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High-performance liquid chromatographic determination of GS4071, a potent inhibitor of influenza neuraminidase, in plasma by precolumn fluorescence derivatization with naphthalenedialdehyde

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Abstract

GS4071 is a potent inhibitor of influenza neuraminidase. A precolumn fluorescence derivatization HPLC method is described for the analysis of GS4071 in rat plasma. Plasma samples were subjected to solid-phase extraction on C_{18} extraction columns. After extraction, GS4071 was derivatized with naphthalenedialdehyde in the presence of potassium cyanide to produce highly fluorescent cyano[*f*]benzoisoindole derivatives. Derivatized samples were stable for >24 h at 4°C. The samples were analyzed by an isocratic HPLC method using fluorescence detection at 420 nm excitation and 470 nm emission wavelength. The method was validated and applied to the analysis of plasma samples from pre-clinical pharmacokinetic studies in rats. The limit of detection for GS4071 was 20 ng/ml. For five replicate samples at 50, 400, and 1000 ng/ml, the within-day precision values were 16.9, 9.4 and 4.5%, respectively, and the between-day precision values were 16.9, 7.9, and 2.1%, respectively. The method was linear from 25 to 1600 ng/ml and the total recovery was >68% over this concentration range.

Keywords: Neuraminidase inhibitor; GS4071; Influenza

1. Introduction

GS4071 [(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid] is a potent and selective inhibitor of influenza virus neuraminidase [1], an enzyme involved in the release of new virus particles from infected cells. GS4071 was highly effective in animal models of infection (mice and ferrets). An orally bioavailable ethyl ester prodrug of GS4071, GS4104 [ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate], is currently undergoing clinical evaluation for the prophylaxis and treatment of influenza A and B [2]. In multiple animal species and in humans, GS4104 has high oral bioavailability and, following absorption from the gastrointestinal tract, undergoes rapid enzymatic conversion to GS4071 [3,4]. Levels of GS4071 achieved in the rat lung after oral administration of GS4104 are sufficient to provide antiviral concentrations in excess of the IC_{90} [5].

There have been no published methods for detection of GS4071, therefore a sensitive and robust method was needed to support pre-clinical toxicokinetic studies. In early pre-clinical studies of GS4071 and the prodrug, an enzymatic assay utiliz-

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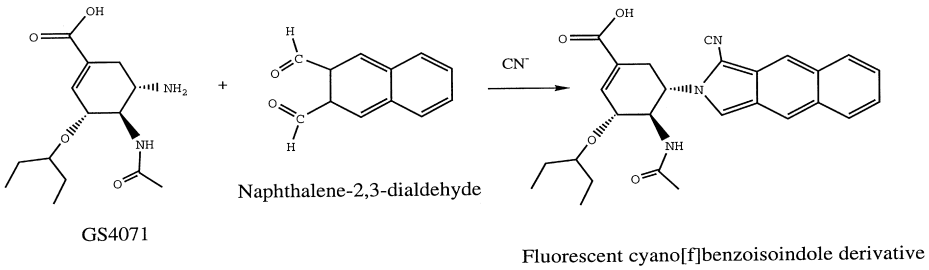

Fig. 1. Schematic of the reaction of NDA in the presence of KCN with the amino neuraminidase inhibitors (conditions for the reaction are given in the Experimental section).

ing viral neuraminidase was developed and used for detection of GS4071 in rat plasma [6]. However, the assay was time-consuming, suffered from a lack of specificity and was difficult to use in routine analyses.

Reversed-phase HPLC offered an attractive alternative to the enzymatic assay. However, GS4071 does not possess a strong chromophore (Fig. 1) and even with extensive sample clean-up, the sensitivity and selectivity of UV detection were not sufficient for the analysis of GS4071 in pre-clinical plasma samples following oral administration of all but the highest doses of the prodrug. Primary amines are known to react with dicarboxyaldehydes in the presence of nucleophiles to form fluorescent derivatives [7–10]. Naphthalene-2,3-dialdehyde (NDA) forms particularly stable and highly fluorescent derivatives with primary amines in the presence of cyanide ions [10,11]. These derivatives are non-polar and well suited to reversed-phase HPLC detection.

In this report, we describe the sensitive HPLC detection of GS4071 in plasma by application of precolumn derivatization with NDA.

2. Experimental

2.1. Materials

GS4071 (RO 64-02/000) and GS4057 [(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-cyclopentanoxy)-1-cyclohexene-1-carboxylic acid] were synthesized by Gilead Sciences (Foster City, CA, USA). GS4057 was used as an internal standard for GS4071. Radiolabeled [2-acetyl-^{14}C] GS4104 was obtained from Moravek Biochemicals (Brea, CA, USA). Radiolabeled [2-acetyl-^{14}C] GS4071 was produced from [2-acetyl-^{14}C] GS4104 by chemical hydrolysis at elevated pH. Naphthalene-2,3-dialdehyde (NDA) and KCN were purchased from Fluka (Ronkonkoma, NY, USA). All other chemicals and solvents (analytical grade) were purchased from Baxter (Muskegon, MI, USA). Water was purified using a Milli-Q UF purification system (Millipore, MA, USA).

2.2. HPLC system

The HPLC system comprised a Model P4000 pump, refrigerated AS3000 autoinjector with column oven (ThermoQuest, San Jose, CA, USA) and F-7480 fluorescence detector (Hitachi, San Jose, CA, USA). The Peak Pro data acquisition system (Beckman, Palo Alto, CA, USA) was used for data acquisition and processing. For radiochromatography, a Radiomatic A515A radioactive flow detector (Packard Instruments, Downers Grove, IL, USA), equipped with a 0.5 ml flow cell and detector splitter set at 50%, was added to the system after the fluorescence detector. Redi-Solv scintillation cocktail (Packard) was delivered at 2 ml/min. Data from the A515A were analyzed using FLO-ONE for Windows data acquisition and processing software (Packard). The analytical column used in all HPLC separations was a Prodigy (ODS-2) 150×4.6 mm, 5 μm (Phenomenex, Torrance, CA, USA).

The most efficient separation of the derivatives of GS4057 (IS) and GS4071 from endogenous compounds in plasma was achieved by using an isocratic reversed-phase HPLC method. The column temperature was set at 40°C, the fluorescence detector was set at 420 nm excitation wavelength and 472 nm

emission wavelength, the injection volume was 40 μl.

The mobile phase consisted of 50 mmol/l sodium acetate in acetonitrile–water (27:73, v/v). The flow-rate was maintained at 2 ml/min and the total run time was 10 min. The retention times were 4.3 min for GS4057 and 5.2 min for GS4071.

2.3. Solutions

Stock solutions (0.25 mg/ml each) of GS4071 and GS4057 were prepared in 50 mmol/l NaH_2PO_4. For working standards, the appropriate volumes of the GS4071 stock solution were diluted with the 50 mmol/l NaH_2PO_4 solution to obtain seven standards with GS4071 concentrations ranging from 25 to 1600 ng/ml. The internal standard solution (1 μg/ml GS4057) was also prepared in the 50 mmol/l NaH_2PO_4 solution. The NDA solution was prepared in acetonitrile (20 mmol/l) and was stable for at least a month when refrigerated in an amber vial. A solution of KCN (20 mmol/l) was prepared in 200 mmol/l phosphate buffer, pH 6.5, and was stable for at least a month when refrigerated.

2.4. Solid-phase extraction

Extraction cartridges (C_{18}, 100 mg, Varian, Harbor City, CA, USA) were conditioned with 1 ml of the elution solvent (75% acetonitrile–25% water solution) followed by 1 ml of the wash buffer (0.01 *M* HCl in 5% acetonitrile–95% water solution). Prior to extraction, plasma samples were acidified with citric acid (25 μl of 1 mol/l solution to 100 μl of plasma). The acidified plasma samples were mixed with 100 μl of the internal standard solution and loaded onto the cartridges. The loaded cartridges were then washed with 1 ml of the wash solvent and the analytes were selectively eluted with 400 μl of the elution solvent into 1.5 ml screw-cap centrifuge tubes.

2.5. Derivatization procedure

A 50 μl volume of the KCN/phosphate solution was added to each tube containing the eluant from the solid-phase extraction cartridges followed by 50 μl of the NDA solution. The tubes were vortex mixed, capped and incubated at 40°C for 45 min. After incubation, the samples were evaporated to dryness under reduced pressure at room temperature (SpeedVac sample concentrator, Savant, Farmingale, NY, USA). Following evaporation, the samples were reconstituted in 100 μl of the 50 mmol/l NaH_2PO_4 solution, briefly centrifuged and transferred into autosampler vials for HPLC analysis.

2.6. Derivatization efficiency and recovery

To determine the derivatization efficiency of the method, the same procedure was employed using [2-acetyl-^{14}C] GS4071. Sufficient amounts of the corresponding unlabeled and labeled compounds were used to obtain a concentration of 400 ng/ml and a specific activity of 1 μCi/ml in plasma standards and aqueous standards. The radiolabeled plasma standards were extracted, derivatized and reconstituted as described above. The processed plasma samples and underivatized aqueous standards were injected into the HPLC system with a Radiomatic scintillation flow detector. To assess the derivatization efficiency, the peak area (cpm) of the desired cyano[*f*]benzoisoindole derivative was compared to the total peak area of derivatized plasma sample chromatograms. To determine the recovery of the method, the total peak areas of the derivatized plasma samples were compared to the peak areas of the corresponding unprocessed aqueous standards.

3. Results and discussion

3.1. Derivatization optimization

Cyano[*f*]benzoisoindole derivatives are well suited for reversed-phase HPLC analysis because they are highly fluorescent and significantly more lipophilic than the original primary amine compounds, which often show tailing on reversed-phase sorbents. Furthermore, the reaction of primary amines with NDA in the presence of KCN is readily adaptable for precolumn derivatization. In order to improve the accuracy and precision of the method, we used a cyclopentyl analog of GS4071 (GS4057) as an internal standard. This analog possesses similar physico-chemical properties to those of GS4071,

allowing its simultaneous derivatization by the NDA reagent to a fluorescent compound as well as good separation from GS4071.

Fig. 2 shows the excitation (A) and emission spectra (B) for the cyano[*f*]benzoisoindole derivative of GS4071. The spectra were taken ''on-the-fly'' after injecting the derivatized 200 ng/ml aqueous standard.

To optimize the conditions for derivatization, several parameters of the reaction were investigated, including concentrations of NDA and KCN, pH, temperature, and organic solvent. The effects of these variables on the reaction were estimated by comparing the relative rate of formation of the derivative by direct injection of the reaction mixture (5 μl) into the HPLC system. The fluorescence responses of the corresponding derivative peaks were monitored at their respective maximum excitation and emission wavelengths. To examine the effect of pH and organic solvents on the reaction yield, various mixtures of phosphate buffers and acetonitrile were used at fixed temperature (40°C). The presence of an organic solvent was necessary to dissolve the NDA in reaction mixture. No apparent change in the reaction yield was detected within the range of 20–75% acetonitrile. The fluorescence response remained constant over the pH range of 7–8, but gradually decreased outside of that range (at pH 5 and pH 9, the fluorescence responses were about 50% of the maximum value). The reaction yield was the highest at pH values around 7.5. However, the rate of chemical hydrolysis of GS4104 to GS4071 also increased at elevated pH (8% conversion at pH 7.5 versus 0.5% conversion at pH 6.4). In order to minimize the conversion to GS4071 while maintaining a reasonable yield, pH 6.5 was used for the reaction.

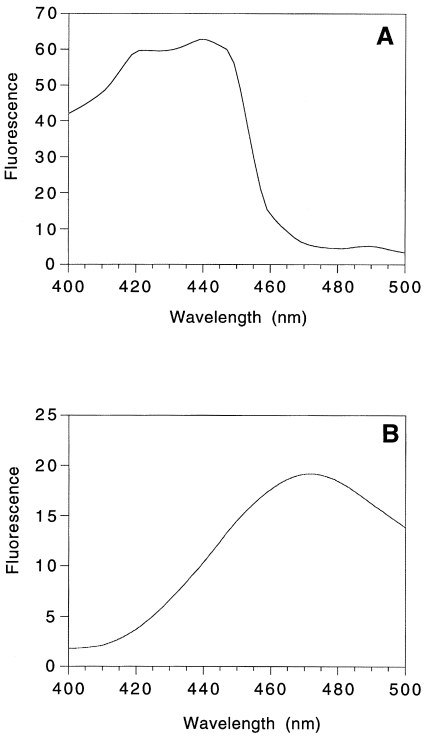

Fig. 2. Excitation–emission spectra of the fluorescent derivative of GS4071 [(A) excitation spectrum, (B) emission spectrum]. Conditions are described in the Experimental section.

The temperature of the reaction had a significant impact on the reaction rate. At 90°C the reaction was complete in about 5 min, and at 40°C the reaction required about 45 min for completion. In rat plasma, GS4104 is rapidly converted to GS4071 by esterases and therefore no unconverted prodrug is anticipated to be present in rat plasma samples [6]. However, in dog, marmoset and human plasma, GS4104 is more enzymatically stable and some unconverted prodrug may be present in plasma samples [6]. In order to make the assay applicable to analysis of both GS4071 and the prodrug in plasma from different species, the reaction conditions were optimized to reduce the possibility of chemical conversion of intact GS4104 to GS4071. Increased temperature may increase the rate of chemical hydrolysis of GS4104 to GS4071, therefore lower temperature with longer reaction time were ultimately selected.

In summary, the highest overall yield for derivatization of GS4071 was achieved in the phosphate buffer, pH 6.5, with 70% of acetonitrile at 40°C for 45 min (Fig. 3). Higher temperatures should allow derivatization to proceed more rapidly (approximately 5 min) but they can only be used when no prodrug is present.

3.2. Solid-phase extraction of GS4071 from rat plasma

Both GS4071 and its internal standard GS4057 possess an ionizable carboxylic acid group and, as a

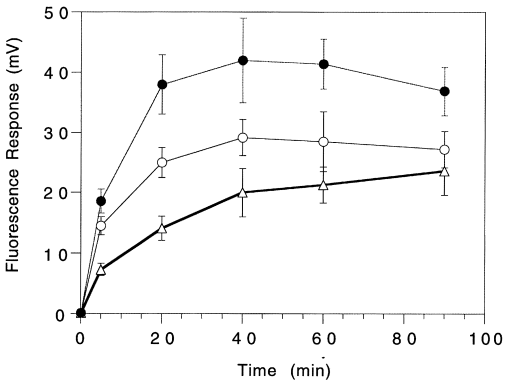

Fig. 3. Time course of the reaction of NDA with GS4071. Values shown are mean±SD, $N=3$ [(△) 40°C, (○) 60°C, (●) 90°C]. The remaining reaction and chromatographic conditions are as described in the Experimental section.

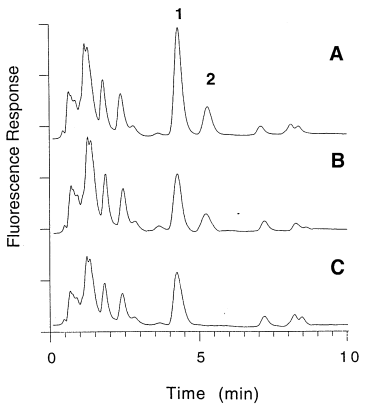

Fig. 4. Representative chromatograms of (A) derivatized plasma sample taken from a rat, 4 h following oral administration of 30 mg equiv./kg GS4104; (B) derivatized plasma sample spiked with 100 ng/ml GS4071 (40 μl injection); (C) derivatized blank rat plasma. Samples in chromatograms A, B and C were spiked with 1 μg/ml IS. Chromatographic and derivatization conditions are as described in the Experimental section. Peak identification: (1) GS4057 (internal standard); (2) GS4071.

result, their retention on reverse-phase sorbents is pH-dependent. Lowering pH below 3 protonates the carboxylic acid group and greatly improves the retention of GS4071 and GS4057. The use of an acidified matrix and acidic wash buffer on C_{18} extraction cartridges allows selective retention of both compounds. The final eluent from the cartridges (75% acetonitrile–25% water) is well suited for direct derivatization after the addition of the KCN/phosphate and NDA solutions.

3.3. HPLC separation of the GS4071 derivative in plasma

Isocratic elution provided good separation of the analyte and IS from endogenous plasma peaks. A representative chromatogram of a derivatized plasma sample spiked with 200 ng/ml GS4071 is displayed in Fig. 4A. A chromatogram of a plasma sample obtained from a rat 4 h after oral administration of 30 mg/kg GS4104 is shown in Fig. 4B. An example of a derivatized drug-free plasma sample is displayed in Fig. 4C. All plasma samples were spiked with 1 μg/ml IS. The total chromatography time was 10 min, the peaks were symmetrical and well resolved, and the baseline was free from interfering peaks. Chromatographic conditions used in this study have been optimized for rapid analysis of GS4071 in a large number of pre-clinical rat plasma samples, but mobile-phase composition, column temperature and length can be easily modified to allow simultaneous analysis of GS4071 and the prodrug, GS4104, in dog, marmoset, or human plasma.

3.4. Assay validation

The method was validated by performing replicate analyses ($n=5$) on three separate days of pooled rat plasma quality control standards spiked with GS4071 (50, 400, and 1000 ng/ml). Concentrations were determined using the respective standard curves prepared on the day of analysis over the concentration range 25–1600 ng/ml. The precision and the overall accuracy of the method were estimated (Table 1). The within-day precision, defined as the mean of the daily coefficients of variation at each concentration ($n=5$), ranged from 4.5 to 16.9%. The between-day precision, expressed as the coefficient of variation of the pooled three-day data at each concentration ($N=15$), was in the range 2.1–16.9%. The accuracy of the method, expressed as the ratio of actual to predicted concentration (C-ratio), was in the range 98.3–104.0%. To confirm stability, the derivatized quality control standards were reinjected after 24 h at 4°C and after 1 week at −20°C. The

Table 1
Accuracy and precision data for the determination of GS4071 in rat plasma using precolumn derivatization with NDA

Concentration (ng/ml)	Day	Assay (mean±SD, N=5) (ng/ml)	Within-day precision[a] (mean±SD, N=5) (%)	Between-day precision[a] (N=15) (%)	Accuracy[a] (mean±sd, N=5) (%)
50	1	60.8±7.3	16.9±3.9	16.9	104.0±6.3
	2	46.5±8.0			
	3	52.0±6.5			
400	1	386.6±19.8	9.4±3.2	7.9	98.3±1.5
	2	391.1±15.4			
	3	407.6±26.1			
1000	1	995.6±25.3	4.5±0.5	2.1	99.8±0.9
	2	1022.0±16.2			
	3	984.8±28.2			

[a]The within-day, between-day precision and accuracy are defined in the text.

derivatives were stable in the 50 mmol/l NaH_2PO_4 solution under the tested conditions.

The specificity of the method was assessed by comparing chromatograms of derivatized blank plasma from rats, mice, ferrets, dogs and humans. All chromatograms were free of interfering peaks and endogenous compounds were well separated from the GS4057 and GS4071 peaks.

3.5. Linearity, limit of detection and limit of quantitation

Peak area ratios of GS4071 to the internal standard were plotted versus drug concentrations and the slopes and intercepts of the standard curves were estimated by least-squares linear regression. Standard curves from 25 to 1600 ng/ml GS4071 were linear and gave correlation coefficients better than 0.997. The method detection limit, determined at a signal-to-noise ratio of 3, was 20 ng/ml. The detection limits on column were 1 ng (ca. 3 pmol) for GS4071. The limit of quantitation of the method defined as the lowest concentration providing at least 80% accuracy and 20% precision was 50 ng/ml (concentration of the low quality control standards). The limit of quantitation can be lowered at least 5- to 10-fold (5–10 ng/ml) for GS4071 in plasma of larger animals or humans by simply increasing plasma volumes used for analyte isolation (0.5–1 ml of plasma can be loaded onto the solid-phase extraction cartridges, whereas volumes of rat plasma available from multiple point pharmacokinetic studies are typically limited to 150–200 μl).

3.6. Recovery and derivatization efficiency

Radiochromatograms of underivatized ^{14}C GS4071 in aqueous solutions were compared with derivatized plasma standards. Over 85% of radiolabeled GS4071 (85.1±8.7%, N=3) was converted to its fluorescent derivative, with no other radioactive reaction products. Comparison of the peak area of the desired derivative to the peak area of the underivatized standards gave the total method recovery of >68% (68.3±11.2%, N=3) over the entire concentration range.

4. Conclusions

In conclusion, a simple, rapid and robust method has been developed and validated for the determination of GS4071 in pre-clinical plasma samples. The selectivity of the method eliminates the need for extensive clean-up of biological samples and, since the NDA reagent is not fluorescent, the assay does not require any additional steps for reagent removal. In addition, the method can be readily modified to allow accurate estimation of the pharmacokinetic parameters of the novel anti-influenza compound in humans following oral administration of its prodrug.

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