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Highly sensitive analysis of the antifolate pemetrexed sodium, a new cancer agent, in human plasma and urine by high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatography method was developed and validated for the quantitation of pemetrexed (LY231514, ALIMTA) in human urine and plasma. Plasma samples were spiked with the internal standard lometrexol and extracted using Certify II columns. Pemetrexed was assayed in diluted urine by an external calibration method. A C_8 column was used for the separation of analytes with a mobile phase composed of sodium formate buffer and acetonitrile. Between- and within-day precision and accuracy were acceptable down to the limit of quantitation of 5 ng/ml in plasma. This method was used successfully for an investigation of the disposition of pemetrexed in patients receiving 500 mg/m^2 as a 10-min infusion.

Keywords: Pemetrexed; LY231514; ALIMTA; Lometrexol

1. Introduction

Pemetrexed (LY231514: *N*-{4-[2-(2-amino-4,7-dihydro-4-oxy-3H-pyrrolo[2,3-D] pyrimidine-5-ly)-ethyl]benzoyl}-L-glutamic acid) is a promising folate-based antimetabolite (Fig. 1) with activity against a variety of tumour types, in particular lung and mesothelioma [1]. Pemetrexed has been shown to inhibit multiple folate-requiring enzymes including dihydrofolate reductase, thymidylate synthase and glycinamide ribonucleotide formyltransferase [2,3].

Three schedules of administration of pemetrexed have been investigated in phase I trials: every 3 weeks as a 10-min infusion, weekly for 4 weeks and a five daily times every 3 weeks protocol [1]. The 3-week regimen was selected for further study on the basis of a superior therapeutic index.

Combination regimens containing pemetrexed are being explored in phase I/II studies and we required a sensitive assay to support such trials. In our hands, we found existing methods to be impractical and lacking in specificity. For this reason we have

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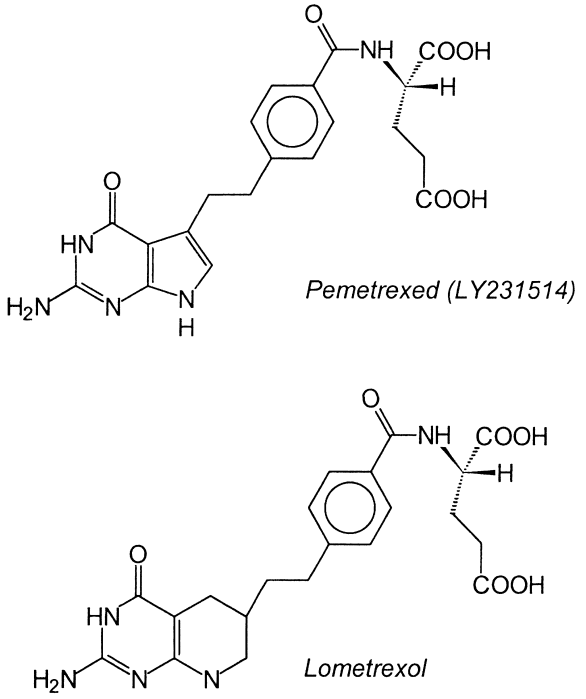

Fig. 1. Chemical structures of pemetrexed (LY231514) and the internal standard, lometrexol, as acids.

developed a highly sensitive and robust method for measuring pemetrexed in the plasma of patients.

2. Experimental

2.1. Chemicals and HPLC reagents

Pemetrexed was obtained from Eli Lilly (IN, USA) as the disodium salt (LY289739, lot 262SB6). Lometrexol (Fig. 1) was used as the internal standard (I.S.) and was supplied by Eli Lilly as the acid (lot 064-2). Stock solutions (5 mg/ml) of pemetrexed and lometrexol were prepared in water and 0.1 *M* NaOH, respectively and stored at −20°C until use. High-grade methanol and acetonitrile (ChromAR-HPLC, Mallinckrodt) were obtained from Biolab Scientific (Melbourne, Australia). Water was of Milli-Q grade (Millipore, Sydney, Australia) and all solutions were filter-degassed using a 0.45 μm filtration system (Millipore). The mobile phase was composed of 0.2% formic acid adjusted to pH 3.08 with 10 *M* NaOH. This was mixed automatically with 100% acetonitrile with the use of a quaternary solvent selection valve in the proportion of 86:14 (v/v) and pumped at 1.0 ml/min. Date expired fresh-frozen plasma was kindly provided by the Royal Prince Alfred Hospital Blood Bank and used as a blank matrix for assay development.

2.2. Apparatus

Separation of analytes was performed at ambient temperature using a Waters Symmetry C_8 reversed-phase column (150×3.9 mm I.D., 4 μm) preceded by a matching Symmetry C_8 guard column (Waters, Sydney, Australia). The HPLC system (Shimadzu Aust., Sydney, Australia) consisted of a LC-10AT pump, FCV-10AL gradient valve, DGU-12A in-line solvent degasser, SCL-10A system controller, SIL-10AXL autoinjector and SPD-10 AV UV–Vis detector. Data were collected and analysed on a personal computer using Class VP software (version 4.2, Shimadzu).

2.3. Preparation of plasma samples

For sample preparation, Bond-Elut Certify II solid-phase extraction cartridges (200 mg, 3 ml Varian, Harbor City, CA, USA) were tested with different elution protocols. In addition to analyte recovery, specific attention was paid to the presence of endogenous compounds eluting in proximity to lometrexol and pemetrexed when blank plasma extracts were injected. Columns were prepared by sequential rinsing with 2 ml methanol and 2 ml of buffer (0.1 *M* sodium acetate, pH 7.0). Samples and standards were prepared by spiking plasma with 10 μl of the appropriate solutions of pemetrexed and lometrexol (internal standard, I.S.) in sterile 10 ml polypropylene tubes (Sarstedt, Adelaide, Australia). For the concentration range of 0.005–1 μg/ml, 1 ml of plasma spiked with 250 ng of lometrexol was extracted, whereas for the high-concentration samples, 0.1 ml plasma and 5 μg of lometrexol were used. The plasma (0.1 or 1 ml) was diluted with 2 ml buffer and vortex-mixed. The mixture was loaded onto the extraction column that was then rinsed with 2×2 ml buffer and 2 ml methanol.

Compounds were eluted with 1.5 ml 20 m*M* trisodiumcitrate into 2 ml Eppendorf tubes (polyurethane tubes) and the solvent evaporated overnight at ambient temperature in a Speed-Vac (Savant, Holbrook, NY, USA). With the high-concentration samples and standards (1–200 mg/ml), the residue was resuspended into 50 μl of water and centrifuged at 8000 *g* for 5 min. The supernatant was transferred to a 100-μl microvolume polypropylene insert (Alltech, Deerfield, IL, USA) and 5 μl injected onto the chromatograph. With the low-concentration samples, the residue was resuspended into 20 μl of water and the sample desalted with the addition of 250 μl of methanol. Following centrifugation at 8000 *g* for 5 min, the supernatant was transferred to an Eppendorf tube and the contents evaporated. The residue was reconstituted in 50 μl of water, centrifuged and 25 μl injected.

The recovery of pemetrexed and lometrexol from plasma was assessed by comparing areas obtained from spiked samples with those from eluting solution (20 m*M* trisodiumcitrate) spiked with pemetrexed and lometrexol and which was dried and processed as per samples.

Replicate samples ($n=5$) containing pemetrexed at three concentrations were processed for both the low- and high-concentration ranges (low: 0.005, 0.025, 0.25 μg/ml, high: 1, 5, 50 μg/ml) on three separate days to assess accuracy and the within-day and between-day imprecision (C.V.).

2.4. HPLC program

With high-concentration standards and samples, the HPLC analysis was performed under isocratic conditions with a run time of 13 min. The retention times of lometrexol and LY231514 were 6.0 and 8.5 min, respectively. However, with the low-concentration samples and standards (<1 μg/ml), occasional late eluting peaks, although very small, sometimes interfered with the quantitation of pemetrexed. Therefore, a timed rinse of the column was added during analysis of low-concentration samples. At 12 min, the acetonitrile was increased to 50%, where it was kept constant for 5 min and reduced back to 14% for a further 8 min prior to the next injection (total run time of 25 min).

2.5. Preparation of urine samples

Urine concentrations of pemetrexed were found to be extremely high in the first few hours following administration. For this reason, urine concentrations were assayed by direct injection of 1/100 dilutions of urine samples in water. An endogenous peak, tentatively identified as hippuric acid eluted close to lometrexol. Hence, an external calibration curve was used. Standards were prepared in 1/100 diluted blank urine with final concentrations of 0.5, 2, 5, 20 and 50 μg/ml.

2.6. Stability of pemetrexed in urine

Pemetrexed has reduced solubility in acid aqueous solutions. Because of the high concentrations present, we were concerned that the recovery of pemetrexed in frozen urine samples might be compromised. A stability/recovery study of pemetrexed was carried out in blank urine with pH adjusted to represent the range of normal values (4.5, 6.4, 7.8) and three storage conditions (ambient temperature, 4°C, −20°C). Pemetrexed was added to each stock urine at a concentration of 0.5 mg/ml. Dilutions (1/100) were immediately carried out and assayed in triplicate. Dilutions were then performed at 2 and 8 h (ambient temperature), 8, 24 and 48 h (4°C) and 1 and 6 weeks (−20°C). Concentrations were calculated from freshly prepared standard curves (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) that were diluted 1/100 with diluted blank urine just prior to analysis.

2.7. Pemetrexed pharmacokinetics

The disposition of pemetrexed in the plasma of patients receiving 500 mg/m^2 (10 min infusion) as second-line treatment of non-small cell lung cancer was studied. The study was approved by the Ethics Committee of the Royal Prince Alfred Hospital. Venous blood (9 ml) was collected into heparinised tubes before, at the middle and end of infusion and then 5, 10, 20, 30 min and 1, 2, 4, 8 and 24 h post-infusion. The plasma was collected and stored at −70°C until analysis. Pemetrexed has been shown to be stable for several months in plasma under these conditions [4].

For the analysis, the plasma samples were thawed

and 0.1 ml transferred to a 10-ml tube. These were processed as for the high-concentration samples along with the appropriate standards (six points, 1–200 μg/ml) and quality control samples (1.5 and 25 μg/ml). Concentrations of pemetrexed (as the disodium salt) were calculated according to the peak area ratio of pemetrexed and lometrexol using a log–log transformation of the standard curve. Samples which yielded concentrations <1 μg/ml were re-processed using 1 ml of plasma which was extracted alongside the appropriate standards (six points, 0.005–1 μg/ml) and quality control samples (0.008 and 0.5 μg/ml).

Plasma concentrations were fitted to the equation:

$$c(t) = \sum_{i=1}^{n} \frac{A_i}{\lambda_i \tau}(e^{-\lambda_i t'} - e^{-\lambda_i t})$$

where A_i and λ_i are the ith coefficient and exponent, respectively, τ is the duration of the infusion and t' is the time after the end of the infusion (and $=0$ when $t<\tau$). Non-linear regression was carried out as per Bowen and Jerman [5] in an Excel spreadsheet environment (Microsoft Excel v. 7.0). The inclusion of three compartments, rather than two or one in the model, yielded superior fits as assessed from Akaike's Information Criteria (AIC) values [6] in all subjects. Other pharmacokinetic parameters (CL, total body clearance; Vd_{SS}, volume of distribution at steady-state, MRT, mean residence time; and $t_{1/2}$, half-lives of the three phases) were calculated according to standard equations following the trapezoidal integration of the concentration and concentration×time curves.

2.7.1. Results and discussion

As illustrated in Fig. 2, the method was able to detect pemetrexed in human plasma at concentrations as low as 5 ng/ml (as the disodium salt). This, however, required the use of a desalting step to remove excess citrate as the latter otherwise resulted in split peaks. In the method of Hamilton and Kirkwood, elution was carried out with 35 m*M* sodium phosphate [4]. We found citrate to be more selective and this was reflected by a cleaner baseline in blank plasma and retention of some coloration on the solid-phase extraction column. We tried using mono-sodium glutamate (20, 30 and 40 m*M*) with the intention of increasing the specificity of elution

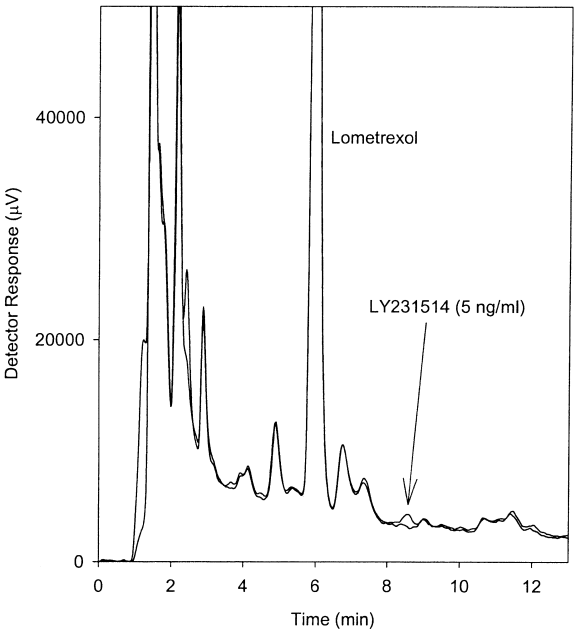

Fig. 2. Representative chromatograms of blank plasma and plasma spiked with pemetrexed (5 ng/ml).

but the recoveries were very low. In any case, the desalting step was relatively rapid and not required for the high-concentration samples (>1 μg/ml) as a result of the small volume injected. Analyte recovery was not dependent on the analyte concentration and averaged 76.9±14.0% and 84.1±3.3% for pemetrexed and lometrexol, respectively. These values are slightly higher than those reported by Hamilton and Kirkwood [4], indicating no loss in recovery in spite of the increased specificity. Avoiding the need for column switching made this technique more suitable for our laboratory.

Typical standard curves of peak area ratio as a function of pemetrexed were linear with $r^2>0.999$ (Fig. 3) regardless of the matrix.

The precision and accuracy data presented in Tables 1 and 2 indicate that the plasma and urine assays were suitable for concentrations ranging from 5 ng/ml to 200 μg/ml and 0.05 to 5 mg/ml, respectively. These ranges were adequate for estimating plasma pharmacokinetics and urinary recovery, respectively, over a period of 24 h (Table 3 and Fig. 4). Importantly, storage of urine did not compromise the analysis and pemetrexed concentrations after the longest periods of storage under

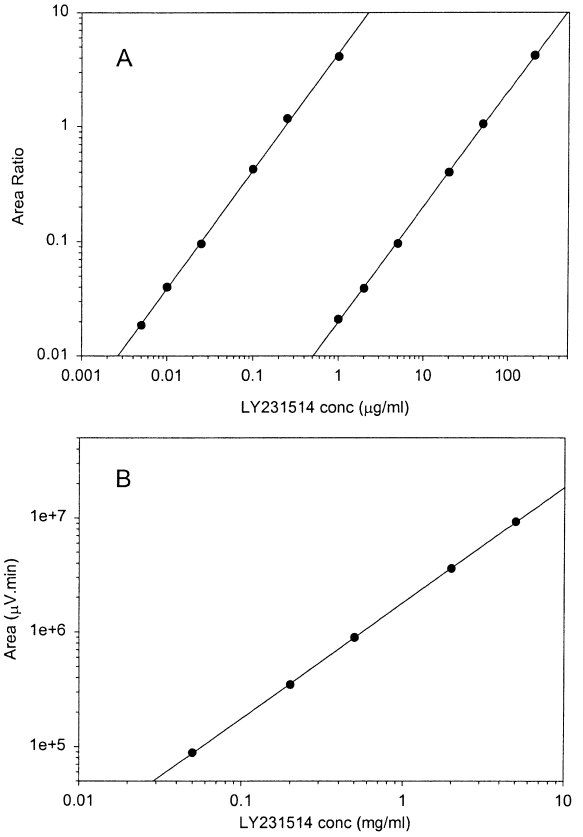

Fig. 3. (A) Low (left) and high (right) concentration calibration curves for pemetrexed (LY231514) in plasma. (B) External calibration curve for pemetrexed in urine as a function of initial concentration (urine was diluted 1/100 prior to assay).

ambient conditions (4 and −20°C) were not significantly different to those determined at time zero (Table 4). In spite of the potential risk of precipitation, stability was not dependent on pH. However, it should be noted that urine samples were vigorously agitated prior to dilution to avoid sampling from discrete layers (supernatant, sediment, etc.).

Table 1
Within-day and between-day precision (C.V.) and overall accuracy (%) of the determination of pemetrexed in human plasma during a 3-day validation ($n=5$ each day)

Concentration	C.V. (%)		Accuracy (%)
	Within-day	Between-day	
5 ng/ml	16.0	8.0	107.3
25 ng/ml	5.2	4.2	90.5
250 ng/ml	5.9	2.9	97.3
1 mg/ml	6.7	4.5	93.7
5 mg/ml	2.2	2.1	101.6
50 mg/ml	3.5	3.7	100.7

Table 2
Within-day and between-day precision (C.V.) and overall accuracy (%) of the determination of pemetrexed in human urine during a 3-day validation ($n=4$ each day)

Concentration	C.V. (%)		Accuracy (%)
	Within-day	Between-day	
0.05 mg/ml	4.6	2.3	100.0
0.2 mg/ml	2.7	2.4	98.7
0.5 mg/ml	1.2	0.6	97.4
2 mg/ml	1.1	2.2	101.2
5 mg/ml	2.2	1.1	100.9

The preliminary pharmacokinetic data indicate that pemetrexed has a distribution volume smaller than the extracellular water volume and that it is excreted rapidly and extensively into the urine. These results are in agreement to those published for a phase I study of pemetrexed using the same regimen [7]. Analysis of urine from patients with lower recoveries of pemetrexed did not reveal additional peaks not present in pre-treatment urine samples. In mice and dogs, the majority of urine radioactivity (90 and 68%, respectively) can be accounted for by LY231514 following administration of ^{14}C-labelled drug [8].

In summary, a sensitive HPLC method was developed to measure pemetrexed in plasma. This method was used successfully in a preliminary study

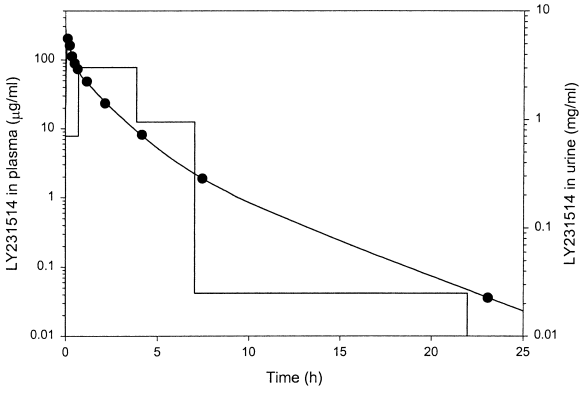

Fig. 4. Disposition of pemetrexed (LY231514) administered at a dose of 500 mg/m^2 as a 10-min intravenous infusion. The curve represents the best fit of the plasma concentration data (circles) by a tri-exponential equation. The urine concentrations of pemetrexed during the corresponding period are shown as a step-function.

Table 3
Pharmacokinetic data from five patients receiving pemetrexed at a dose of 500 mg/m^2 over 10 min

	Mean	C.V. (%)	Minimum	Maximum
CL (L/h/m^2)	2.27	28.7	1.36	3.26
Vd_{ss} (L/m^2)	5.17	21.17	3.49	6.68
$t_{1/2}$ a (h)	0.15	32.2	0.09	0.23
$t_{1/2}$ b (h)	0.98	12.5	0.76	1.11
$t_{1/2}$ c (h)	2.90	6.5	2.55	3.09
MRT (h)	2.35	15.3	1.81	2.78
% Urinary recovery (24 h)	81.7[a]	11.1	72.0	93.6

[a] $n=4$.

Table 4
Recovered pemetrexed after storage (0.5 mg/ml) under indicated conditions (mean±S.D.)

Time, temperature	pH of stored urine aliquot		
	4.5	6.4	7.8
8 h, ambient	0.54±0.023	0.51±0.004	0.51±0.008
48 h, 4°C	0.53±0.009	0.51±0.013	0.51±0.010
6 weeks, −20°C	0.46±0.030	0.50±0.02	0.49±0.005

of the disposition of pemetrexed in patients treated with 500 mg/m^2 every 3 weeks.

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