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High-performance liquid chromatographic analysis of new triazole antifungal agent SYN-2869 and its derivatives in plasma☆

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Abstract

A simple reversed-phase high-performance liquid chromatography (HPLC) method with UV detection was developed and validated for the quantitation of SYN-2869, a novel triazole antifungal agent and its analogs in rat plasma. The method involved a simple precipitation of plasma protein with acetonitrile (1:10 ratio). The reconstituted sample after evaporation to dryness was injected onto a HPLC column. SYN-2869 and its analogs were separated from the matrix components on a symmetry C18 column using an aqueous mobile phase of acetonitrile and water with a flow rate of 1 ml min^{-1}. A step gradient of 40–80% acetonitrile eluted all four compounds. The run time was 30 min. The linear range was 0.5–10 μg ml^{-1} ($r^2 > 0.999$). The limit of quantitation was 0.5 μg ml^{-1}. The inter-day precision and accuracy for SYN-2869 standard concentration were from 1.9 to 8.5% and from −1.4 to +4.4%, respectively. The precision and accuracy of intra-day quality control samples were from 4.6 to 5.2% and from 4.6 to 12%, respectively.

Keywords: Azoles; Antifungals

1. Introduction

SYN-2869, (2R,3R)-2-(2,4-difluorophenyl)-1-(1H-1,2 triazole-1-yl)-3-[4-{4-[2-(4-trifluromethoxy-benzyl)-2H-1,2,4-triazol-3-one-4-yl] phenyl} piperazin-1-yl] butan-2-ol, is a wide spectrum orally active antifungal agent that is being evaluated for treatment against *Candida* and *Aspergillus* infections particularly in pulmonary invasive aspergillosis [1]. SYN-2869 and its derivatives exhibits a wide spectrum of antifungal activity in vitro, comparable to itraconazole and other leading azoles. Efficacy of an Itraconazole–cyclodextrin solution against *Aspergillus fumigatus*

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has been assessed in an immunosuppressed, temporarily leukopenic rabbit model of invasive aspergillosis [2]. Hydroxypropyl-β-cyclodextrin formulated SYN-2869 or its amorphous form, made by co-precipitation with polyvinylpyrrolidone K 30 and Polyethylene glycol (Average molecular weight, 3350), were well absorbed and exhibited efficacy in systemic and invasive mycoses, especially against lung invasive aspergillosis. The efficacy of the formulated SYN-2869 was observed to be significantly superior to that of the cyclodextrin formulated itraconazole, which is the most orally absorbable form of this compound [3]. Several methods have been reported on itraconazole analysis, a triazole antifungal drug involving liquid–liquid extraction [4,5]. The objective of this study was to develop and validate a simple assay for determination of SYN-2869 and analogs in this new series of novel triazole antifungal agents in plasma suitable for further preclinical and clinical pharmacokinetic studies (Fig. 1).

2. Experimental

2.1. Chemical and reagents

SYN-2869 and its derivatives SYN-2836, SYN-2903, SYN-2921 and the internal standard SYN-2506 were synthesized at SynPhar Laboratories (Edmonton, AB, Canada). Unless stated otherwise, all solvents and reagents used were of HPLC grade. Frozen heparinized control rat plasma was supplied by Harlan.

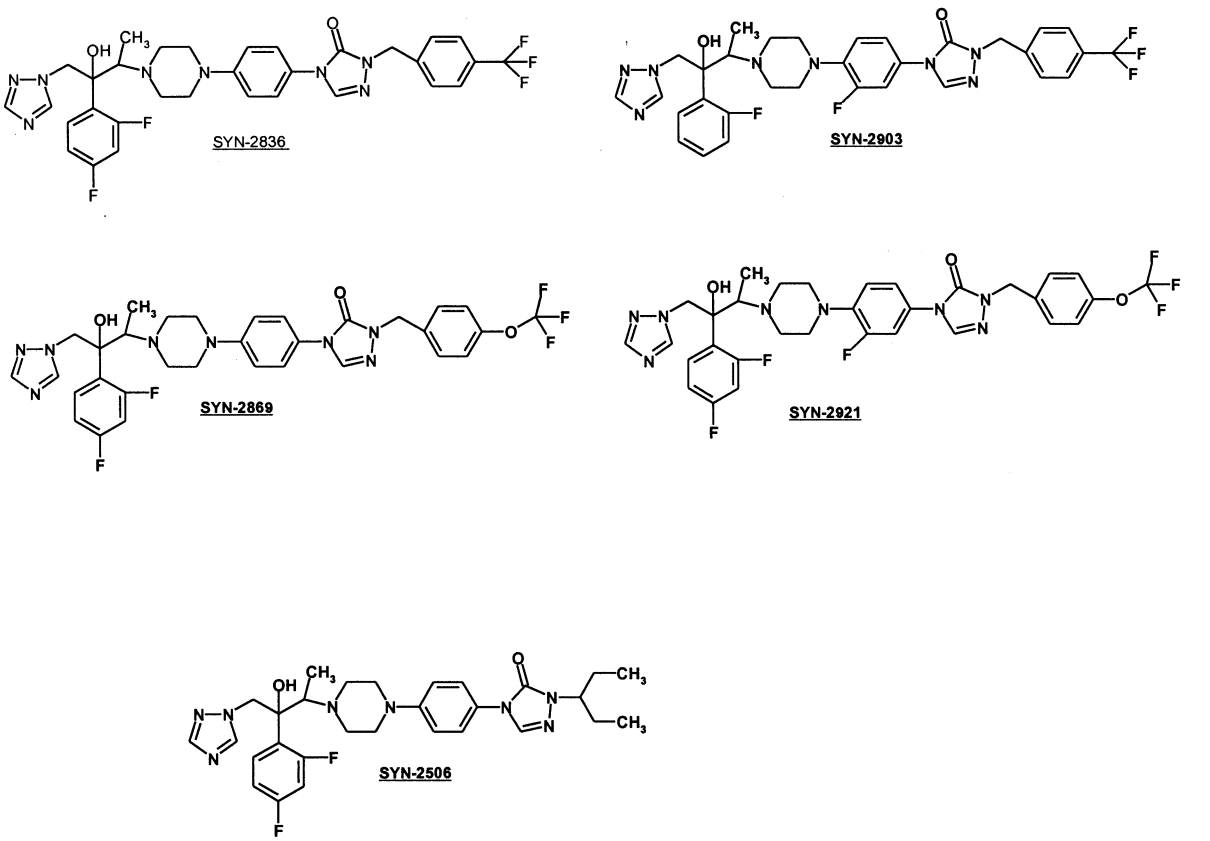

Fig. 1. Structure of novel triazole antifungal agent SYN-2869 and its derivatives.

2.2. Standard solutions

Primary standard solutions of SYN-2869, SYN-2836, SYN-2903 and SYN-2921 were made in acetonitrile of nominal concentrations of 1 mg ml^{-1}. Working standard solutions containing a mixture of SYN compounds and internal standard SYN-2506 were used for the preparation of plasma standards. These were prepared by diluting aliquots from each stock solution with acetonitrile to yield nominal concentrations over a range 0.1–25 μg ml^{-1}. All primary and working stock standard solutions were stored at 4°C. Plasma standard solutions were prepared daily by adding 10–25 μl of working standard solutions (concentration range 1–100 μg ml^{-1}) to 100 μl of plasma. The samples were vortexed for 5 min to incorporate all components. Quality control samples (QCs) were prepared from separate weighing and sub stock solution of 1 and 10 μg ml^{-1} was used to produce concentration of 0.25, 0.5 and 1.0 μg ml^{-1} in plasma.

2.3. Sample collection and processing procedures

For intravenous administration, the mice were put under anesthesia for 5 min by making them inhale methoxyflurane (Metofane®). After securing the animal properly, absolute alcohol was applied on the tail to dilate the vein and disinfect the injection site. After 1 min of application of alcohol, mild pressure was applied with the index finger near the base of the tail over the vein to secure it properly and the needle was then gently introduced into the vein and solution was injected slowly. The syringe used was a 1-ml graduated hypodermic syringe with a 27.5 gauge disposable needle. For oral administration, the mice were not anesthesized and fasted overnight. At each time point (5, 15, 30 min and 1, 2, 4, 6, 8, 12, 16 and 24 h), three animals were euthanised by carbon dioxide and immediately blood was removed and plasma samples were obtained and stored at −20°C until analysed. Sprague-Dawley rats were dosed through the jugular vein and the same site was also used for withdrawing blood samples (5, 15, 30 min and 1, 2, 4, 6, 8, 12, 16 and 24 h) after flushing the catheter to avoid contamination. For oral administration, a gavage was used to administer the drug and samples were collected through the jugular vein. To prepare the sample for analysis, 1 ml of ice cold acetonitrile was added to each sample to allow the protein to precipitate and then vortexed for 10 min. The samples were than centrifuged at $3500 \times g$ for 10 min and the supernatant transferred into clean culture tubes. The clear solution was then dried under vacuum and reconstituted by adding 200 μl of methanol.

2.4. Instrumentation

The HPLC system comprised a Waters 717 autosampler, Waters 996 photo diode array detector and Waters 600 series solvent delivery system. Millenium Chromatography Manager Ver. 2.1 was used for data management. The injection volume was 50 μl and 2D chromatograms were generated from photo diode array data with an extraction wave length of 263 nm. The flow rate was 1.0 ml min^{-1}. Mobile phase A was 100% acetonitrile and B was water. The elution program involved the following: Initial condition of 40% A with step gradient of 50% A in 5 min, 70% A in 10 min, 80% A in 15 min maintained at 80% A until 20 min and finally to 40% A in 25 min with an equilibration of 5 min. The separation was performed by Waters Symmetry C_{18} analytical column (5 μm particle size, 150×3.9 mm i.d.)

2.5. Assay validation

2.5.1. Linearity and range

Three determination ($n = 3$) from a minimum of five concentration levels (0.25, 0.5, 1, 2.5, 5 and 10 μg ml^{-1}) of the analyte were made. A detector response was correlated against analyte concentration by least-squares regression. A weight of $1/x$ was used to determine slopes, intercepts and correlation coefficients. The minimum acceptable coefficient to establish linearity was set at 0.99.

2.5.2. Method precision and percent recovery

Precision of the assay was established by analysis of nine replicates ($n = 9$) of a standard solution of the analyte at the following concentrations: 0.5, 2.5, 1, 2.5, 5 and 10 μg ml^{-1}. To determine

intra-day precision of the assay, replicate ($n = 5$) samples of three different concentrations were analyzed. The percent coefficient of variation of the assay results was determined for three consecutive days.

Extraction efficiency was determined by comparing replicate ($n = 5$) peak area ratio of the analyte and internal standard of extracted plasma samples versus unextracted acetonitrile samples for 0.5, 2.5 and 10 μg ml^{-1} concentrations. Stability of SYN-2869 and its analogs in plasma was evaluated by comparing the mean peak area of three 100 μg ml^{-1} standards for 24 h.

2.5.3. Accuracy

Method standards in the concentration range 0.5–10 μg ml^{-1} from nine different runs performed over 3 days were used to check for accuracy. The mean of the nine runs was calculated and compared to the spiked value to determine the percentage difference between the mean and the spiked value.

2.6. Pharmacokinetic study

Studies were designed to investigate the pharmacokinetics of SYN-2869 and its analogs in Balb C mice and Sprague Dawley rats. The protocols were approved by the Health Sciences Animal Welfare Committee at the University of Alberta. SYN-2869 and its analogs in a microemulsion formulation containing cremophore were administered intravenously (20 mg kg^{-1}) and orally (50 mg kg^{-1}) to mice and rats. At selected time points, plasma samples were collected and stored at -20°C. The total area under the plasma concentration–time curve from time zero to time infinity ($AUC_{0-\infty}$) was calculated by the linear trapezoidal rule extrapolation, $AUC_{last} + C_n/\lambda z$ where C_n denotes either the observed or predicted concentration at the last sample time. λ_z in the first order rate constant associated with the terminal (log-linear) portion of the curve and is estimated via linear regression of time versus log concentration. Terminal half-life was calculated as $-\ln(2)\lambda z$. Pharmacokinetic parameters were calculated by non-compartmental analysis using Winnonln computer program.

Statistical analysis: All results were reported as mean ± SD. For kinetic parameters, a one way ANOVA was used to evaluate the difference with different compounds. When a difference was detected, Duncan's multiple comparison test was used to evaluate the differences among groups. The level of significance was set at $P = 0.05$. SPSS computer software was used for all statistical calculations.

3. Results and discussion

3.1. Resolution

Fig. 2 represents chromatograms of extracted blank plasma, a calibration standard containing SYN-2869 and its analogs and unknown sample. The assay was found to be specific for SYN-2869 and well resolved from its analogs and internal standard. Moreover, the peaks of SYN-2869 and internal standard were sufficiently separated with typical retention times ranged between 17.20–17.35 min for SYN-2869 ($n = 51$) and 15.2–15.3 min for internal standard SYN-2506 ($n = 51$). The retention time range for analogs were between 16.92 and 16.96 for SYN-2836 ($n = 21$), 17.76–17.82 for SYN-2903 ($n = 21$) and 18.22–18.37 for SYN-2921 ($n = 21$).

3.2. Extraction recovery

After the comparison of the ratio of SYN-2869 and internal standard SYN-2506 in extracted plasma samples and acetonitrile samples, the mean extraction recovery was found to be 84.02 ± 17.37, 74.15 ± 6.48 and 105.7 ± 4.17% for 0.5, 2.5 and 10 μg ml^{-1} standards, respectively. The mean recovery for SYN-2836, SYN-2903 and SYN-2921 was 102.3 ± 12.4, 97.4 ± 6.17, and 99.6 ± 1.19%, respectively. The high variability of extraction values especially at the lower concentration of SYN-2869 may be due to its lipophilic nature and some degree of non-specific binding.

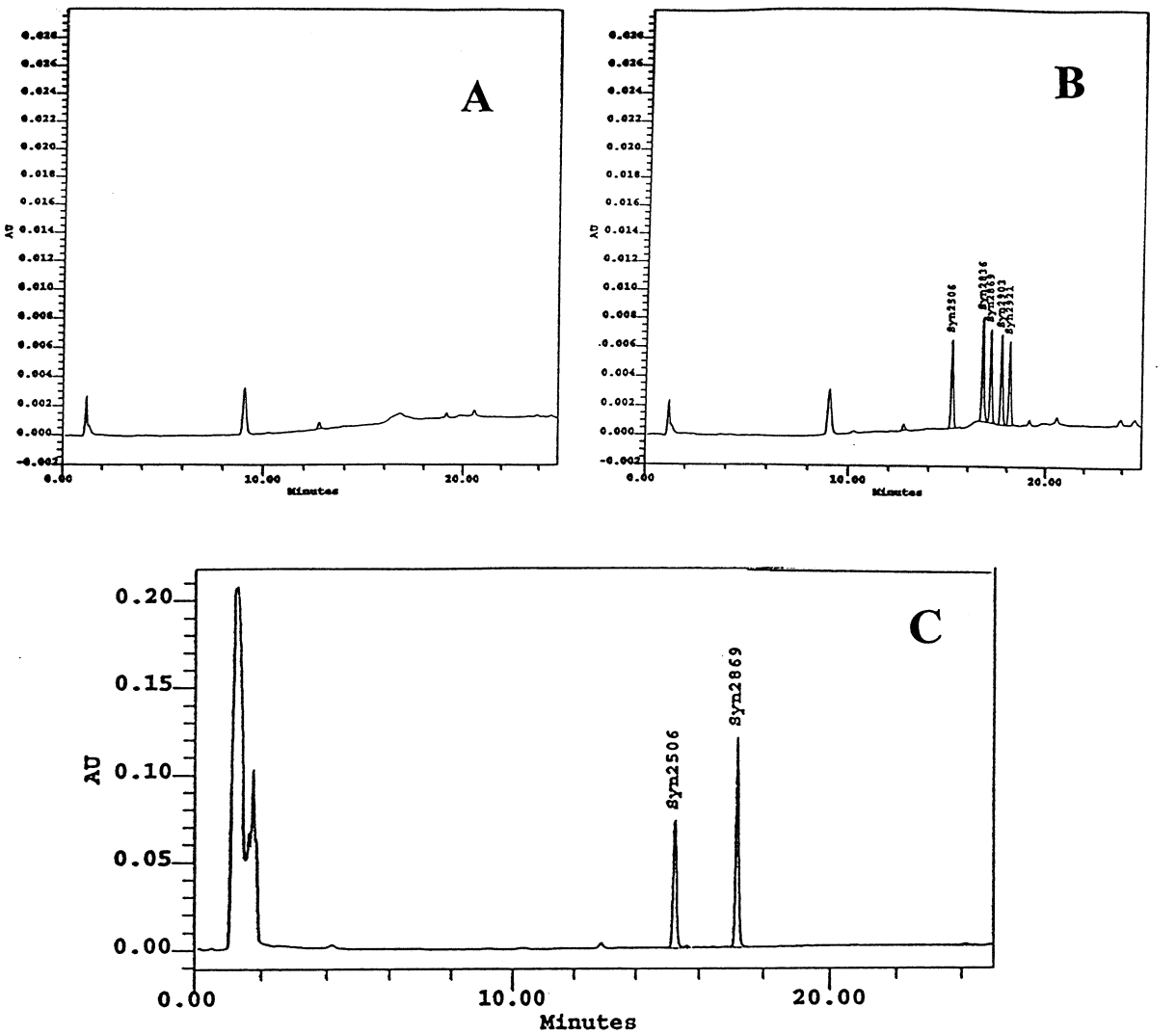

Fig. 2. Representative chromatogram of: (A) Extracted blank plasma samples; (B) Extracted plasma samples spiked with SYN-2836, SYN-2869, SYN-2921 and internal standard SYN-2506; and (C) unknown mouse plasma sample after intravenous administration of SYN-2869.

3.3. Validation and precision

The standard curves for SYN-2869 showed linearity over the selected concentration range (0.5–10 μg ml^{-1}) with reproducible slopes and excellent correlation coefficients ($r^2 > 0.99$) through out the validation runs. The analogs of SYN-2869 also had excellent correlation coefficient ($r > 0.99$) in all intra-day validation runs. The intra-day and inter-day precision data for SYN-2869 in rat plasma are listed in Tables 1 and 2. The intra-day CV was 6.6% or less for all standards whereas inter-day precision was 8.5% or less. The intra-day and inter-day accuracy, as indicated by RE, ranged from −1.40 to 12% for SYN-2869. The precision of the assay expressed as the CV was $< 6.6\%$ for all concentrations of SYN 2869 and was very similar for all the analogs

Table 1
Inter-day precision and accuracy for SYN-2869 standard concentration

	SYN-2869 (μg ml^{-1})				
	0.5	1	2.5	5	10
Mean (μg ml^{-1})	0.522	1.003	2.582	4.930	9.932
CV (%)	8.5	5.7	3.1	3.7	1.9
RE (%)	+4.44	+0.33	+3.29	−1.40	−0.68
n	9	9	9	9	9

Table 2
Intra-day precision and accuracy for quality control samples

	SYN-2869 (μg ml^{-1})		
	0.5	2.5	10
Intra-day			
Mean (μg ml^{-1})	0.56	2.51	10.46
CV (%)	5.21	6.63	4.64
RE (%)	12.0	0.4	4.60
n	5	5	5

of SYN-2869 (Table 3). The fluctuation of the %RE for SYN 2903 and SYN-2921 at lower concentration may be due to highly lipophilic nature of these compounds with non-specific binding, SYN 2921 being more lipophilic than SYN 2903.

3.4. Pharmacokinetic studies

SYN-2836, SYN-2869, SYN-2903 and SYN-2921 were rapidly absorbed into the systemic circulation and reached a maximum concentration of 7.31 ± 2.53, 6.28 ± 0.85, 6.16 ± 0.39, 3.41 ± 0.34 μg ml^{-1}, respectively in mice after an oral dose of 50 mg kg^{-1}. There was no significant difference in $AUC_{0-\infty}$ which varied from 48.13 to 63.43 μg h^{-1} ml^{-1}. The half-life

Table 3
Intra-day precision and accuracy for SYN-2836, SYN-2903 and SYN-2921 standard concentration

	SYN-2836 (μg ml^{-1})				
	0.5	1	2.5	5	10
Mean (μg ml^{-1})	0.513	1.057	2.707	4.997	9.427
CV (%)	4.9	8.9	4.3	1.8	1.7
RE (%)	+2.67	+5.67	+8.27	−0.07	−5.73
n	3	3	3	3	3
SYN-2903 (μg ml^{-1})					
Mean (μg ml^{-1})	0.623	1.160	2.725	4.883	9.140
CV (%)	3.70	6.84	2.34	4.20	3.46
RE (%)	+24.67	+16.0	+9.0	−2.33	−8.60
n	3	3	3	3	3
SYN-2921 (μg ml^{-1})					
Mean (μg ml^{-1})	0.680	1.2	2.65	5.02	8.827
CV (%)	6.41	1.67	3.83	2.95	12.35
RE (%)	+36.0	+20.0	+6.0	0.40	−11.73
n	3	3	3	3	3

after single intravenous dose of 20 mg kg^{-1} was in the range 4.5–6 h. The bioavailbility of SYN-2836, SYN-2869, SYN-2903 and SYN-2921 in mice was 71.58, 60.26, 45.89 and 83.92%, respectively. No significant difference in $AUC_{0-\infty}$ were observed for SYN-2836 and SYN-2869 after a single i.v. dose of 20 mg kg^{-1} in rats but oral bioavailability for SYN-2836 was less than 1% compared to 14.7% for SYN-2869.

4. Conclusion

The method described is a simple, specific and robust assay for the determination and quantitation of a novel series of triazole antifungal agents. This method has been successfully applied to several preclinical pharmacokinetic and tissue distribution studies in the integrated drug discovery program to select a lead candidate compound based on PK–PD relationship in respiratory tract infection animal models.

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