HPLC–ESI-MS was performed in selected-ion monitoring (SIM) mode using target ions at $[M+H]^+$ m/z 459.1 for raltitrexed and $[M+H]^+$ m/z 397.1 for IS.

2.4. Preparation of standard solutions

The stock solutions of raltitrexed (1.0 mg/ml) and internal standard (1.0 mg/ml) were prepared in methanol and stored at −20 °C. Standard solutions of raltitrexed with concentrations of 100 μg/ml, 10 μg/ml, 1 μg/ml, 100 ng/ml, were prepared by serial dilution of raltitrexed stock solution with methanol in separate 10 ml volumetric flasks. A solution containing 10 μg/ml internal standard was also obtained by further dilution of IS stock solution with methanol.

2.5. Sample preparation

To a 1-ml aliquot plasma sample, 50 μl of internal standard (10 μg/ml benazeprilat) was added. The sample mixture was deproteinized with 5 ml of methanol and vortex-mixed for approximate 2 min, allowed to stand for 10 min, and the mixture was centrifuged for 8 min at 4000 rpm. Then 4.5 ml of supernatant was transferred and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was reconstituted with 160 μl of mobile phase, and a 5-μl aliquot of this solution was injected onto the HPLC–ESI-MS for analysis.

2.6. Preparation of calibration curves and quality control samples

Calibration standards of raltitrexed were prepared by spiking appropriate amounts of the standard solutions in 1 ml blank plasma obtained from the volunteers. Standard curves were prepared in the range of 2.0–3000 ng/ml for raltitrexed at concentrations of 2.0, 10.0, 30.0, 100.0, 300.0, 1000, 2000 and 3000 ng/ml. The calibration curve was prepared and assayed along with quality control (QC) samples. QC samples were prepared in 1 ml blank plasma at concentrations of 5.0, 250.0 and 2500 ng/ml for raltitrexed, and stored at −20 °C.

2.7. Assay validation

2.7.1. Selectivity

The selectivity of the assay was checked by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Each blank plasma sample was tested using the protein precipitation procedure and HPLC–ESI-MS conditions to ensure no interference of raltitrexed and IS from plasma.

2.7.2. Linearity of calibration curves and lower limit of quantification

Calibration standards of eight raltitrexed concentration levels at 2.0, 10.0, 30.0, 100.0, 300.0, 1000, 2000 and 3000 ng/ml were extracted and assayed. To evaluate the linearity, calibration curves were prepared and assayed on five days. The calibration curve was constructed by plotting the peak-area ratios of raltitrexed to the IS versus the concentrations of raltitrexed, using weighted least squares linear regression (weighting factor was $1/C$). The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within ±20% [11], and it was established using five samples independent of standards. The QC samples were assayed along with clinical samples to monitor the performance of the assay and to assess the integrity and validity of the result of the unknown clinical samples analyzed.

2.7.3. Precision and accuracy

The validation samples were prepared and analyzed on three different days (one batch per day) to evaluate the accuracy, intra-batch and inter-batch precision of the analytical method. The accuracy, intra-batch and inter-batch precisions of the method were determined by analyzing five replicates at 5.0, 250.0 and 2500 ng/ml of raltitrexed along with one calibration curve on each of three batches. Assay precision was calculated using the relative standard deviation (R.S.D.%). The accuracy of an

analytical method describes the closeness of mean test results obtained by the method to the true value of the analyte [11]. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (R.E.%). It was calculated using the formula: R.E.% $= (E - T)/T \times 100\%$.

2.7.4. Extraction recovery

The extraction recovery of raltitrexed was evaluated by analyzing five replicates at 5.0, 250.0 and 2500 ng/ml of raltitrexed. The recovery was calculated by comparison of the peak areas of raltitrexed extracted from plasma samples with those of injected standards.

2.7.5. Stability

The stability of raltitrexed in plasma was studied under a variety of storage and handling conditions using the low (5.0 ng/ml) and high (2500 ng/ml) QC samples. The short-term temperature stability was assessed by analyzing QC samples that were kept at ambient temperature for 6 h. The stability of samples in autosampler was conducted reanalyzing extracted QC samples kept under the autosampler conditions (10 °C) for 12 h. Freeze–thaw stability (−20 °C in plasma) was checked through three cycles. The QC samples were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions and thawed unassisted at room temperature. The freeze–thaw cycles were repeated three times, and then analyzed on the third cycle. The long-term stability was performed at −20 °C in plasma for 8 weeks.

2.7.6. Matrix effects [12]

The matrix effect (ME) was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample [11]. In this paper, the matrix effect was assessed by using a method similar to that reported by Matuszewski et al. [12]. It was examined by comparing the peak areas of the analytes and IS between two different sets of samples. In set 1, the analytes were resolved in the blank plasma sample's reconstituted solution, and the obtained peak areas of the analytes were defined as A. In set 2, the analytes were resolved in mobile phase, and the obtained peak areas of the analytes were defined as B. ME was calculated by using the formula: ME (%) $= A/B \times 100$. The matrix effect of the assay was evaluated at three raltitrexed concentration levels of 5.0, 250.0 and 2500 ng/ml and the IS concentration level of 500.0 ng/ml. Five samples at each level of the analytes were analyzed. The blank plasma samples used in this study were five different batches of human blank plasma. If the ME values exceed the range of 85–115%, an exogenous matrix effect is implied.

2.7.7. Application

The method described above was applied to a pharmacokinetic study of raltitrexed in which plasma concentrations of raltitrexed in eight Chinese colorectal cancer patients (5 men, 3 women) were determined up to 336 h after receiving a single 3 mg/m^2 intravenous dose of ratitrexed injection. The intravenous infusion of the ratitrexed injection was designated to finish within 15 min. The blood was sampled pre-dose and at 0.083, 0.25, 0.417, 0.583, 0.75, 1.25, 2, 4, 8, 12, 24, 48, 72, 120, 168, 240 and 336 h post-dose. The clinical pharmacokinetic study was approved by the Ethic Committee of the 81st Hospital of Chinese PLA. The volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki.

The raltitrexed plasma concentrations of these samples were determined, and the pharmacokinetics of the drug in Chinese colorectal cancer patients was evaluated. Pharmacokinetic parameters were calculated for raltitrexed. The maximum plasma concentration (C_{max}) was noted directly. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. The elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve from the start of the infusion to the time of the last determined concentration (AUC_{0-336}) was calculated using the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as follows: $AUC_{0-\infty} = AUC_{0-336} + C_{336}/k_e$, in which the C_{336} was the plasma concentration of raltitrexed at 336 h post-dose.

3. Results and discussion

3.1. Selection of IS

As a proper internal standard, it should be structurally or chemically similar to the analyte. And it should also have similar retention to the analyte, be well resolved from the analyte and other peaks, and mimic the analyte in any sample preparation steps [13]. Benazeprilat was chosen as the internal standard for

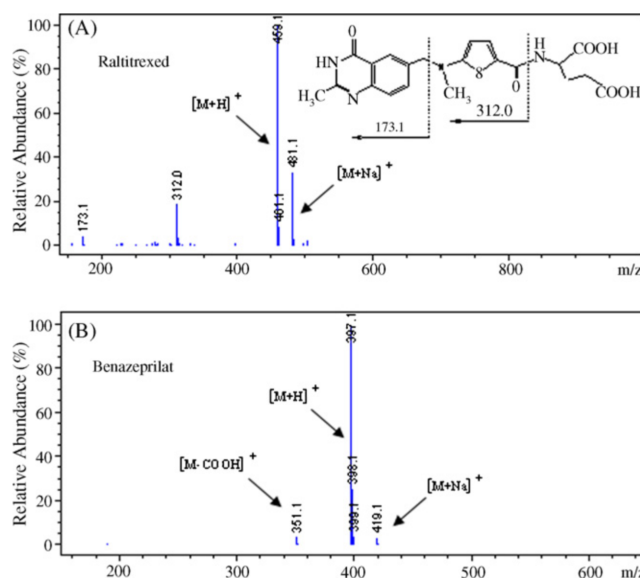

Fig. 2. Mass spectra of the positive ions of raltitrexed (A) and benaze prilat (B) at 140 V fragmentor voltage.

the assay because of its similarity of structure (Fig. 1B), retention and ionization to the analyte.

3.2. Sample preparation

The polar and amphoteric character of raltitrexed causes difficulty in extracting it from plasma by liquid–liquid extraction (LLE) or ion-pair extraction. Protein precipitation (PPT) method often provided a higher recovery compared with LLE for the analytes with amphoteric character. Thus, the PPT method was applied to prepare the plasma samples. Three kinds of precipitation reagents (acetone, acetonitrile and methanol) were tested as the protein precipitation reagents. The test results showed that acetone caused serious interference to the IS, and acetonitrile gave low recoveries of the analytes, while methanol not only gave a higher recoveries of the analytes, but also caused no interference to the analytes. So, methanol was finally chosen as the precipitation reagent in the experiment.

3.3. Conditions for ESI-MS

Because raltitrexed had both amino and carboxylic groups in its structure, it had mass spectrometric response either in the positive ion mode or in the negative ion mode. The test result showed that the signal intensity of the analytes obtained in the positive mode was much higher than that in the negative mode. So, the positive monitoring mode was selected in the MS detection. Usually, the electrospray ionization (ESI) was used for medium- to high-polarity analytes, and the ESI was adopted for the assay of analytes for their high-polarity property. In the HPLC–ESI-MS, there are several kinds of ions, such as fragment ions [14], deprotonated molecule ions [15,16] and protonated molecule ions [17,18], which may be selected as the target ions of the analytes in SIM. By adjusting the fragmentor voltage to different values, the different base peaks (the highest ion peak in the mass spectrum, which can be selected as the target ions of the analytes) were obtained. As the fragmentor voltage was set at lower values, the base peak obtained in the mass spectrum of

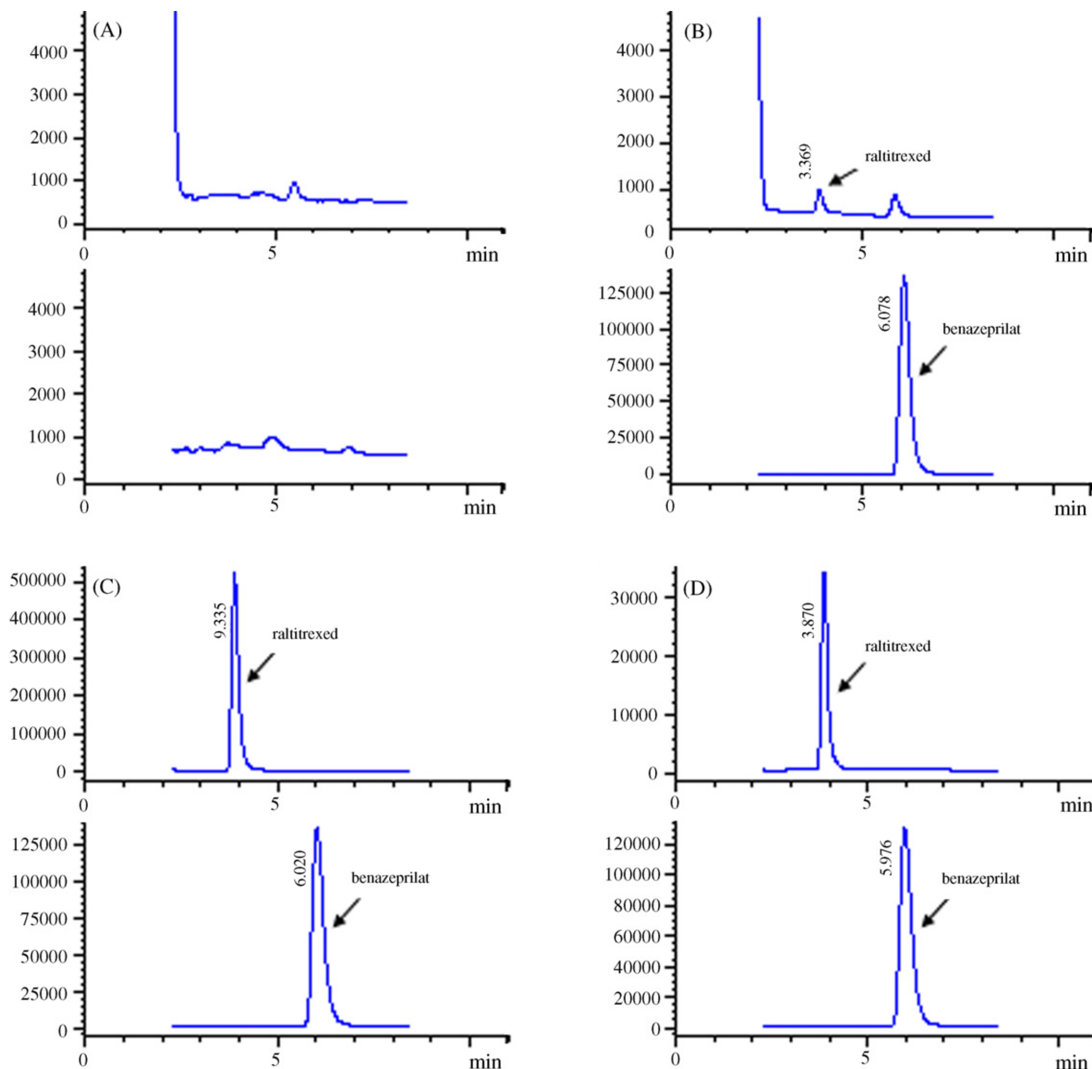

Fig. 3. Typical SIM mass chromatograms of blank plasma (A), LLOQ for raltitrexed in plasma (2.0 ng/ml) and benazeprilat (B), plasma spiked with raltitrexed (3000 ng/ml) and benazeprilat (C) plasma obtained from a patient at 45 min after receiving a single 3 mg/m^2 intravenous dose of raltitrexed injection, the plasma concentration of raltitrexed was estimated to be 220.2 ng/ml (D).

raltitrexed is the protonated molecular $[M+H]^+$ at m/z 459.1. When the fragmentor voltage exceeded 170 V, the intensity of the fragmentor ion at m/z 312.0, which is produced by loss of the group of 2-amino-1,5-pentane dioic acid, increased obviously, and became the base peak at 190 V fragmentor voltage. When the fragmentor voltage exceeded 170 V, selecting the fragment ion at m/z 312.0 as the target ion could achieve the higher sensitivity. However, this sensitivity was still less than the one achieved by selecting the protonated molecular $[M+H]^+$ at m/z 459.1 as the target ion with the fragmentor voltage less than 170 V. So the protonated molecular $[M+H]^+$ at m/z 459.1 was finally selected as the target ion of raltitrexed in the assay. In order to determine the optimal fragmentor voltage, the intensities of raltitrexed protonated molecular m/z 459.1 were compared at fragmentor voltages of 30, 50, 70, 90, 110, 120, 130, 140, 150, 170, 190, 200, 220 and 250 V in the SIM mode. The test result showed that the highest sensitivities could be obtained by choosing a fragmentor voltage of 140 V. At this fragmentor voltage, the base peak in the mass spectrum of IS was the protonated molecular $[M+H]^+$ at m/z 397.1. So, the protonated molecular $[M+H]^+$ at m/z 397.1 was selected as the target ion for the IS in the assay. The fragmentations of raltitrexed and IS are shown in Fig. 2.

3.4. Conditions of HPLC

The selection of mobile phase components was a critical factor in achieving good chromatographic peak shapes and resolution. At first, a basic elution system of water–acetonitrile was employed as the mobile phase. The experimental results showed that acidifying the aqueous portion of the mobile phase with formic acid could not only improve peak shapes of raltitrexed and IS, but also increase the MS sensitivity. Different concentrations of formic acid at levels of 0.05%, 0.1% and 0.2% were tested in the aqueous portion of the mobile phase. The result showed that adding 0.1% formic acid in the aqueous portion could sufficiently make the chromatographic peaks sharp and symmetric, and achieve a higher MS sensitivity for raltitrexed and IS. The ion suppression or enhancement due to the presence of unintended analytes or other interfering substances in the sample was usually observed in the HPLC–MS assay, and the same problem occurred during the process of development of the method. This problem had not been resolved by changing the ratio of the aqueous portion to the acetonitrile portion of the mobile phase. So, the addition of some different organic solvents, such as methanol, isopropyl alcohol, tetrahydrofuran, to the aqueous portion of the mobile phase was tried to improve the chromatographic separations of the analytes. The test results showed that the addition of 2% methanol in the aqueous portion could separate raltitrexed from the interference of the endogenous substances. Finally, the acceptable retention and separation of raltitrexed was obtained by using an elution system of acetonitrile–water containing 0.1% formic acid and 2% methanol (21.9:78.1, v/v) as the mobile phase. Representative selected-ion chromatograms are shown in Fig. 3. Typical retention times were about 3.8 min for raltitrexed and 6.0 min for IS.

Table 1
Results of five representative stand curves for raltitrexed HPLC–ESI-MS determination

	Back calculated standard results								Standard curve results		
	Standard								Slope ($\times 10^{-4}$)	Intercept ($\times 10^{-4}$)	r
	STD 1 2.0 ng/ml	STD 2 10.0 ng/ml	STD 3 30.0 ng/ml	STD 4 100.0 ng/ml	STD 5 300.0 ng/ml	STD 6 1000 ng/ml	STD 7 2000 ng/ml	STD 8 3000 ng/ml			
Run ID	Found concentration (ng/ml)										
I	2.242	8.642	28.98	101.5	334.2	940.9	2013	3037	7.638	0.04401	0.9994
II	1.723	9.835	32.53	100.9	327.9	1029.2	1942	3025	7.781	1.720	0.9996
III	2.225	9.602	27.82	102.2	304.4	1004.6	1951	3070	6.233	1.652	0.9998
IV	2.223	9.445	27.36	104.7	307.8	984.6	2019	3019	8.033	1.176	0.9999
V	2.326	9.512	29.16	93.25	298.3	1014	2025	3002	7.803	−0.7509	0.9999
Mean	2.148	9.407	29.17	100.5	314.5	994.7	1990	3031	7.498	0.77	0.9997
S.D.	0.24	0.45	2.03	4.30	15.65	34.13	39.99	25.19	0.72	1.08	0.0002390
R.S.D.%	11.2	4.8	7.0	4.3	5.0	3.4	2.0	0.8	9.62	NA	0.02
R.E.%	7.4	−5.9	−2.8	0.5	4.8	−0.5	−0.5	1.0			

Note: Calibration curves were weighted 1/conc. STD, standard; R.S.D., relative standard; R.E., relative error; NA, not applicable.

Table 2
Accuracy and precision for the analysis of LLOQ ($n = 5$).

Concentration level (ng/ml)	Calculated concentration (ng/ml)	Mean (ng/ml)	R.S.D. (%)	R.E. (%)
2.0	1.725			−13.8
2.0	2.380			19.0
2.0	2.285	2.086	12.6	14.3
2.0	1.952			−2.4
2.0	2.089			4.5

Note: R.S.D., relative standard; R.E., relative error; *n*, number of replicates.

3.5. Assay validation

3.5.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Fig. 3 shows the typical chromatograms of a blank, a spiked plasma sample with raltitrexed (2.0 ng/ml) and IS, a spiked plasma sample with raltitrexed (3000 ng/ml) and IS, and a plasma sample from the patient. There was no significant interference from endogenous substances observed at the retention times of the analytes.

3.5.2. Calibration curve and sensitivity

Five calibration analyses were performed on five days and the back-calculated values for each level were recorded (see Table 1). The calibration curves did not exhibit any non-linearity within the chosen range. The back-calculated results showed good day-to-day accuracy and precision. The LLOQ for raltitrexed in plasma was 2.0 ng/ml. The data of LLOQ is shown in Table 2.

3.5.3. Assay precision and accuracy

Table 3 summarizes the intra- and inter-batch precision and accuracy for raltitrexed evaluated by assaying the QC samples. The precision was calculated by using one-way ANOVA. In this assay, the intra-batch precision was 6.7% or less, and the inter-batch precision was 10.3% or less for each QC level of raltitrexed. The results above demonstrate that the values are within the acceptable range and the method is accurate and precise.

Table 3
Accuracy and precision for the analysis of raltitrexed (in prestudy validation, three batches, five replicates per batch)

Parameter	Concentration (ng/ml)		
	5.0	250.0	2500
Batch 1 (mean ± S.D.)	5.031 ± 0.41	261.6 ± 14.58	2356 ± 107.58
Batch 2 (mean ± S.D.)	4.612 ± 0.36	243.1 ± 24.05	2443 ± 211.51
Batch 3 (mean ± S.D.)	4.965 ± 0.19	250.6 ± 7.04	2328 ± 39.01
Overall mean	4.870	251.8	2376
Intra-batch R.S.D. (%)	6.7	6.7	5.8
Inter-batch R.S.D. (%)	10.3	8.3	5.6
Overall accuracy (R.E.%)	−2.6	0.7	−4.9

Note: S.D., standard deviation; R.S.D., relative standard; R.E., relative error.

3.5.4. Recovery

The choice of methanol as the extraction solvent may not only eliminate the interference of endogenous substances, but also meet the requirement of sensitivity for the assay. The recovery of raltitrexed, determined at three concentration levels of 5.0, 250.0 and 2500 ng/ml were 85.2 ± 5.8%, 89.8 ± 3.3% and 91.1 ± 2.2% ($n = 5$), respectively.

3.5.5. Stability

The stability results (see Table 4) showed that no significant degradation occurred at ambient temperature for 6 h. Samples in autosampler were stable for at least 12 h. And there were also no significant degradation occurred during the three freeze–thaw cycles for raltitrexed plasma samples. Raltitrexed in plasma at −20 °C was stable for 8 weeks.

3.5.6. Matrix effects

The test results of matrix effects are summarized in Table 5. The results obtained were within the acceptable limit, and it indicated that there was no matrix effect of the analytes observed in this study.

3.6. Application

The assay was successfully applied in a pharmacokinetic study in which plasma concentrations of raltitrexed in eight Chinese colorectal cancer patients (5 men, 3 women) were determined up to 336 h after receiving a single 3 mg/m^2 intravenous dose of ratitrexed injection. The mean plasma concentration–time curve of raltitrexed is shown in Fig. 4. The maximum plasma concentration (C_{max}) was 616.2 ± 212.0 ng/ml. The elimination half-life ($t_{1/2}$) was 163.0 ± 63.3 h. The AUC_{0-336} and $AUC_{0-\infty}$ were 1333.1 ± 458.8 ng h ml^{-1} and 1999.9 ± 653.8 ng h ml^{-1}, respectively. Beale et al. [8] reported the pharmacokinetic profiles of ratitrexed in UK patients with advanced solid malignancies after receiving a single dose of 3.0 mg/m^2 [^{14}C]-raltitrexed. The observed C_{max} was 700.6 ± 165.3 ng/ml. The elimination half-life ($t_{1/2}$) was 257 ± 62.9 h at 3.0 mg/m^2. The $AUC_{0-\infty}$ was 2341.7 ± 941.1 ng h ml^{-1}. The elimination half-life ($t_{1/2}$) of raltitrexed was not similar between the Chinese and European patients, but the C_{max} and $AUC_{0-\infty}$ were comparable.

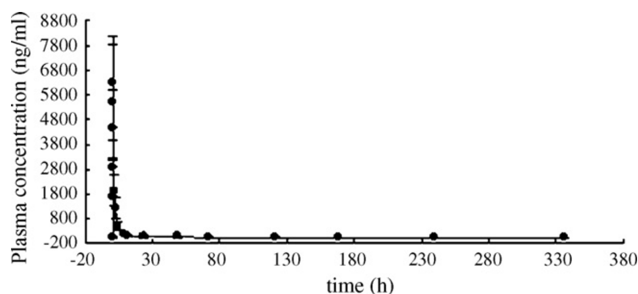

Fig. 4. Mean plasma concentration–time profile of raltitrexed in eight Chinese colorectal cancer patients after receiving a single 3 mg/m^2 intravenous dose of raltitrexed injection.

Table 4
Stability data of raltitrexed in human plasma under various storage conditions ($n = 3$)

Storage conditions	Concentration level (ng/ml)	Calculated concentration (ng/ml) (mean ± S.D.)	R.E. (%)
Ambient temperature for 6 h	5.0	5.553 ± 0.17	11.1
	2500	2615 ± 165.38	4.6
Autosampler for 12 h	5.0	4.890 ± 0.60	−2.2
	2500	2357 ± 67.88	−5.7
Three freeze–thaw cycles	5.0	4.599 ± 0.14	−8.0
	2500	2357 ± 61.80	−0.4
8 weeks at −20 °C	5.0	5.261 ± 0.24	5.2
	2500	2662 ± 102.50	6.5

Note: S.D., standard deviation; R.E., relative error; *n*, number of replicates.

Table 5
Matrix effects data for raltitrexed and benazeprilat (IS) in five different lots of human plasma ($n = 5$)

Samples	Concentration level (ng/ml)	*A* (mean ± S.D.)	*B* (mean ± S.D.)	Matrix effect (%)
Raltitrexed	5.0	21869 ± 2877	22370 ± 1228	97.8
	250.0	1280085 ± 203467	1304858 ± 122604	98.1
	2500	11386643 ± 885708	11728716 ± 907354	97.1
Benazeprilat	500.0	7284158 ± 375849	6760029 ± 373118	107.8

Note: S.D., standard deviation; *n*, number of replicates.

4. Conclusion

The assay achieved good sensitivity and specificity for the determination of raltitrexed in human plasma. No significant interferences caused by endogenous compounds were observed. This simple and sensitive assay is suitable for pharmacokinetic studies of raltitrexed in human subjects.

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