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Liquid-Liquid Extraction and HPLC-UV Method for Determination of Pralatrexate in Human Plasma

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Abstract

Pralatrexate, a novel antifolate chemotherapeutic agent, approved for the treatment of relapsed or refractory peripheral T-cell lymphoma (PTCL). The purpose of the present study was to develop a simple and sensitive high-performance liquid chromatographic method by UV detection for quantification of pralatrexate concentration in human plasma. After a liquid-liquid extraction with diethyl ether, pralatrexate and zidovudine (internal standard) are separated on YMC ODS C18 (250×4.6, 5µm) column using a simple binary mobile phase of methanol: water containing 0.1 % orthophosphoric acid (60:40, v/v). Samples were eluted isocratically at a flow rate of 0.6 ml/min and UV detection at 242 nm. The calibration was linear in the range 202.69-10134.3 ng/ml. Intra and inter-day coefficients of variation were less than 7 %. This method is selective, accurate and precise. It can be successfully applied for pharmacokinetics. Furthermore, the sensitive and simplicity of the method suggests the validity of method for routine clinical analysis.

Key words: Pralatrexate, Peripheral T-cell lymphoma, HPLC, Liquid-liquid extraction, human plasma.

1. INTRODUCTION

Peripheral T-cell lymphoma (PTCL), a subset of non-Hodgkin lymphoma (NHL), comprises a spectrum of rare and usually aggressive T-cell disorders with a generally poor prognosis. Pralatrexate (PDX) (Figure 1) is an antimetabolite for the treatment of relapsed or refractory peripheral T-cell lymphoma. It competitively inhibits dihydrofolate reductase (DHFR) and thymidylate synthase. Subsequent depletion of thymidine monophosphate (TMP) occurs so that the cancer cell is unable to synthesize DNA and RNA. As a result, the cancer cell cannot proliferate and is forced to undergo apoptosis [1-3]. Pralatrexate is more effective against cells that are actively dividing. Several clinical trials have quantitatively measured the pralatrexate in plasma for pharmacokinetic analyses, either as single-agent therapy [4] or in combination with such drugs as carboplastin [5], romidepsin [6], and 5-flurouracil [7].

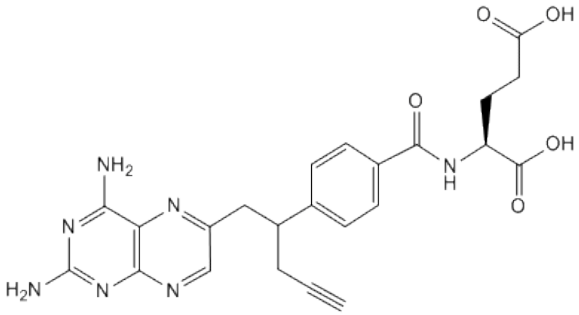

Fig. 1: Chemical structure of Pralatrexate

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods are currently available to determine pralatrextae in human plasma [6]. However, these methods were not officially published. LC-MS compatible HPLC method was developed for the identification and characterization of injection degradation products [8]. Such equipment is not available in most clinical laboratories; high performance liquid chromatography (HPLC) with ultraviolet detection represents an alternative. To our knowledge, no HPLC method has been still published for the determination of pralatrexate concentration in human plasma. The aim of this study was to develop and validate an accurate, precise and sensitive HPLC method to monitor pralatrexate in human plasma.

2. MATERIALS AND METHODS

Chemicals and reagents

Pralatrexate (purity 98.00% w/w) was purchased from Gland Pharma Limited, Hyderabad, India. Zidovudine (used as internal standard, purity 99.5% w/w) is purchased from (Hetero drugs Ltd., Hyderabad, India). Methanol, acetonitrile (HPLC grade), Orthophosphoric acid (AR grade) and diethyl ether were purchased from Merck Ltd., Mumbai, India. All the other reagents used were analytical grade. All aqueous solutions were prepared using deionized water, processed through a Milli-Q water purification system (Millipore, USA). Human k_3 EDTA plasma was collected from Sri Laxmi Sai Clinicals, Hyderabad, India. Methanol: Water (50:50 v/v) used as diluent in experimentation.

Instrument and Chromatographic conditions

The chromatographic system consisted of a Shimadzu Class VP Binary pump LC 10ATvp, SIL-10ADvp Auto sampler, CTO-Avp Column Temperature Oven, SPD-10Avp UV Detector. All the components of the system were controlled using SCL-10Avp system controller. Data acquisition was done using LC Solutions software. The detector is set at a wavelength of 242 nm. Chromatographic separations were accomplished using an YMC ODS C18, 250 × 4.6 mm i.d., 5 µm column. The mobile phase was composed of methanol-water containing 0.1% ortho-phosphoric acid (60:40 v/v). The mixture was

filtered through 0.22 µm membrane (Millipore, Bedford, MA, USA) under vacuum, and then degassed by flushing with nitrogen for 5 min. The mobile phase was pumped isocratically at a flow rate of 0.6 ml/min during analysis, at ambient temperature.

Preparation of stock solutions, plasma calibration standards and quality controls

The stock solution containing 1 mg/ml of pralatrexate (PDX) were prepared in sodium hydroxide (0.01 N) and 1 mg/ml of zidovudine (IS) were prepared in methanol and stored at 4 ºC. From stock solution, further dilutions were made with diluent to get working standards with concentrations of 4.05, 8.11, 40.54 121.61, 162.15, 202.69 µg/ml. Aliquots of 475 µL of blank human plasma were spiked with 25 µL of the working standard solutions to get calibration curve standards containing 202.69-10134.3 ng/ml of pralatrexate. Each of these standard solutions were distributed into disposable polypropylene micro centrifuge tubes (2.0 ml, eppendorf) and the tubes were stored at -20 ºC until analysis. The quality control samples were similarly prepared in plasma such that the final concentrations were 202.69, 608.06, 4940.47, 7144.68 ng/ml, respectively and labeled as lower limit of quantification (LLOQ), low quality control (LQC), median quality control (MQC) and high quality control (HQC). Finally, a working solution of IS (25 µg/ml) was also freshly prepared with diluent.

Sample preparation

The extraction of the plasma samples involved liquid-liquid extraction (LLE) process. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 200 µL was then transferred to prelabeled 2.0 ml polypropylene centrifuge tubes. Internal standard solution, 25 µL (25 µg/ml) was then added and mixed. Extraction solvent, 0.6 ml, was then added to extract the drug and internal standard. After an agitation of 10 min with a mechanical shaker, the tubes were centrifuged 10 min at 4000 rpm at room temperature. The supernatant was transferred into prelabeled polypropylene tubes and was allowed to evaporate to dryness under nitrogen at constant temperature of 40 ºC. The dried residue was then dissolved in 200 µL of mobile phase and volume of 20 µL was injected into system for analysis.

Method validation

The validation of an analytical method confirms the characteristics of the method to satisfy the requirements of the application domain [9]. The initial assay was fully validated for PDX analysis in human plasma according to FDA guidelines [10].

Selectivity

The interference by the endogenous compound was assessed by comparing the chromatograms obtained from the samples containing pralatrexate and the internal standard with those obtained from the blank samples.

Linearity and Lower Limit of Quantification

Qualitative analytical results are highly influenced by the quality of the calibration curve. Six different concentrations of pralatrexate with fixed concentration of IS in blank plasma were processed and the linearity of the calibration curve for pralatrexate was assessed in the range of 202.69-10134.3 ng/ml in the plasma samples. The calibration curve was plotted between the ratio of the peak areas of pralatrexate to IS and concentration of pralatrexate. The straight line regression was presented with its correlation coefficient.

Lower limit of quantification (LLOQ) was defined as the lowest concentration that could be measured with an inter-day coefficient of variation (CV) of < 20 % and accuracy between 80 and 120 %.

Precision and accuracy

The intra and inter-day precision of the assay was assessed by calculating the coefficients of variation (% CV) and accuracy was determined by comparing the calculated concentrations to known concentration with calibration curves. Intra and inter-day precision and accuracy were evaluated by analysis of QCs at four levels (LLOQ, LQC, MQC and HQC) of six replicates each.

Recovery

The recovery of pralatrexate was calculated by comparing the peak area of the analyte from the extracted plasma standard with that obtained from an unextracted standard at the same concentration for the quality control samples containing 608.06, 4904.47, 7144.68 ng/ml for pralatrexate. Internal standard recovery was tested (25 µg/ml) by comparing six extracted and un-extracted samples at each concentration.

Stability studies

The stability of pralatrexate was determined by measuring concentration change in control sample overtime. Stock solution stability was performed by comparing the area response of analyte and internal standard in the stability sample, with the area response of sample prepared from fresh stock solution. The stability of stock solutions were tested and established at room temperature for 6 h and under refrigeration (2−8 ºC) for 15 days. The quality control standards containing 608.06 and 7144.68 ng/ml of pralatrexate were subjected for detection of stability of the drug in plasma. Samples were retrieved from the deep freezer after freeze-thaw cycles and frozen at -20 ºC in three days. Bench top stability studied for 6 h period at room temperature with control concentrations. Auto-sampler stability was studied following 48 h at 4 ºC storage period. The amount of the drug in the stability samples was estimated and the % nominal concentration and coefficient of variation (% CV) were calculated.

3. RESULTS AND DISCUSSION

Method optimization was achieved by monitoring varying chromatographic conditions in terms of appropriate chromatographic columns, mobile phases and their constitution, extraction solvents, and solvent for

reconstitution before arriving at suitable conditions that gave satisfactory results. The development of the method started with the evaluation of three chromatographic columns against different composition of mobile phase system to identify a suitable stationary phase. Both Agilent Zorbax C18 and YMC C18 columns resulted in sharp peaks, while a phenomenex C18 (150 × 4.6 mm, 5 µm) column produced broad peaks. The YMC C18 was eventually chosen based on the peak symmetry and retention time obtained with the optimization of the mobile phase. The choice of mobile phase for analysis is important in order to achieve good resolution between the peaks and to produce analyte with distinct sharp peaks without interference from endogenous substances. It was observed that a mobile phase consisting of methanol: water containing 0.1% orthophosphoric acid solution was most appropriate for good resolution, elution and peak shape. Extraction methods were initially attempted using protein precipitation technique. Organic solvents such as methanol and acetonitrile were used as reagents for protein precipitation. It was found that the analyte recovery was less with these precipitating agents. When liquid-liquid extraction was performed using different solvents (tert-butyl methyl ether, ethylacetate, dichloromethane, hexane and diethylether), it was observed that both the analyte and IS were extracted and good recovery was obtained with diethyl ether. The ability of LLE to produce a much cleaner extract compared to protein precipitation technique and relatively inexpensive when compared to other sample preparation methods [11]. Once the chromatographic and analytical conditions were optimized, the choice of IS was problematic. Since structurally related compound were tested. Either the extraction yield or the chromatographic retention, were not adequate. Hence various structurally unrelated compounds such as valsartan, telmisartan, olaparib, paracetamol and zidovudine were tested. Zidovudine as an internal standard was easily separated and eluted along with the analyte. The best resolution and sensitivity of the method was obtained at 242 nm with 0.6 ml/min flow rate of the mobile phase.

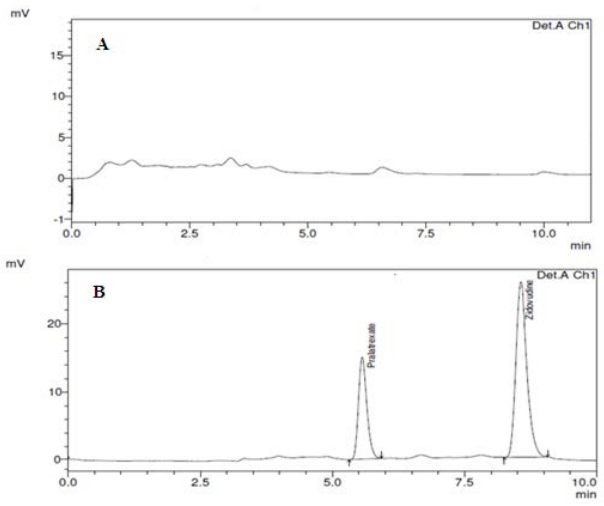

Fig. 2: Chromatogram of (A) extracted blank plasma sample (B) plasma spiked with pralatrexate and zidovudine

Selectivity

Figure 2 shows typical chromatograms of blank plasma in comparison to plasma spiked with PDX and the internal standard. The retention times of the PDX and internal standard were 5.55 and 8.80 min, respectively. No endogenous compounds appear at the retention time of pralatrexate and internal standard to interfere with their peaks. The base line was relatively free from drift.

Linearity

The calibration curves were obtained by plotting the peak area ratio of PDX to IS against the concentrations of PDX. The linearity of the calibration curve showed good reproducibility and found to be linear over an analytical range of 202.69-10134.3 ng/ml of pralatrexate with the regression coefficient value of 0.9989 (Figure 3). A typical calibration plot obtained during plasma analysis could be described by the linear equation, y = 0.00006 x - 0.0038, where y is peak area and x is concentration (ng/ml).

Limit of detection and limit of quantification

Limit of detection (LOD) was defined as the lowest concentration that produces a peak distinguishable from background noise (minimum ratio of 3:1). The approximate LOD was 20 ng/ml. The LLOQ has been accepted as the lowest points on the standard curve with a relative standard of less than 20 % and signal to noise ratio of 5:1. Results at lowest concentration studies (202.69 ng/ml) met the criteria for the LLOQ (Table 1).

Table 1: Linear regression data for calibration curve (n = 3).

Parameter	Values
Absorption maxima (nm)	242
Linearity range (ng/ml)	202.69-10134.3
Quantification limit (ng/ml)	202.69
Linear regression equation	Y = 0.00006x-0.0038
Correlation coefficient	0.9989
Slope	0.00006
Intercept	0.0038

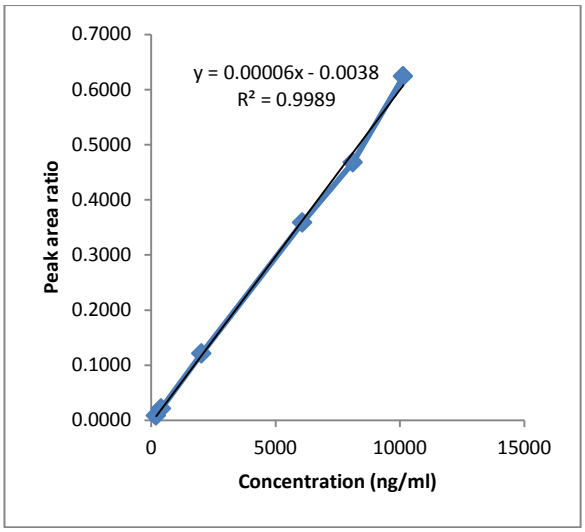

Fig. 3: Calibration curve of pralatrexate

Table 2: Intra-day and Inter-day precision and accuracy (n = 6)

	Intra-day			Inter-day		
Conc. Added (ng/ml)	Conc. calculated	% CV	% Accuracy	Conc. calculated	% CV	% Accuracy
202.69	190.23	3.03	93.85	191.52	1.32	94.48
608.06	645.66	5.37	106.18	653.62	5.20	107.49
4940.47	4780.97	3.76	97.48	4761.8	2.67	97.09
7144.68	7586.76	2.62	106.18	7597.34	2.40	106.33

CV: Coefficient of variation

Precision and Accuracy

The precision and accuracy data of the method at four different concentrations are shown in table 2. Intra and inter-day % CV values was less than 7 % and obtained mean concentration was within 15% of the nominal values for the QC samples, except for the LLOQ were within 20%. Acceptance criteria for precision and accuracy were met in all cases.

Recovery

The mean ± S.D. absolute recovery values of pralatrexate at 608.06, 4904.47 and 7144.68 ng/ml were 51.13 ± 2.86%, 66.53 ± 2.52% and 67.47 ± 1.49 %, respectively. The overall mean recovery of the IS was 39.74 ± 4%. The results were summarized in the table 3.

Table 3: Recovery of pralatrexate at three concentrations (n = 6)

Concentration (ng/ml)	Mean Recovery ± SD	% CV
608.06	51.13 ± 2.86	5.60
4904.47	66.53 ± 2.52	3.79
7144.68	67.47 ± 1.49	2.21

SD: Standard deviation; CV: Coefficient of variation.

Carryover effect

A critical issue with the analysis of many drugs is their tendency to get adsorbed by reversed phase octadecyl-based chromatographic packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank plasma solutions were injected immediately following the highest calibration standard.

Table 4: Results of stability studies for pralatrexate (n = 3).

QC Level	Freeze-thaw stability		Stability at 4 º C		Stability at RT	
	% Nominal	% CV	% Nominal	% CV	% Nominal	% CV
LQC	94.86	5.79	95.3	3.69	101.24	2.46
HQC	101.3	6.11	102.3	2.67	104.96	4.96

CV: Coefficient of variation.

Stability studies

The stock solutions of pralatrexate and zidovudine stored for 6 h and at 2−8 ºC for 15 days were compared to the freshly prepared solution, the CV % for pralatrexate and zidovudine was ≤ 1.20, indicating that stock solutions were stable at least for 15 days. The results of freeze thaw, bench top and autosampler stability are presented in table 4. From the stability studies, it can be concluded, that for each stability study, % nominal concentration was between 93 and 105 and % CV was less than 15, which confirm the stability of pralatrexate in plasma samples under different storage conditions. It revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

4. CONCLUSION

A simple, economical, sensitive, accurate HPLC method was developed and validated for determination of pralatrexate in human plasma using liquid-liquid extraction. The method involves simple extraction procedure, short analysis time, small plasma volume (0.2 ml), good precision and reproducibility. Hence the proposed method can be used for routine bioanalysis of pralatrexate from plasma to support pharmacokinetic and bioequivalence studies.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper

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