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Liquid chromatographic–electrospray tandem mass spectrometric determination of novel antifungal agents in plasma

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Abstract

A rapid, selective, sensitive and reproducible HPLC–electrospray tandem mass spectrometric method has been developed for the analysis of novel triazole antifungal agents, SYN-2869 and its derivatives (SYN-2836, SYN-2903 and SYN-2921), in rat plasma using SYN-2506 as an internal standard. Isolation of these compounds from plasma and sample desalting were performed by a simple extraction procedure involving protein precipitation, vacuum-drying and reconstitution with acetonitrile. For all the agents, linearity was observed over the range of 10–10 000 ng/ml ($r \geq 0.996$) and the limit of quantitation was 10 ng/ml using a 100-μl plasma volume. A measurement rate of 400–500 samples/day/instrument could be achieved using this method.

Keywords: Antifungal agents; SYN-2869

1. Introduction

SYN-2869, SYN-2836, SYN-2903 and SYN-2921 (Fig. 1) are currently under development as a class of novel triazole antifungal agents [1] for the treatment of systemic and superficial fungal infections [2–4]. For pharmacokinetic studies, a high-performance liquid chromatography (HPLC) method with UV detection was developed to quantify the four compounds in plasma [5]. However, this HPLC assay was time-consuming and lacked the necessary sensitivity when low dosages were involved.

HPLC coupled with atmospheric pressure ionization (API) tandem mass spectrometry (MS–MS) has become an ideal alternative to the repertoire of bioanalytical methods, which allows scientists to develop ultrasensitive assays of drugs and drug candidates in biofluids [6–9]. As a common belief, use of LC–MS–MS can practically guarantee assay specificity with simplified sample preparation along with no or little chromatographic separation. Therefore, chromatographic run times of less than 3 min using short (≤5 cm) analytical columns are commonly employed, leading to high-throughput (20–40 samples/h/instrument) quantitation of analytes in complex biological samples [10–14].

This paper describes a rapid, sensitive and selective LC–MS–MS assay employing an electrospray interface with multiple reaction monitoring (MRM) for the determination of SYN-2869, SYN-2836, SYN-2903 and SYN-2921 in rat plasma. A simple extraction procedure was carried out using SYN-2506 as the internal standard (I.S.). This method was validated mainly using SYN-2869 as a model com-

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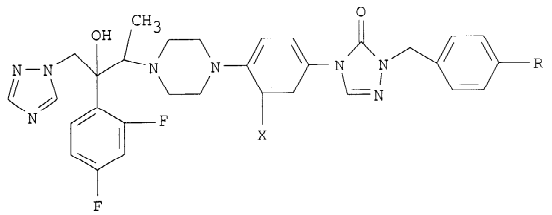
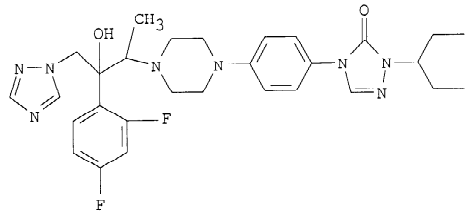


Compound	X	R	M_r (Da)
SYN-2836	H	CF3	654
SYN-2869	H	OCF3	670
SYN-2903	F	CF3	672
SYN-2921	F	OCF3	688



SYN-2506

Fig. 1. Structural formulae of the new antifungal compounds and internal standard, SYN-2506.

pound considering the similarity in physicochemical properties of the four triazole derivatives.

2. Experimental

2.1. Chemicals

SYN-2836, SYN-2869, SYN-2903, SYN-2921 and SYN-2506 were synthesized at SynPhar Labs. (Edmonton, Canada). HPLC-grade acetonitrile was received from EM Science (Gibbstown, NJ, USA). Ammonium acetate was purchased from Sigma (St. Louis, MO, USA).

2.2. Sample preparation

Individual stock and diluted solutions of the four antifungal agents and I.S. were prepared in acetonitrile and stored at 4°C. Individual calibration standards in plasma, over the concentration range of 10–10 000 ng/ml, were prepared by mixing aliquots (≤100 μl) of the diluted solutions, along with 50 μl of I.S. solution (1 μg/ml), with 100 μl of control (drug-free) rat plasma followed by addition of 1 ml of acetonitrile. The samples were vortexed for 5 min to incorporate all components, and centrifuged at 3500 *g* for 10 min to precipitate proteins. The supernatants were transferred into 100×13 mm borosilicate glass culture tubes, and dried using a vacuum drier at a high drying rate. The residues were reconstituted by adding 200 μl of acetonitrile followed by vortexing for 10 min. The solutions were transferred to polypropylene microcentrifuge tubes, centrifuged at 3500 *g* for 5 min, and transferred into autosampler vials at 4°C for injection (10 μl). Similarly, quality control (QC) samples of SYN-2869 were prepared at concentrations of 20, 200 and 2000 ng/ml ($n=6$) on three separate days.

2.3. LC–MS–MS conditions

The Waters 2690 separations module HPLC system (Milford, MA, USA) was operated at ambient temperature (22±2°C) using a Phenomenex Columbus C_{18} column (50×2.0 mm I.D., 5 μm particle size, Torrance, CA, USA) with an isocratic mobile phase composed of acetonitrile–ammonium acetate (5 m*M*) (90:10, v/v) at a flow-rate of 200 μl/min. Using a post-column split of 1:3, the effluent was delivered into the electrospray interface (positive-ion mode ES^+, source temperature 100°C, capillary voltage +3.5 kV) of a Quattro II mass spectrometer (Micromass, Manchester, UK). Nitrogen served as the drying gas and nebulizing gas at flow-rates of 220 and 20 l/h, respectively. Under these conditions, full-scan spectra of the compounds of interest were recorded. For collision-induced dissociation (CID), argon was used as the target gas at a pressure of $5 \cdot 10^{-4}$ mbar. Quantification was performed by multiple reaction monitoring (MRM, dwell time 0.08 s) of the protonated molecular (precursor) ions ($[MH]^+$) and their corresponding product ions (Table 1) using an internal standard calibration method with peak area ratios and $1/x$ weighting. All data were processed by Masslynx software (Micromass).

2.4. Recovery measurement

Extraction efficiency of the four antifungal agents and I.S. was determined by comparing replicate ($n=$

Table 1
Precursor/product ions, cone voltages and collision energies used for the multiple reaction monitoring of different triazole compounds

Compound	Precursor/product ion (m/z)	Cone voltage (V)	Collision voltage (eV)
SYN-2836	655/586	40	25
SYN-2869	671/602	45	25
SYN-2903	673/604	45	25
SYN-2921	689/620	45	25
SYN-2506 (I.S.)	567/498	40	20

6) peak area ratios of extracted plasma samples versus unextracted standards at 20, 200 and 2000 ng/ml.

2.5. Validation procedures

This LC–MS–MS assay was validated for the linearity, limit of quantification, recovery and stability of SYN-2836, SYN-2869, SYN-2903 and SYN-2921, respectively, in rat plasma. Intra- and inter-day accuracy and precision were determined only for SYN-2869. The accuracy and precision of the method were assessed by analyzing replicates of the QC samples.

3. Results and discussion

3.1. LC–MS–MS optimization

To detect these antifungal agents using MRM, full-scan spectra and product ion spectra for each agent and I.S. were investigated. Under the mass spectral conditions stated above, the ES^+ full-scan spectra of all the compounds, obtained by direct infusion, indicated the protonated molecule ($[MH]^+$) to be the most abundant ion. $[MNa]^+$ adduct was also observed at an inferior abundance. As an example, the full-scan spectrum of SYN-2869 is shown in Fig. 2A.

The $[MH]^+$ ion of each compound was therefore selected as the precursor ion for CID fragmentation to find the most abundant product ion. Such a product ion spectrum of SYN-2869 is given in Fig. 2B. The precursor/product ions chosen for the MRM detection of different triazole compounds are summarized in Table 1. All the precursor/product ion pairs show identical m/z difference [$=m/z$ (precursor)$-m/z$ (product)] of 69 which arose from the neutral loss of the triazole moiety, $C_2H_3N_3$, from the $[MH]^+$ ions (Fig. 1).

To increase the sensitivity of MRM detection, cone voltage and collision energy were found to be very significant parameters for optimization. In this study, the two parameters were first optimized for each compound by using the commonly used direct infusion at a flow-rate of 5 μl/min. When the optimized cone voltage and collision energy were used to perform LC–MS–MS analysis, the sensitivity was not as high as expected (data not shown). For this reason, the real LC–MS–MS system was used to re-optimize cone voltage and collision energy, achieving much higher sensitivity for each individual compound. The resultant cone voltages and collision energies for MRM are given in Table 1, which were used in all quantitative experiments throughout this study.

Due to the high selectivity of MRM detection, it was easy to distinguish each triazole compound from I.S. without the necessity for a complete chromatographic baseline separation. This opened the possibility for rapid analysis of these antifungal agents. The chromatographic conditions were evaluated by achieving a maximum response (peak area) and a minimum baseline noise along with a run time as short as possible. As a result, a short reversed-phase microbore column and an isocratic mobile phase consisting of acetonitrile–ammonium acetate (5 m*M*) (90:10, v/v) were found to be optimal for this purpose. Furthermore, a post-column splitter was used to reduce the flow-rate of eluate from 200 to 50 μl/min, which ensured the whole LC–MS–MS system to generate the optimum performance. Fig. 3 shows the representative LC–MS–MS profiles of SYN-2836, SYN-2869, SYN-2903 and SYN-2921

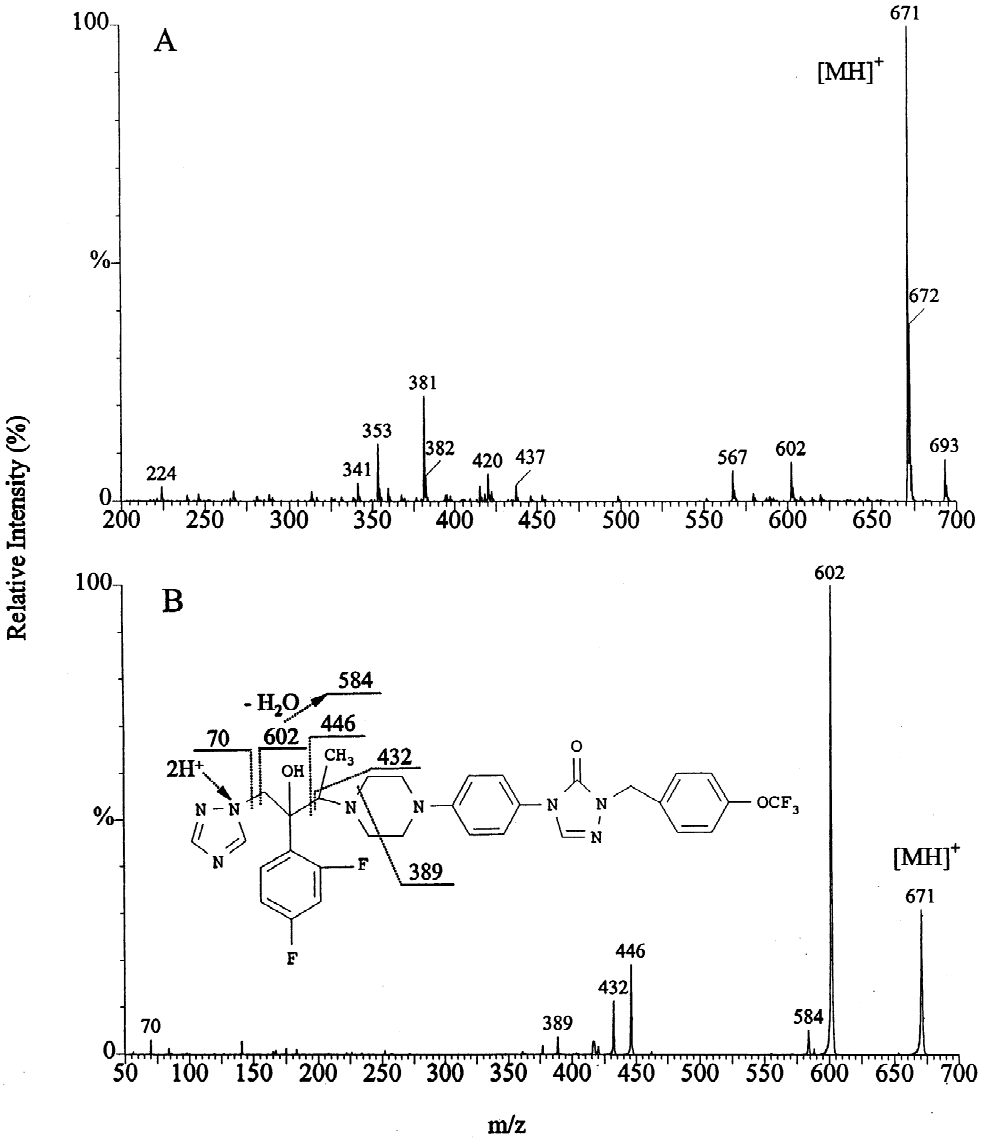


Fig. 2. (A) Positive-ion electrospray Q1 mass spectrum of SYN-2869; (B) full-scan product ion spectrum of SYN-2869 with its protonated molecular ion ($[MH]^+$) at m/z 671 as the precursor ion.

extracted from plasma containing 20 ng/ml of each analyte, I.S. extracted from plasma containing 500 ng/ml, under the experimental conditions described in Section 2.3. Similarly, chromatogram of control rat plasma shows that all antifungal agents were free from interference from endogenous species (Fig. 3).

3.2. Linearity and lower limit of quantification

The calibration range was based on the concentrations expected in plasma samples to be analyzed. The concentration range of 10–10 000 ng/ml proved to be sufficient for the analysis of SYN-2836, SYN-

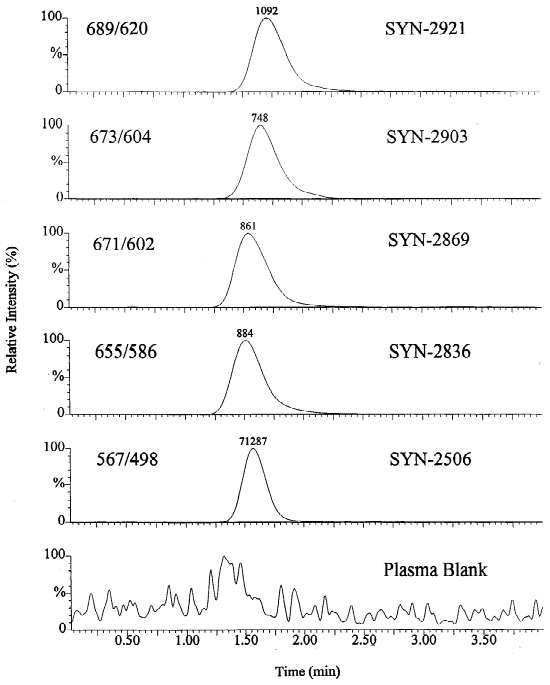


Fig. 3. Representative MRM chromatograms of SYN-2836, SYN-2869, SYN-2903 and SYN-2921 extracted from plasma containing 20 ng/ml of each analyte, SYN-2506 (I.S.) extracted from plasma containing 500 ng/ml, and total ion current MRM profile of plasma blank recorded for the above five compounds.

2869, SYN-2903 and SYN-2921 in rat plasma samples. Control plasma was spiked with the respective analyte to give concentrations of 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 and 10 000 ng/ml. I.S. was added to each sample at 500 ng/ml prior to extraction. For all four compounds, excellent linearity was achieved in the specified concentration range with the correlation coefficients for the calibration regression lines being 0.996 or greater, and all other regression data are shown in Table 2.

The lower limit of quantification (LLQ) is defined as the lowest concentration on the calibration curve for which an acceptable accuracy of 100±20% [(mean observed concentration/nominated concentration)×100] and a precision of 20% [relative standard deviation (RSD)] is obtained. The current assay has LLQ of 10 ng/ml for SYN-2836, SYN-2869, SYN-2903 and SYN-2921 in rat plasma based on a 100-μl plasma volume.

3.3. Recovery

Because of their similarity in structure, the four antifungal agents were extracted from rat plasma with similar recoveries. The overall recoveries of SYN-2836, SYN-2869, SYN-2903 and SYN-2921 were determined to be 91, 86, 82 and 94%, respectively, representing the average value of the recoveries at 20, 200 and 2000 ng/ml. Similarly, the overall recovery of SYN-2506 was determined to be 89%.

3.4. Method precision and accuracy

In this study, method precision and accuracy were assessed only for SYN-2869 as a model considering the similarity in physicochemical properties of SYN-2836, SYN-2869, SYN-2903 and SYN-2921. The intra-day precision and accuracy for the method were determined by analyzing six QC replicates at 20, 200 and 2000 ng/ml on each of three days. The accuracy of the method was determined by calculating relative error (RE) and the precision by calculating RSD. Table 3 summarizes the intra-day accuracy and

Table 2
Linearity parameters for determination of the analytes extracted from rat plasma (n=6)

Compound	Slope		Intercept	
	Mean±SD	RSD (%)	Mean±SD	RSD (%)
SYN-2836	0.003983±0.000247	6.2	0.000212±0.000208	98.3
SYN-2869	0.004269±0.000209	4.9	0.000328±0.000297	90.5
SYN-2903	0.004065±0.000178	4.2	0.000107±0.000077	71.6
SYN-2921	0.004527±0.000262	5.8	0.000285±0.000234	82.1

Table 3
Intra-day validation data for SYN-2869 in rat plasma

Day	Parameter	QC sample level (ng/ml)		
		20	200	2000
1	Mean (ng/ml)	20.79	189.42	1972.33
	SD (ng/ml)	2.29	9.68	150.60
	Accuracy (RE, %)	3.76	−5.29	−1.38
	Precision (RSD, %)	11.01	5.11	7.64
	n	6	6	6
2	Mean (ng/ml)	20.58	195.54	2015.97
	SD (ng/ml)	2.07	11.68	128.80
	Accuracy (RE, %)	2.90	−2.23	0.80
	Precision (RSD, %)	10.06	5.97	6.39
	n	6	6	6
3	Mean (ng/ml)	21.37	195.89	2217.69
	SD (ng/ml)	1.00	2.17	124.04
	Accuracy (RE, %)	6.85	−2.06	10.88
	Precision (RSD, %)	4.68	1.11	5.59
	n	6	6	6

precision data for SYN-2869 in rat plasma. The inter-day precision and accuracy data for SYN-2869 in rat plasma are shown in Table 4. The inter-day accuracy ranged from −3.2% to 4.6% of nominated values with precision ranging from 1.8% to 6.4% over the three concentrations evaluated.

3.5. Stability

The stability of SYN-2836, SYN-2869, SYN-2903 and SYN-2921 in rat plasma was evaluated at ambient temperature (22°C) over a 4-h period using plasma spiked with the individual compounds at a concentration of 1000 ng/ml. The concentrations of these compounds found in plasma incubated for 4 h were compared with the values observed in the same plasma without incubation. The recoveries ranged from 91 to 108% ($n=4$) for all four agents, indicating that they were stable for at least 4 h in rat plasma stored at ambient temperature. In addition, the stability study at different concentrations (i.e., 20, 1000 and 5000 ng/ml) did not show pronounced concentration dependence (data not shown). This may be due to the high stability of the antifungal compounds in plasma resulting in minor degradation within the test period. These compounds also demonstrated stability up to four freeze–thaw cycles (−20/+22°C) in plasma. After four cycles, the recovery of the four agents from plasma ranged from 90 to 106% ($n=4$). Following sample preparation, these compounds were stable up to 10 days in autosampler vials at 4°C.

Table 4
Inter-day validation data for SYN-2869 in rat plasma

Parameter	QC sample level (ng/ml)		
	20	200	2000
Mean (ng/ml)	20.91	193.62	2068.66
SD (ng/ml)	1.76	3.64	130.90
Accuracy (RE, %)	4.55	−3.19	3.43
Precision (RSD, %)	1.97	1.88	6.33
n	18	18	18

4. Conclusions

The straightforward LC–MS–MS assay for the quantitation of SYN-2836, SYN-2869, SYN-2903 and SYN-2921 offers a number of unique features. First, the extraction procedure is relatively simple and requires only 100 μl of plasma. Second, the assay has excellent sensitivity and selectivity for the four compounds in rat plasma. It is likely that the assay could be amenable to the determination of these agents in plasma from other species (e.g., rabbit, dog and human) and even in other types of biological matrices (e.g., urine, bile, feces and tissues) with no or minor modification to the sample preparation procedure. Finally, this is a rapid method with a measurement rate of 400–500 samples/day/instrument. In practice, the pre-column filter and analytical column were cleaned once after a 24-h run, and the API source was disassembled and cleaned once after one-week run, in order to maintain the HPLC–API-MS–MS system in excellent working condition. Considering the analogy of the four antifungal agents, the precision and accuracy data of this method are provided only for SYN-2869 as a model in this paper. Thus the assay was not fully validated for SYN-2836, SYN-2903 and SYN-2921 pursuant to full validation guidelines. The rapid and rugged method proved to be capable of quantifying the four new antifungal agents in plasma in support of pharmacokinetic evaluation.

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