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Determination of LY295501 in human plasma by reverse phase HPLC with UV detection

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Abstract

A high performance liquid chromatographic (HPLC) assay was developed and validated for the quantitative determination of LY295501 in human plasma. A structural analog, LY186641, was selected as the internal standard. The samples were processed by protein precipitation with acetonitrile followed by concentration of the supernatants and reconstitution. Chromatographic resolution of LY295501 from endogenous plasma components was accomplished with a Waters Novapak C18 HPLC column (3.9 × 150 mm, d_p 4 mm). Detection was by absorbance at 260 nm. The linear dynamic range was from 5 to 400 μg ml^{-1} of human plasma using a 0.25 ml aliquot. The inter-day precision (%RSD) and accuracy (%RE) in plasma ranged from 2.4 to 4.7, and −4.9 to 1.4, respectively. This assay is both simple and rapid, and has been used to successfully analyze over 1500 samples from human clinical trials.

Keywords: LY295501; Sulfonylurea; Anti-tumor drug; High performance liquid chromatography; Reverse phase chromatography; UV detection

1. Introduction

Compound LY295501 (Fig. 1) is one of a series of sulfonylureas that has shown potent in vivo antitumor activity in mouse models and is currently being evaluated in Phase 1 clinical trails. LY295501 is a novel oncolytic agent [1–3] that is related to the compound sulofenur [4–7]. LY295501 shows the same broad spectrum of activity as sulofenur in mouse tumor models, but is significantly more potent in cell cytotoxicity assays [1–3].

Previous methodology used in the determination of LY295501 was presented by Ehlhardt et. al. [8], in a paper that discussed the disposition and metabolism of LY295501 in mouse, rat, and monkey. This high performance liquid chromatography (HPLC) method used a Zorbax RX-C8 column and an acetonitrile protein precipitation procedure to determine LY295501 in the animal matrices listed, validation data were not presented. Modification of this assay reduced the instrument run time from 20 to 8 min, and the assay was validated to determine LY295501 concentrations in human plasma.

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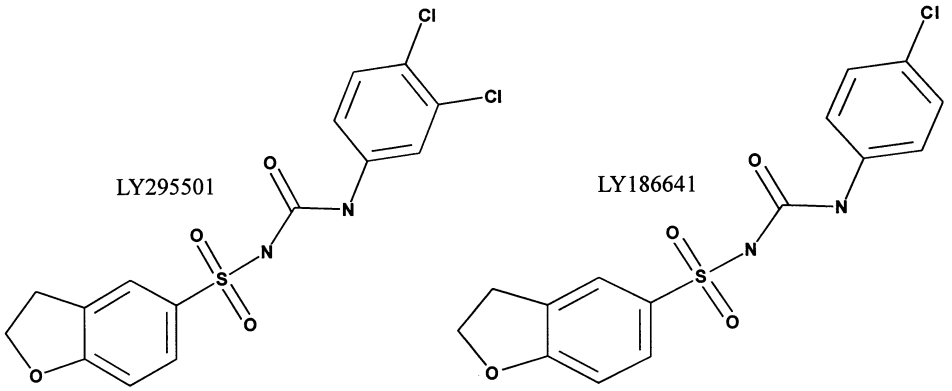

Fig. 1. Chemical structures of LY295501 and LY186641 (internal standard).

In this article, an HPLC assay is presented for the quantitative determination of LY295501 in human plasma. A structural analog, LY186641, was used as the internal standard (Fig. 1). This method was developed to analyze study samples from human clinical trials [9]. Based upon the results of initial clinical studies, this assay was validated over the concentration range of 5–400 μg ml^{-1}.

2. Experimental

2.1. Chemicals and materials

Compounds LY295501 and LY186641 (internal standard) were obtained from Eli Lilly and Company (Indianapolis, IN). Control human plasma was obtained from human health services at Eli Lilly and Company. HPLC grade acetone and acetonitrile were obtained from Mallinckrodt (Paris, KY), and water was from a Milli-Q system. Analytical reagent grade phosphoric acid (85% w/w) and Chempure brand sodium hydroxide (5.0 N) was from Curtis Matheson Scientific (Houston, TX).

2.2. HPLC equipment and analytical conditions

The HPLC system consisted of the following components: two Shimadzu LC-10AD pumps; SCL-10A system controller; GT-104 solvent degasser; and SIL-10A autosampler. Detection of the analytes was accomplished with a Kratos Spectroflow 783 programmable absorbance detector adjusted to 260 nm. The chromatographic system consisted of a Waters Novapak C18 HPLC column (3.9 × 150 mm, d_p 4 μm) and used a mobile phase, delivered isocratically at 1 ml $min.^{-1}$, that consisted of the following components: A-sodium phosphate buffer (pH 7.0; 20

Table 1
Intra-day validation statistics for LY295501 in human plasma

Day	Parameter	Validation sample level (μg ml^{-1})		
		5	50	400
1	Average (ng ml^{-1})	4.8	49.3	400.6
	Accuracy (RE%)	−3.2	−1.4	0.1
	Precision (RSD%)	6.0	5.2	2.0
	n	5	5	5
2	Average (ng ml^{-1})	5.0	51.5	373.4
	Accuracy (RE%)	0.0	3.0	−6.7
	Precision (RSD%)	1.1	0.4	0.5
	n	5	5	5
3	Average (ng ml^{-1})	4.6	51.3	366.8
	Accuracy (RE%)	−7.6	2.6	−8.3
	Precision (RSD%)	8.0	1.9	1.5
	n	5	5	5

Table 2
Inter-day validation statistics for LY295501 in human plasma

Day	Parameter	Validation sample level (μg ml^{-1})		
		5	50	400
1–3	Average (ng ml^{-1})	4.8	50.7	380.3
	Accuracy (RE%)	−4.0	1.4	−4.9
	Precision (RSD%)	4.2	2.4	4.7
	n	15	15	15

mM); B-acetonitrile (70:30, v/v). The autosampler was setup to inject 10 μl sample aliquots every 8 min.

Data collection and manipulation was performed using Perkin Elmer's Access* Chrom data system.

2.3. Standard solutions

A standard stock solution of LY295501 was prepared by dissolving ≈50 mg of LY295501 in acetone such that the final concentration was 10 mg ml^{-1}. This solution was diluted to give a series of working solutions with concentrations of 8000, 2000, 1000, 500, 250, 125, 100, 50, and 25 μg ml^{-1}. The working solutions were diluted with sodium phosphate buffer (pH 7.0, 20 mM)-acetone (50:50, v/v). The standard stock solutions were prepared in duplicate from separate weighings, one set was used to prepare standard samples and one set was used to prepare either validation or quality control (QC) sample pools. A standard stock solution of LY186641 (internal standard) was prepared by dissolving ≈5 mg of LY186641 in acetone so that the final concentration was 0.5 mg ml^{-1}. The working solutions were placed in polypropylene vials and stored at approximately 4°C when they were not in use. Under these conditions, the working solutions were stable for at least 16 days.

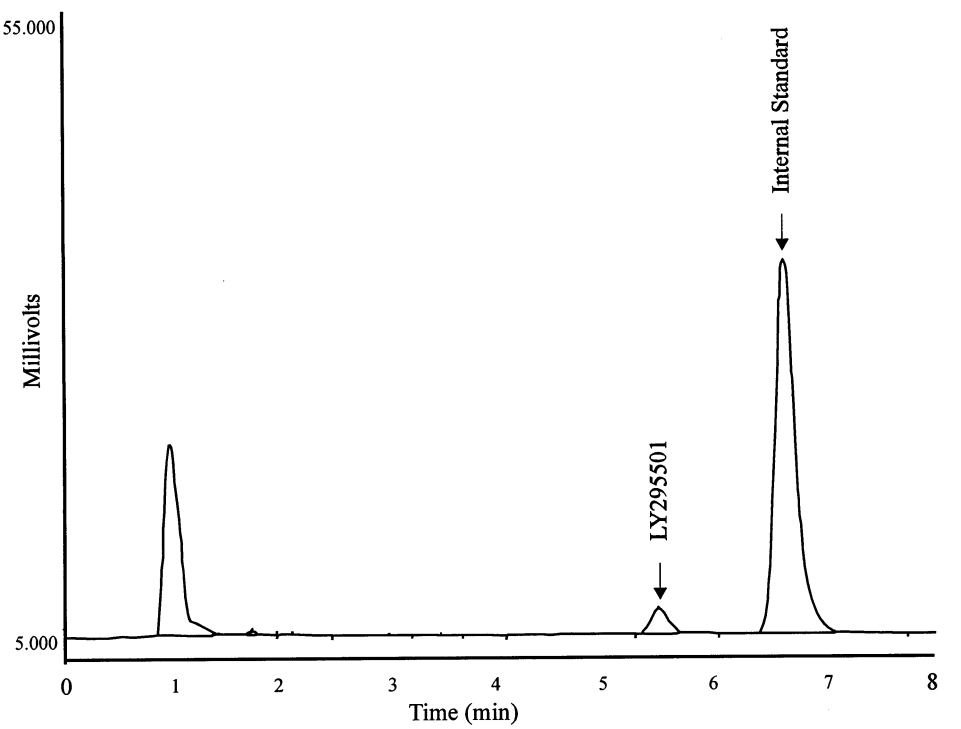

Fig. 2. Representative chromatogram of a 5 μg ml^{-1} standard extracted from human plasma.

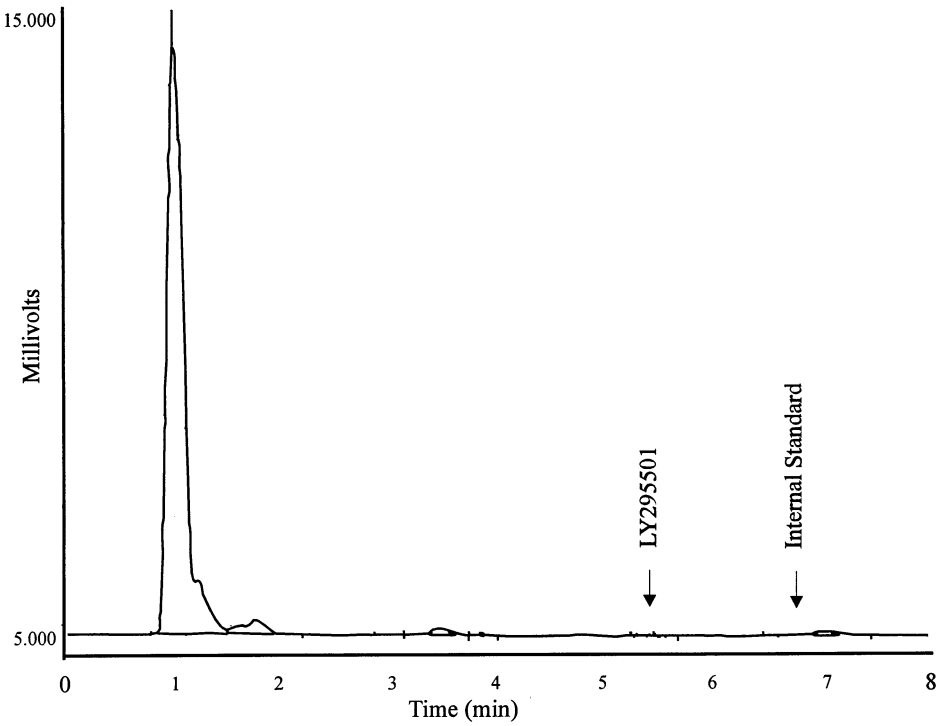

Fig. 3. Representative chromatogram of a double blank extracted from human plasma.

2.4. Sample preparation

The standard curve samples were prepared by placing 250 μl of plasma into a polypropylene centrifuge tube and spiking 50 μl of the appropriate standard working solution followed by 50 μl of the internal standard working solution. Standards were prepared for each analysis during the validation and study sample analysis at the following concentrations: 400, 200, 100, 50, 25, 10, and 5 μg ml^{-1}.

The validation samples, used to evaluate accuracy and precision during the validation, and the QC samples, used during the analysis of study samples, were prepared by spiking known quantities of LY295501 into control human plasma. During the validation, five replicates at each of the following concentrations were prepared and analyzed on each of 3 days: 400, 50, and 5 μg ml^{-1}. During the analysis of study samples, QC samples were prepared in duplicate (or more) at the following concentrations and run with each analysis: 400, 50, and 5 μg ml^{-1}. The validation and QC samples were prepared by spiking 0.5 ml of the appropriate stock solution into a 10 ml volumetric flask and diluting to mark with control human plasma. Aliquots (0.25 ml) were placed in polypropylene centrifuge tubes and stored at −70°C until analysis.

A double blank sample (no internal standard or standard) was prepared and run with each analysis and was included in both validation and study sample analyses. The double blank was prepared by spiking 50 μl of working solution diluent (see Section 2.3) into 250 μl of control human plasma.

A blank sample (no standard) was prepared and run with each analysis and was included in both validation and study sample analyses. The blank was prepared by spiking 50 μl of working solution diluent and 50 μl of the internal standard working solution into 250 μl of control human plasma.

The study samples were prepared by adding 250 μl of the sample, 50 μl of working solution dilu-

ent, and 50 μl of the internal standard working solution to a polypropylene centrifuge tube.

All samples were extracted by adding 2 ml of acetonitrile followed by vortexing, and centrifugation at $\approx 3000 \times g$ for 15 min. The sample supernatants were concentrated to dryness using a Savant SpeedVac, and the dry residues were reconstituted by adding 250 μl of acetonitrile and 500 μl of sodium phosphate buffer (pH 7.0, 20 mM). Finally, the samples were transferred to 1.5 ml Waters Ultrafree-MC centrifuge filters and centrifuged at $\approx 3000 \times g$ for 5 min. The filtered samples were transferred to autosamplers vials and injected onto the HPLC for analysis.

2.5. Calibration

One replicate of each of the following LY295501 concentrations: 5, 10, 25, 50, 100, 200, and 400 mg ml^{-1} was prepared and run at the beginning of each analysis. The peak area ratio of LY295501 to the internal standard, LY186641, was related to concentration using a linear regression with $1/Y^2$ weighting. During the validation, the residual error (back calculated standards) of the calibration graph was better than $\pm 10\%$ at all concentrations, and the correlation coefficients were at least 0.997.

2.6. Validation procedures

Validation samples were prepared and analyzed to evaluate the intra-day and inter-day accuracy and precision of the analytical method in human plasma. A total of five replicates of each of the validation concentrations (5, 50, and 400 μg ml^{-1}) were analyzed along with a set of standard samples and blanks on each of 3 days using the same instrument.

The extraction efficiency of LY295501 and the internal standard was determined by comparing the peak areas of extracted samples to the peak areas of extracted blanks spiked with standard and internal standard. This was performed by

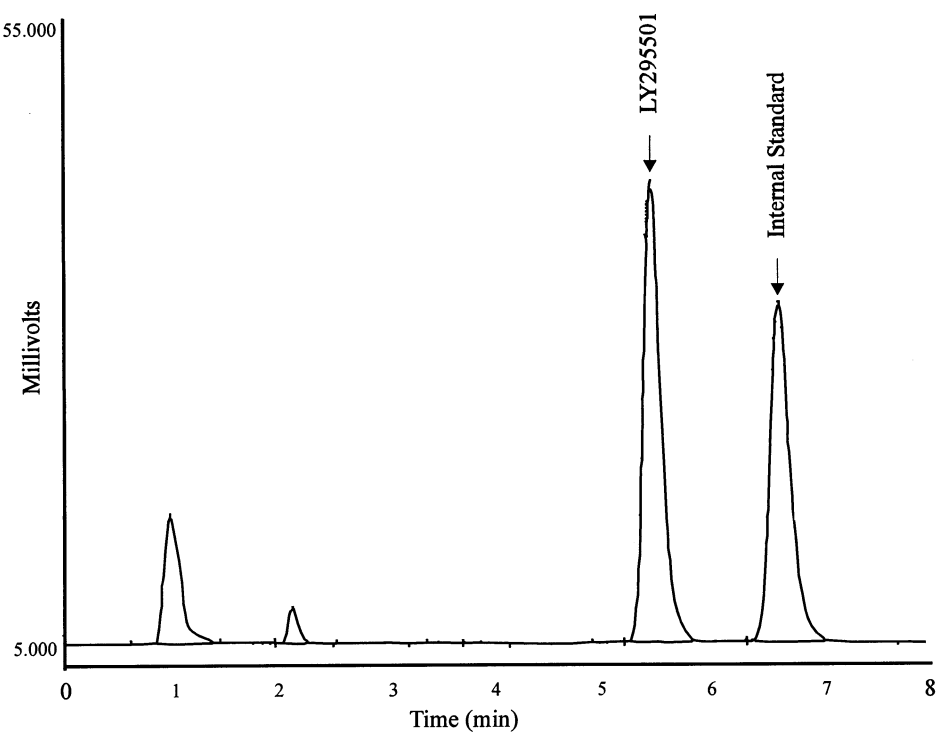

Fig. 4. Representative chromatogram of a study sample from a human clinical trial.

analyzing three replicates at each of two concentrations, 5 and 400 μg ml^{-1} (the internal standard concentration was 100 μg ml^{-1}). The selectivity of the assay was investigated by processing and analyzing blanks prepared from six independent lots of control plasma; the blanks were surveyed for interfering peaks.

The stability of LY295501 in human plasma was studied under a variety of storage and process conditions. The freezer stability (-70°C) was evaluated by preparing three validation samples at 5, 50, and 400 μg ml^{-1} and analyzing them after being stored at -70°C for a period of 1, 2, and 4 weeks. The stability of the analytes in the injection solvent (extract stability) was studied to verify that the compounds would not degrade over the course of an analysis. This was accomplished by extracting validation samples, storing them overnight at room temperature, and injecting them onto the HPLC system the following day with a fresh standard curve. The effect of three freeze/thaw cycles on the stability of the analytes was determined by analyzing triplicate validation samples at each concentration after 1, 2, and 3 freeze/thaw cycles. Room temperature stability was studied by analyzing triplicate validation samples at each concentration after being incubated in the biological matrix, prior to extraction, for a period of 4 h. Finally, the stability of the stock solutions was tracked by analyzing a neat system suitability sample prior to each analysis at 50 μg ml^{-1}. The same stock solutions were sampled for over 2 weeks to ensure instrumental accuracy and sensitivity for the day, as well as stock solution stability.

3. Results and discussion

3.1. Assay precision and accuracy

The data for the intra-day assay precision and accuracy for human plasma, determined by analyzing five replicates at 5, 50, and 400 μg ml^{-1} on each of 3 days, are reported in Table 1. The inter-day assay precision and accuracy data for human plasma are reported in Table 2. The accuracy of the method was determined by calculating the percent relative error (%RE), and the precision was determined by calculating the percent relative standard deviation (%RSD).

In human plasma, the inter-day precision ranged from 2.4 to 4.7, and the inter-day accuracy ranged from -4.9 to 1.4, over the three concentrations evaluated.

3.2. Stability

Standard stock solutions of LY295501 and the internal standard were found to be stable for 16 days when prepared as described and stored in polypropylene vials at ≈ 4°C when not being used. Also, LY295501 was found to be stable at ≈ -70°C (in human plasma) for at least 30 days.

No degradation of LY295501 or the internal standard was observed in the reconstitution solvent during the period the samples were on the autosampler waiting to be injected (up to 24 h), during three freeze/thaw cycles, or during incubation at room temperature (up to 4 h).

3.3. Assay specificity

The specificity of the assay was demonstrated by the absence of endogenous substances, in drug free matrices, that interfere with the quantitation of LY295501 at the lower limit of quantitation (LLOQ). In addition, no obvious interfering peaks were observed during the analysis of over 1500 study samples. Representative chromatograms of a 5 μg ml^{-1} standard, a double blank, and a study sample from a human clinical trial are presented in Figs. 2–4, respectively.

3.4. Limits of quantitation and extraction efficiency

The lower and upper limits of quantitation (LLOQ and ULOQ) are defined as the lowest and highest concentrations on the calibration graph at which an acceptable accuracy of at least $100 \pm 20\%$ {(mean assay conc./theoreti-

cal) × 100} and precision of at least 20% (RSD) were obtained. The LLOQ and ULOQ of this assay are 5 and 400 μg ml^{-1}, respectively.

The extraction efficiency of LY295501 was ≈ 83%, and the extraction efficiency of the internal standard, compound LY186641, was ≈81%.

4. Conclusion

This article describes a validated HPLC assay for the determination of LY295501 in human plasma. This assay has been used to support phase 1 human clinical trials, and has been successfully applied to the analysis of over 1500 samples. Sample preparation involves a simple and rapid acetonitrile protein precipitation procedure, and requires only 0.25 ml of human plasma. It would be easy to analyze 100 samples, including standards and QCs, in a single day. The freezer, freeze/thaw, analysis, and room temperature stability of LY295501 was investigated, and no significant degradation was observed when the outlined procedures were followed. This assay is both accurate and precise, and no interfering peaks were observed while investigating assay specificity or during study sample analysis.

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