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A rapid and reliable solid-phase extraction–LC/MS/MS assay for the determination of two novel human leukocyte elastase inhibitors, SYN-1390 and SYN-1396, in rat plasma

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Abstract

A rapid and rugged solid-phase extraction-liquid chromatographic-electrospray tandem mass spectrometric method has been developed to quantitate two novel human leukocyte elastase inhibitors, SYN-1390 and SYN-1396, in rat plasma. A reversed-phase column and an isocratic mobile phase consisting of acetonitrile-water-formic acid (70:30:0.2, v/v/v) were used. The mass spectrometer was operated in the multiple reaction monitoring mode. For both analytes, standard curves were linear over a working range of 0.1–20 μg ml^{-1} ($r \geq 0.995$) and the limit of quantitation was 0.1 μg ml^{-1} with a 150 μl plasma volume. This assay proved to be useful for the determination of SYN-1390 and SYN-1396 in plasma samples from pharmacokinetic study.

Keywords: Human leukocyte elastase inhibitors; SYN-1390; SYN-1396; Solid-phase extraction; Liquid chromatography/tandem mass spectrometry

1. Introduction

Human leukocyte elastase (HLE), a serine protease released by the polymorphonuclear leukocytes, has been implicated in the extracellular degradation of structural proteins such as elastin and collagen. As a result, inhibition of HLE has been targeted as a potential therapy in diseases such as cystic fibrosis [1] and emphysema [2].

During the past decade, numerous studies have been reported concerning low molecular weight nonpeptidyl HLE inhibitors [3–6]. In this area, SynPhar has developed a number of novel compounds based on cephalosporin skeleton [7,8]. It

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was observed by preliminary evaluation that these compounds are potent HLE inhibitors in lung injury model and show good safety profiles in rats [9,10]. As a further development, two of such inhibitors, SYN-1390 and SYN-1396 (Fig. 1), have been selected for pharmacokinetic evaluation for which an appropriate analytical method was needed.

The compounds of interest are analytically challenging due to their highly polar nature which may induce a strong affinity or covalent binding of the agents to plasma proteins. An HPLC method with UV detection was investigated first, but the preliminary results (data not shown) revealed that the HPLC method lacked sufficient sensitivity and selectivity for pharmacokinetic study. Therefore, a rapid and reliable liquid chromatographic–electrospray tandem mass spectrometric (LC/MS/MS) [11–13] assay using solid-phase extraction (SPE) has been developed for the determination of SYN-1390 and SYN-1396 in rat plasma.

2. Experimental

2.1. Chemicals

SYN-1390 and SYN-1396 were synthesized at SynPhar Laboratories (Edmonton, Alberta, Canada). HPLC-grade methanol and acetonitrile were received from EM Science (Gibbstown, NJ, USA). Analytical-grade orthophosphoric acid and formic acid were purchased from BDH (Toronto, Ontario, Canada).

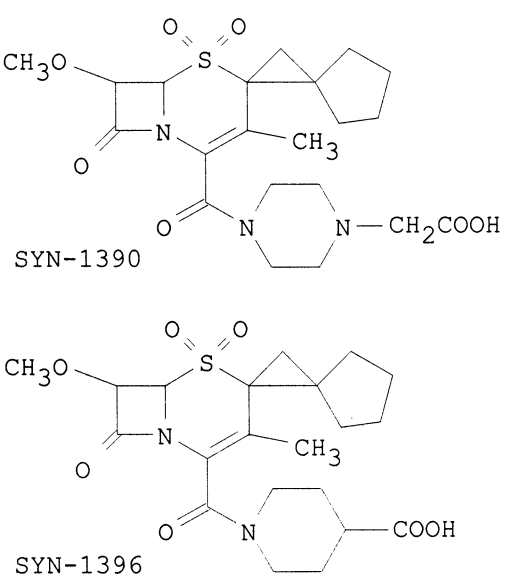

Fig. 1. Structures of SYN-1390 and SYN-1396

2.2. Sample preparation

Individual stock and diluted solutions of SYN-1390 and SYN-1396 were prepared in acetonitrile–water–formic acid (50:50:0.2, v/v/v) and were stored at 4 °C. SYN-1396 was used as an internal standard (IS) for SYN-1390 while SYN-1390 was used as an IS for SYN-1396. Isolation of both compounds from plasma and sample desalting were performed by SPE technique using the new Waters Oasis™ HLB extraction cartridges (Milford, MA, USA) on an ASPEC® (Gilson, Villiers-le-Bel, France). To prepare the standards and quality control (QC) samples, control (drug-free) rat plasma (150 μl) was spiked with SYN-1390 and SYN-1396, and water was added to the spiked plasma samples to reach a volume of 1 ml. The samples were acidified by the addition of 20 μl of concentrated orthophosphoric acid, vortex-mixed for 5 min to incorporate all components, and clarified by centrifugation at $3{,}500 \times g$ for 5 min. The supernatants were loaded onto the HLB cartridges which had been activated with 1 ml of methanol followed by 1 ml of water. The loaded cartridges were washed with 1 ml of methanol-water (5:95, v/v) which was subsequently discarded. The analytes were eluted with 1 ml of methanol. The eluates were evaporated to dryness at 37 °C by nitrogen, and the residues were reconstituted by adding 200 μl of mobile phase consisting of acetonitrile-water-formic acid (70:30:0.2, v/v/v). The final solutions were transferred into autosampler vials at 4°C for injection (10 μl). The same method was used for preparation of calibration standards in plasma over the concentration range of 0.1–20 μg ml^{-1} with the IS concentration of 3.5 μg ml^{-1}, and QC samples in plasma were prepared at levels of 0.2, 1 and 10 μg ml^{-1} ($n = 6$) on three separate days.

2.3. HPLC/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-water-formic acid (70:30:0.2, v/v/v) at a flow rate of 200 μl min^{-1}. Using a post-column split of 1:3, the effluent was delivered into the electrospray interface (positive-ion mode ES^+, source temperature 80 °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 250 and 25 l h^{-1}, respectively. For collision-induced dissociation (CID), argon was used as the target gas at a pressure of 5×10^{-4} mbar. Quantification was performed by multiple reaction monitoring (MRM, dwell time 0.08 s) of the precursor/product ions at m/z 468/145 for SYN-1390 and m/z 453/336 for SYN-1396 using an internal standard calibration method with peak area ratios and 1/x weighting. All data were processed by Masslynx software (Micromass, Manchester, UK).

2.4. Recovery measurement

Recovery of SYN-1390 and SYN-1396 was determined by comparing replicate ($n = 6$) peak area ratios of extracted plasma samples versus unextracted standards at 0.2, 1 and 10 μg ml^{-1}.

2.5. Validation procedure

This SPE-LC/MS/MS method was validated for the linearity, limit of quantification, recovery, as well as intra- and inter-day accuracy and precision of SYN-1390 and SYN-1396, respectively, in rat plasma. The accuracy and precision of the assay were assessed by analyzing replicates of the QC samples. The concentrations were determined using the calibration equations.

3. Results and discussion

3.1. LC/MS/MS optimization

SYN-1390 and SYN-1396 were determined using MRM detection. To find appropriate MRM conditions, full-scan mass spectra and product ion spectra for each analyte were carried out. The ES^+ full-scan spectra of both agents, generated using direct infusion under the conditions described above, showed the protonated molecules ($[MH]^+$) as the most abundant ion (data not shown). The negative-ion (ES^-) full-scan spectra of SYN-1390 and SYN-1396 were also recorded by direct infusion using acetonitrileammonium acetate (pH 7.0; 5 mM) (70:30, v/v) as solvent (data not shown). It was demonstrated that the ES^- spectra of the individual compounds suffered from a lower total ion current than the ES^+ spectra. Similar behavior was also reported by Moder and co-workers for acylcarnitines [14]. Therefore, ES^+ mode was exclusively employed in this study.

The $[MH]^+$ ions of SYN-1390 and SYN-1396 were selected as the precursor ions to record their product ion spectra (Fig. 2). From Fig. 2, the ideal precursor/product ion pairs chosen for the MRM detection were determined to be m/z 468/145 for SYN-1390 and m/z 453/336 for SYN-1396.

For MRM detection, cone voltage (CV) and collision energy (CE) need to be carefully optimized to achieve high sensitivity [15]. In this study, the two parameters were first optimized for each analyte using direct infusion at a flow rate of 5 μl min^{-1}. When the MRM detection was performed with these pre-optimized CVs and CEs for SYN-1390 and SYN-1396, it did not yield as high sensitivity as expected (data not shown). For this reason, the MRM operation was used to re-optimize the parameters. This was performed by varying CVs, which was easily carried out by multi-channel acquisition following a single injection, at the pre-optimized CE to find the optimal CV, and by varying CEs, which was executed in the same way as varying CVs, at the optimal CV to determine the optimal CE for each analyte. Significantly higher sensitivity was subsequently obtained utilizing the finally-optimized CV and CE for each analyte (data not shown). This result is consistent with that observed for highly lipophilic antifungal agents [15]. The optimal CVs and CEs for MRM were 40 V and 23 eV for

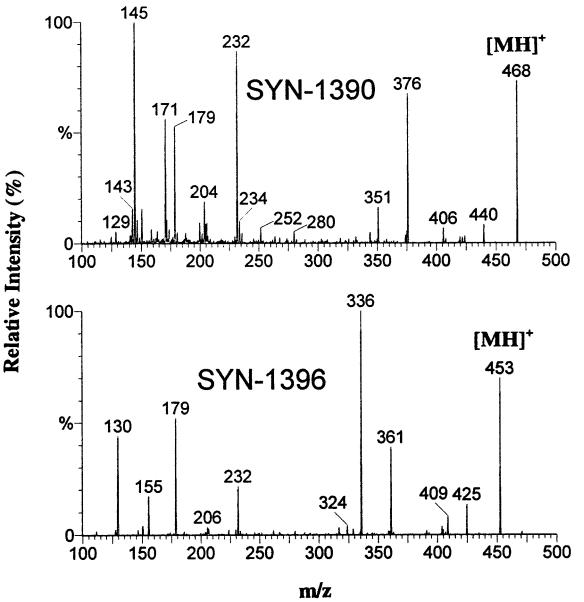

Fig. 2. Full-scan product ion spectra of SYN-1390 and SYN-1396 with their protonated molecular ions ($[MH]^+$) at m/z 468 and 453, respectively, as the procursor ions.

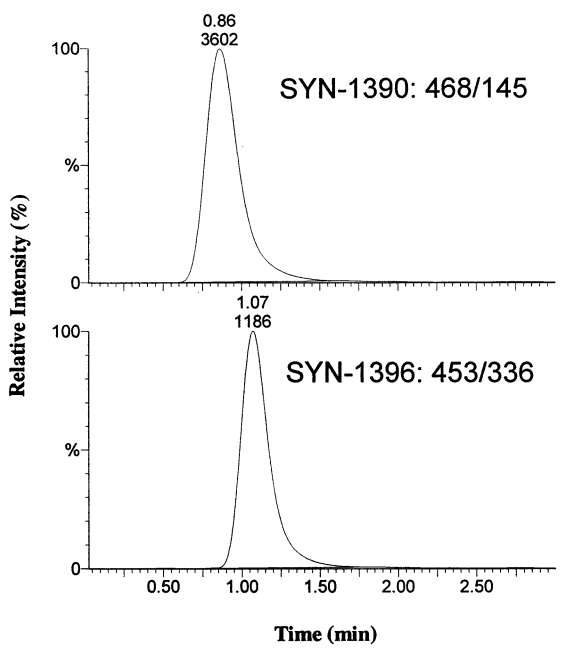

Fig. 3. MRM chromatograms of SYN-1390 and SYN-1396 extracted by SPE from plasma containing 2 μg ml^{-1} of each analyte.

SYN-1390 and 40 V and 17 eV for SYN-1396, which were used in all quantitative experiments throughout this study.

MRM detection was so specific that it was able to distinguish SYN-1390 and SYN-1396, despite their structural analogy, without the necessity for a complete chromatographic baseline separation. This provided the prerequisite for both compounds to be rapidly analyzed. 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 analytical column (50 × 2.0 mm I.D.) and an isocratic mobile phase composed of acetonitrile-water-formic acid (70:30:0.5, v/v/v) were found to be optimal for this purpose. Furthermore, a post-column splitter was used to cut the flow rate of effluent from 200 to 50 μl min^{-1}, ensuring the whole LC/MS/MS system was working optimally. The operation using the lower flow rate greatly reduced the contamination of the atmospheric pressure ionization source without losing sensitivity. Fig. 3 shows the typical LC/MS/MS profiles of SYN-1390 and SYN-1396 extracted by SPE from plasma containing 2 μg ml^{-1} of each analyte under the experimental conditions described in the Experimental section. Similarly, chromatograms of control rat plasma show that both elastase inhibitors were free from interference from endogenous species (data not shown).

3.2. Linearity and lower limit of quantification

The calibration range was based on the concentrations expected in the plasma samples to be analyzed. The concentration range of 0.1–20 μg ml^{-1} proved to be sufficient for the determination of SYN-1390 and SYN-1396 in rat plasma samples. Control plasma was spiked with the respective analyte to give concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 μg ml^{-1} with the IS concentration of 3.5 μg ml^{-1}. For both agents, calibration curves were linear over this concentration range with correlation coefficients of 0.995 or greater. All other regression parameters are given in Table 1.

The lower limit of quantification (LLQ) is defined as the lowest concentration on the calibra-

tion curve for which an acceptable accuracy of $100 \pm 20\%$ [(mean observed concentration/nominal concentration) $\times$ 100] and a precision of 20% [relative standard deviation (RSD)] is obtained. This assay has LLQ of 0.1 μg ml^{-1} for both SYN-1390 and SYN-1396 in rat plasma using a 150 μl plasma volume.

3.3. Recovery

The overall recoveries ranged from 31 to 43% for SYN-1390 and from 52 to 71% for SYN-1396 at concentrations of 0.2, 1 and 10 μg ml^{-1}. Before developing the SPE procedure, SYN-1390 and SYN-1396 were extracted from plasma samples by a simple extraction method used for new antifungal agents [15]. Unfortunately, the simple method gave a recovery of less than 10%, indicating that both compounds, due to their high polarity, may have strong interactions (affinity and/or covalent binding) with plasma proteins. An SPE method was developed to efficiently isolate the agents from the plasma proteins. As indicated above, SYN-1390 has a lower recovery than SYN-1396, suggesting that the former may have a stronger interaction with the plasma proteins than the latter.

3.4. Assay accuracy and precision

The intra-day precision and accuracy of the method were evaluated by analyzing six QC replicates at concentrations of 0.2, 1 and 10 μg ml^{-1} on each of three days for both SYN-1390 and SYN-1396. The accuracy of the method was determined by calculating relative error (RE) and the precision by calculating RSD. The intra-day accuracy and precision data for both agents in rat plasma are reported in Table 2. The intra-day accuracy ranged from -6.7% to 10.5% for SYN-1390 and from -9.5% to 9.5% for SYN-1396 with precision ranging from 2.8% to 11.5% for SYN-1390 and from 3.6% to 12.2% for SYN-1396. Table 3 summarizes the inter-day precision and accuracy data for both agents in rat plasma. The inter-day accuracy ranged from 1.3% to 4.6% for SYN-1390 and from -3.1% to 1.8% for SYN-1396 with precision ranging from 2.5% to 7.7% for SYN-1390 and from 1.9% to 6.2% for SYN-1396.

Table 1
Calibration curve parameters for SYN-1390 and SYN-1396 extracted from rat plasma ($n = 6$)

Compound	Slope		Intercept	
	mean ± SD	%RSD	mean ± SD	%RSD
SYN-1390	0.361 ± 0.019	5.4	−0.00274 ± 0.00217	79.2
SYN-1396	0.367 ± 0.027	7.3	−0.00301 ± 0.00260	86.3

3.5. Stability

The stability of SYN-1390 and SYN-1396 in rat plasma was evaluated at ambient temperature (22°C) over a 3 h period using plasma spiked with 5 μg ml^{-1} SYN-1390 and SYN-1396, respectively. The concentrations of SYN-1390 and SYN-1396 found in plasma incubated for 3 h were compared with the values observed in the same plasma without incubation. Such recoveries were determined to be $97 \pm 4\%$ and $96 \pm 5\%$ ($n = 5$) for SYN-1390 and SYN-1396, respectively, indicating that both agents are stable for at least 3 h in rat plasma stored at ambient temperature. SYN-1390 and SYN-1396 also demonstrated stability up to three freeze-thaw cycles ($-20/+22$°C) in plasma. After three cycles, the proportion of SYN-1390 and SYN-1396 remaining, relative to the initial analysis, was $95 \pm 6\%$ and $102 \pm 8\%$ ($n = 5$), respectively. Once prepared for analysis by SPE, the samples may be analyzed immediately or after storage at 4°C for up to a week without degradation of the analytes.

4. Conclusion

A validated LC/MS/MS method for the determination of SYN-1390 and SYN-1396 in rat plasma has been developed. The SPE procedure was employed to extract both compounds from plasma with reasonable recoveries. Considering its excellent sensitivity and selectivity, this assay may

Table 2
Intra-day validation results for SYN-1390 and SYN-1396 in rat plasma

Day	Parameter	QC sample level (μg ml^{-1})					
		SYN-1390			SYN-1396		
		0.20	1.0	10.0	0.20	1.0	10.0
1	Mean (μg ml^{-1})	0.221	0.933	10.53	0.182	1.049	9.87
	SD (μg ml^{-1})	0.016	0.027	0.488	0.022	0.065	0.527
	Accuracy (%RE)	10.50	−6.69	5.32	−9.03	4.87	−1.33
	Precision (%RSD)	7.24	2.89	4.63	12.11	6.20	5.34
	n	6	6	6	6	6	6
2	Mean (μg ml^{-1})	0.187	1.049	10.61	0.219	1.055	10.22
	SD (μg ml^{-1})	0.010	0.075	0.667	0.017	0.039	0.612
	Accuracy (%RE)	−6.51	4.87	6.13	9.49	5.54	2.18
	Precision (%RSD)	5.33	7.14	6.29	7.68	3.67	5.99
	n	6	6	6	6	6	6
3	Mean (μg ml^{-1})	0.219	1.061	9.51	0.181	0.943	10.46
	SD (μg ml^{-1})	0.025	0.051	0.467	0.017	0.049	0.535
	Accuracy (%RE)	9.53	6.06	4.92	−9.48	−5.66	4.61
	Precision (%RSD)	11.43	4.76	4.91	9.56	5.23	5.11
	n	6	6	6	6	6	6

Table 3
Inter-day validation results for SYN-1390 and SYN-1396 in rat plasma

Parameter	QC sample level (μg ml^{-1})					
	SYN-1390			SYN-1396		
	0.20	1.0	10.0	0.20	1.0	10.0
Mean (μg ml^{-1})	0.209	1.014	10.22	0.194	1.016	10.18
SD (μg ml^{-1})	0.015	0.035	0.262	0.012	0.020	0.390
Accuracy (%RE)	4.51	1.38	2.23	−3.01	1.64	1.78
Precision (%RSD)	7.65	3.44	2.56	6.18	1.96	3.83
n	18	18	18	18	18	18

be used to determine SYN-1390 and SYN-1396 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 extraction procedure. In addition, a measurement rate of 400–500 samples/day/instrument can be achieved using this assay. The reliable and high-throughput bioanalytical method proved to be capable of quantifying SYN-1390 and SYN-1396 in plasma for the purpose of pharmacokinetic studies.

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