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Short Communication

Column-switching high-performance liquid chromatographic method for the determination of a thymidylate synthase inhibitor, LY231514, an investigational agent for the treatment of solid tumors, in human plasma

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Abstract

A reversed-phase, column-switching high-performance liquid chromatographic (HPLC) method is described for the determination of a new thymidylate synthase inhibitor in human plasma. The compound and an internal standard are extracted from plasma using a Certify II solid-phase cartridge. Extracts are evaporated to dryness and the residue is reconstituted with mobile phase buffer. The analytes are separated from polar interferences and buffer salts originating from the elution step on a 4-mm YMC Basic pre-column. The fraction containing the analytes is further separated on a 25-cm YMC Basic column. The analytes are detected by their absorbance at 250 nm. The limit of quantitation is 10 ng/ml. The method is linear from 10 ng/ml to 80 μg/ml using three standard curve ranges. Validation studies for all three ranges show the method to be reproducible. The method has been successfully used to support pharmacokinetic studies.

1. Introduction

N-[4-[2-[2-Amino-4,7-dihydro-4-oxy-3H-pyrrolo[2,3-D]pyrimidine-5-ly)ethyl]benzoyl]-L-glutamic acid (LY231514, I, Fig. 1) is currently undergoing early clinical trials as a solid-tumor oncolytic. It is a pyrrolopyrimidine antifolate and a specific inhibitor of thymidylate synthase (TS), a target of the established oncolytic agent, 5-fluorouracil (5-FU) [1,2]. Of the 40-plus oncolytic agents currently available for the general practice of clinical oncology, 5-FU is one of only two agents targeting TS. A number of agents, most of which lack single agent cytotoxic activity, are being evaluated in clinical trials with 5-FU. The toxicity associated with 5-FU as well as its marginal activity argue for the development of a more active single agent. I has excellent cytotoxic activity and directly inhibits TS.

In support of clinical trials, a sensitive and selective method has been developed and validated for the determination of I in human plasma. This paper presents the scientific rationale for the development of the method together with its validation prior to its use in clinical studies.

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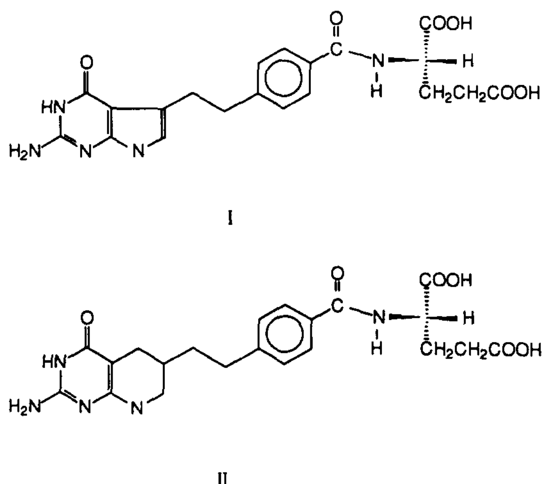

Fig. 1. Structures of LY231514 (I) and the internal standard (II).

2. Experimental

2.1. Chemicals and reagents

Compound I and the internal standard (II), (6R)-N-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-D]pyrimidine-6-yl)ethyl]benzoyl]-L-glutamic acid (Fig. 1), were obtained from Lilly Research Laboratories (Eli Lilly and Company, Indianapolis, IN, USA). HPLC-grade water (Milli-Q System, Millipore) was used in all aqueous solutions. Methanol and acetonitrile were HPLC grade; all other chemicals were of analytical reagent grade. Control (blank) human plasma was obtained from healthy volunteers.

2.2. Liquid chromatography

The HPLC system consisted of two isocratic pumps, a variable wavelength UV absorbance detector (Spectroflow 783, Kratos Division, ABI Analytical, Ramsey, NJ, USA), an autosampler, a column heater and a six-port automated column-switching valve with a programmable controller.

The analytical column was a YMC Basic column (250 × 4.6 mm I.D., 5 μm, YMC, Morris Plains, NJ, USA). The pre-column was a YMC Basic guard column (23 × 4 mm I.D., 5 μm). The mobile phase for the analytical column was 50 m*M* sodium phosphate (pH 3)–acetonitrile (86:14, v/v). The wash solvent for the pre-column was 100% 50 m*M* sodium phosphate (pH 3). The flow-rate for both columns was 0.8 ml/min. The temperatures were ambient for the pre-column and 30°C for the analytical column.

On-line data acquisition and calculations were performed by an Access*Chrom GC/LC data system (Perkin-Elmer Nelson Systems, Cupertino, CA, USA). The equipment configuration is illustrated in Fig. 2.

2.3. Preparation of standard solutions

A 100 μg/ml stock was prepared in water for the low (10–200 ng/ml) and medium (200–4000 ng/ml) curves. A diluted stock (1 μg/ml) in water was also prepared for the low curve. A

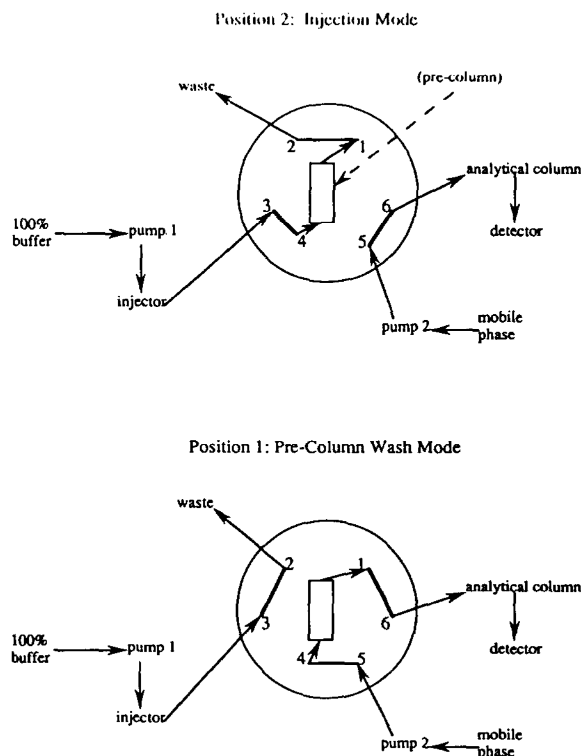

Fig. 2. Configuration of column-switching valve and equipment.

4000 μg/ml stock solution and a diluted stock (400 μg/ml) in water were prepared for the high standard curve (4–80 μg/ml). All concentrations of I are free-base concentrations.

Internal standard stocks were prepared in water at concentrations of 1, 20 and 400 μg/ml for the low, medium and high curves, respectively.

Plasma standards were prepared for each curve range by diluting appropriate amounts of each stock with blank plasma. The low curve concentrations were 10, 25, 50, 100, 150 and 200 ng/ml. The medium curve concentrations were 200, 800, 1600, 2400, 3200 and 4000 ng/ml. The high curve concentrations were 4, 10, 20, 40, 60 and 80 μg/ml.

2.4. Sample preparation procedure

Aliquots (1 ml) of plasma samples or standards were dispensed into disposable glass culture tubes. Following the addition of 200 μl of the appropriate internal standard solution, samples were extracted using Bond Elut Certify II solid-phase cartridges (Varian, Harbor City, CA, USA). The cartridge was conditioned with 2 ml of methanol followed by 2 ml of 35 m*M* sodium phosphate (pH 7) prior to loading the sample. Following sample load (load only 0.1 ml of high curve samples), the cartridge was washed with 2 ml of 35 m*M* sodium phosphate (pH 7) and 2 ml of methanol. The analytes were then eluted into silanized glass culture tubes using 35 m*M* sodium phosphate (pH 7)–acetonitrile (60:40, v/v). The eluent was evaporated to dryness under nitrogen and the residue was reconstituted with mobile phase buffer as follows: 150 μl for the low curve, 200 μl for the medium curve and 0.1 ml for the high curve. Samples were vortex-mixed and transferred to glass HPLC autosampler vials.

2.5. Calculations

A least-squares calibration curve was obtained by plotting the concentrations of the plasma standards *vs.* the peak-height ratios. The peak-height ratios were calculated by dividing the peak height of I by the peak-height of the internal standard. The concentration of I was determined from the peak-height ratio relative to the calibration curve.

2.6. Determination of recovery, precision and accuracy

The extraction efficiency (recovery) of the sample preparation was determined by comparing the peak heights obtained from the chromatograms of aqueous standards (not extracted) to those of extracted plasma samples which had been spiked with compound. Recovery was determined at nine concentrations (0.015, 0.10, 0.15, 0.25, 1.2, 3.6, 5.0, 36 and 72 μg/ml).

The precision and accuracy of the method were determined by performing replicate analyses of six pools of plasma spiked with known concentrations of I for each curve range. These concentrations included the limit of quantitation, pools within the standard curve range and pools between 2–8 times the highest standard for each curve. The latter pools were diluted ten-fold with blank plasma to bring the concentration within the appropriate standard curve range. Five replicates of each pool were analyzed on three different days by the same analyst on the same instrument for the low curve range. Five replicates of each pool were analyzed on one day for the medium and high curve ranges. All pools were analyzed in random order. Two standard curves were included each day, one at the beginning and one at the end of the run.

2.7. Determination of stability

The stability of the compound in plasma was determined by preparing pooled plasma with known amounts of compound (0.40, 3.6 and 36 μg/ml). The pools were aliquoted (4 ml) into polypropylene plastic tubes, capped and stored at −70°C, −20°C, 4°C and room temperature. Five replicates of each pool were assayed from each storage condition at set time points following initial storage.

Processed sample stability of the compound was evaluated by reinjecting a set of pools starting 24 h after the first run was initiated.

2.8. Analysis of samples from a clinical study

Plasma samples from volunteers in a clinical study were frozen at −70°C prior to analysis. On thawing, 1-ml aliquots were pipetted into glass culture tubes and processed as described in *Sample preparation procedures*. Three concentrations of controls for each curve range were prepared from a separately weighed stock. The controls were aliquoted and frozen. Controls were included with each sample run.

3. Results and discussion

3.1. Method development and chromatography

The assay conditions described in this report evolved from the evaluation and optimization of the sample preparation and chromatography of I and the internal standard.

Due to the polar nature of I, a solid-phase extraction scheme for clean-up was explored. Reversed-phase cartridges including C_{18}, C_{18}-OH, C_2, CN and phenyl (Analytichem Bond Elut) gave low recoveries. Dual phase cartridges (*i.e.* reversed-phase/anion-exchange) including SAX and Certify II (Analytichem Bond Elut) which exploit both the reversed-phase and the charged regions of I gave higher recoveries and eliminated endogenous interferences. The best results were obtained using the Certify II cartridges.

In evaluating the chromatographic separation, several columns were screened for optimal peak shape and sensitivity. The columns included various brands of C_{18} (Zorbax ODS, Jones C_{18}, Supelcosil LC-18-DB, Vydac C_{18} endcapped and YMC Basic) as well as cyano columns (Zorbax CN and SB-CN). The YMC Basic column yielded superior peak shapes.

Following the optimization of the sample clean-up and chromatography, there were ruggedness problems which resulted from the salt present in the samples following the solid-phase elution step. The salts were shielding available binding sites on the head of the column causing the analytes to elute earlier than observed for absolute standards. Also, the salt from the extraction buffer was precipitating out on the head of the column causing uneven flow and, consequently, split peaks. This salt buildup also caused high back pressure. Attempts to use other solvents containing volatile salts to elute

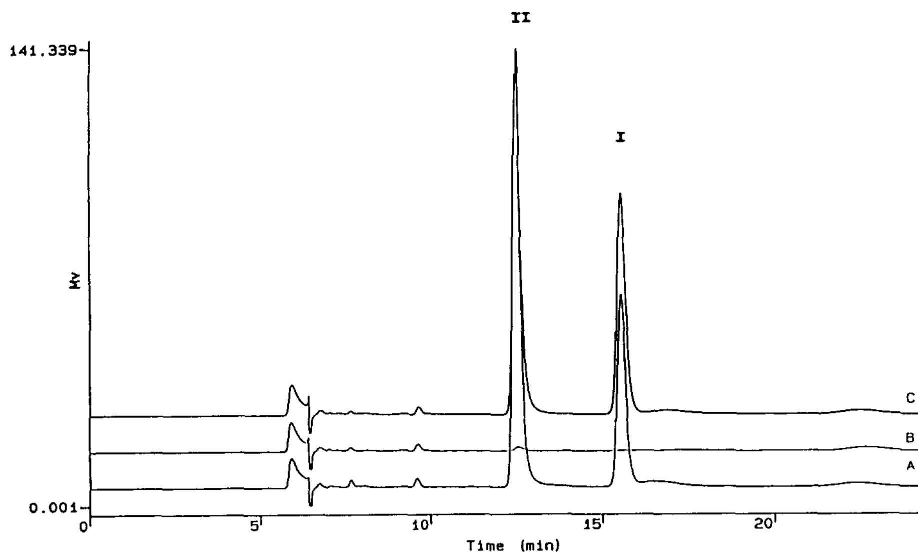

Fig. 3. Representative chromatograms from a patient administered a single i.v. dose (10 mg/m^2) of I. (A) Plasma standard (2400 ng/ml); (B) patient sample, pre-dose; (C) patient sample, 5 min post-dose. Peaks: I = LY231514, II = internal standard.

the analytes from the solid-phase extraction cartridge resulted in substantially lower recoveries.

Column-switching was therefore used to eliminate the salts. The sample was injected onto a pre-column equilibrated with mobile-phase buffer. Salts and any other polar interferences were washed off to waste. After one minute, the valve was rotated thereby allowing mobile phase through the pre-column carrying the analytes onto the analytical column for further separation. After 20 min, the valve was rotated back to the inject position to allow the columns to be equilibrated for the next injection.

Representative chromatograms of extracted patient samples including a pre-dose, post-dose and a standard, are shown in Fig. 3. The chromatogram obtained from the pre-dose sample indicates the absence of any interferences at the retention times of the analytes.

3.2. Sample recovery

Recoveries of I and II from spiked plasma *versus* absolutes are shown in Table 1. The recovery is concentration dependent in the low standard curve range, but constant at *ca.* 73% for the middle and high-curve ranges.

Table 1
Recovery of I and II

Concentration	Recovery (%)	
	I	II
Low-curve range (ng/ml)		81
15	40	
100	69	
150	71	
Medium-curve range (ng/ml)		72
250	73	
1200	83	
3600	76	
High-curve range (μg/ml)		73
5	73	
36	73	
72	73	

3.3. Precision and accuracy

The determination of I was evaluated for precision and accuracy by replicate analyses of plasma pools spiked with I at various concentrations over the three curve ranges. Replicates of the same pools were evaluated on three separate days so that both within-day and between-day precision and accuracy could be determined for the low curve range. Only within-in day precision and accuracy were determined for the medium and high curve ranges. Overall, the precision and accuracy of the method were good; relative standard deviations were typically ≤15% for the low curve range and ≤10% for the medium and high curve ranges (Tables 2–4).

3.4. Sensitivity

The limit of quantitation of the assay is 10 ng/ml.

3.5. Linearity

The linearity of the responses was determined over the concentration range of 10 ng/ml to 80 μg/ml using three curve ranges (10–200 ng/ml, 200–4000 ng/ml and 4–80 μg/ml). Sample concentrations up to 300 μg/ml can be accommodated by ten-fold dilutions with blank plasma prior to extraction.

3.6. Stability

Compound I is stable in plasma for at least 48 h at room temperature, for at least two weeks at 4°C, and for at least six months at −20°C and −70°C (Table 5). Plasma samples are stable through at least 3 freeze/thaw cycles. Processed samples are stable for at least 24 h. Standard stock solutions are stable for at least 3 months at 4°C.

3.7. Application of the method in pharmacokinetic studies

The validated procedure was used to provide pharmacokinetic data for I in human plasma

Table 2
Precision and accuracy data for I: low-curve range

Day		Theoretical concentration				
		10.0 ng/ml	60.0 ng/ml	120 ng/ml	180 ng/ml	1000 ng/ml
1	Mean ($n = 5$)	10.3	56.0	111	170	960
	R.S.D. (%)	8.2	3.3	4.4	3.3	3.0
	Percentage of theory	103	93.3	92.5	94.4	96.0
2	Mean ($n = 5$)	10.3	51.5	102	158	887
	R.S.D. (%)	7.3	5.1	2.28	6.7	6.1
	Percentage of theory	103	85.8	85.0	87.8	88.7
3	Mean ($n = 5$)	10.8	55.4	110	172	925
	R.S.D. (%)	13.2	3.0	2.9	8.8	2.2
	Percentage of theory	108	92.3	91.7	95.6	92.5
Overall	Mean ($n = 15$)	10.5	54.2	108	167	924
	R.S.D. (%)	10.3	5.7	5.2	7.6	5.4
	Percentage of theory	105	90.3	90.0	92.8	92.4

following the administration of single doses. Plasma samples were obtained at defined time intervals post-administration, extracted and analyzed. Analysis of pre-dose samples showed no interference from endogenous substances.

4. Conclusions

The method reported here provides a sensitive and selective method for the determination of I in human plasma. The use of column-switching

Table 3
Precision and accuracy data for I: medium-curve range

Day		Theoretical concentration					
		202 ng/ml	610 ng/ml	1.22 μg/ml	2.43 μg/ml	3.65 μg/ml	30.4 μg/ml
1	Mean ($n = 5$)	207	571	1.15	2.30	3.45	29.0
	R.S.D. (%)	1.8	3.0	4.7	3.4	4.3	2.8
	Percentage of theory	102	93.6	94.3	94.7	94.5	95.4

Table 4
Precision and accuracy data for I: high-curve range

Day		Theoretical concentration					
		3.99 μg/ml	16.0 μg/ml	35.9 μg/ml	75.8 μg/ml	160 μg/ml	299 μg/ml
1	Mean ($n = 5$)	4.22	15.9	35.3	71.0	164	296
	R.S.D. (%)	2.2	2.4	3.1	1.6	3.1	2.1
	Percentage of theory	106	99.4	98.3	93.7	103	99.0

Table 5
Stability of I in human plasma

Theoretical concentration (ng/ml)	Storage time	Mean ($n = 5$) assay concentration (ng/ml)	R.S.D. (%)	Percentage of theory	Storage temperature
404.8	Initial	400.6	5.7	98.9	NA
3636		3479	6.0	95.7	
36 360		35 530	10	97.7	
404.8	48 h	444.2	8.0	109	Room temperature
3636		3475	3.4	95.6	
36 360		34 600	1.3	95.2	
404.8	2 weeks	380.6	3.1	94.0	4°C
3636		3497	3.4	96.2	
36 360		34 300	3.2	94.3	
404.8	6 months	445.4	3.6	110	−20°C
3636		4096	2.5	113	
36 360		40 750	4.8	112	
404.8	6 months	448.0	2.9	110	−70°C
3636		3862	3.0	106	
36 360		40 380	6.4	111	

has eliminated the problem of salt buildup on the analytical column thus allowing reliable, consistent separation of I and internal standard. Based on the results of pharmacokinetic data to this point, the method has proven to be sensitive enough for present needs.

5. Acknowledgements

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6. References

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