ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1995) 191–201

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Determination of the novel sialic acid analog GG167 (GR121167X) in human urine by liquid chromatography: direct injection with column switching☆

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Received for review 25 April 1995, revised manuscript received 21 July 1995

Abstract

GG167 is a novel compound which selectively inhibits viral neuraminidase and has demonstrated activity against influenza A and B. A liquid chromatography (LC) method for the determination of GG167 in human urine has been developed and validated.

The method allows direct injection of urine (7 μl) using LC column switching followed by UV detection. Initial chromatography is performed using a Nucleosil-Diol column (7 μm, 250 mm × 4.6 mm), eluted with 20 mM phosphate buffer (pH 2.5):acetonitrile (18:82, v/v) at 2.0 ml min^{-1}. GG167 is "heart-cut" to a Spherisorb-SCX column (5 μm, 100 mm × 4.6 mm) and eluted with 35 mM phosphate buffer (pH 2.5):acetonitrile (50:50, v/v) at 1.5 ml min^{-1} for final separation. GG167 is detected by UV absorbance at $\lambda = 238$ nm. UV detection and peak shape are enhanced at pH < 2.5. The quantitation range of the assay is 0.3–100 μg ml^{-1}. The method has demonstrated sufficient ruggedness to be used in support of GG167 clinical trials.

Keywords: GG167; Neuraminidase inhibitor; Influenza virus; Urine; Polar solid phase; Ion exchange; Direct injection; Column switching

1. Introduction

GG167 (Fig. 1), 5-acetylamino-4-guanidino-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enoic acid, is a novel sialic acid analog and 4-guanidino derivative of 2-deoxy-2,3-didehydro-D-*N*-acetyl-neuraminic acid and has been shown to be a potent and highly specific inhibitor of influenza neuraminidase activity and virus replication in vitro [1–5]. Studies of experimental murine and ferret influenza have found that topically applied GG167 has antiviral activity in vivo [1,6]. Intranasal administration has been determined to be safe and effective for both prevention and early treatment of experimental human influenza [7]. Clinical trials to establish the potential therapeutic use of GG167 for treatment of patients with naturally acquired infection are in progress.

☆ Presented at the Sixth International Symposium on Pharmaceutical and Biomedical Analysis, April 1995, St. Louis, MO, USA.

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SSDI 0731-7085(95)01626-0

A method for the analysis of GG167 concentrations in human urine was desired that would have sufficient sensitivity and ruggedness to support clinical trials. Analogues of *N*-acetylneuraminic acid have typically been chromatographed on reverse phase silica-based columns [8–10]. However, due to the extreme polarity of GG167, these more conventional approaches proved difficult. Analysis of GG167 in urine has been previously accomplished using porous graphitic carbon-filled capillary liquid chromatography, with reverse phase gradient elution, followed by electrospray mass spectrometry over the range of 1–50 μg ml^{-1} [11]. Alternative approaches were desired which would extend the concentration range and allow the use of conventional LC equipment. Efforts using polar supports and mixed mode separations seemed to offer greater latitude for the chromatography of GG167. Initial attempts with direct injection on LC of urine spiked with GG167 using C_1, Diol, and Cation-Exchange supports, all yielded chromatography which was not free of interferences and was variable in terms of sensitivity. To support clinical trials a rugged method was required which would use the same advantages of direct injection but with on-line (automated) sample pretreatment to enhance the sensitivity and reproducibility of the assay. LC column switching using a combination of chromatography on polar supports and ion exchange supports allowed the requirements above to be fulfilled.

The concepts and utility of column switching and the use of multiport valves to accomplish the switching are well known [12–16]. An automated method for the determination of GG167 in human urine using two six-port column switching valves to perform sample pretreatment and final quantitative chromatography is described. The valve configuration allowed rapid method development and simple automation. The method performed direct injection of urine with LC and UV detection. The method was linear over the range 0.3–100 μg ml. Validation of the method demonstrated acceptable reproducibility, sample throughput, and freedom from interferences.

2. Experimental

2.1. Instrumentation

The LC system consisted of a Waters 600 controller and fluidics unit (Millipore, Milford, MA) modified to accommodate a Waters column heater (Millipore, Milford, MA) and two Model 7000 six-port valves with pneumatic actuators and solenoid valves (Rheodyne, Cotati, CA). The Waters 600 controller served to perform the column switching through programmed external events. The Waters 600 fluidics unit was designated pump 1 for pumping mobile phase 1 (20 mM phosphate (pH 2.5):acetonitrile (ACN) (18:82, v/v)). A Waters 510 pump (Millipore, Milford, MA) was designated as pump 2 for pumping mobile phase 2 (35 mM phosphate (pH 2.5):ACN (50:50, v/v)). Injections were made by a Waters model 715 Ultra-WISP (Millipore, Milford, MA). The Nucleosil-Diol (250 mm × 4.6 mm, 7 μm) and Spherisorb-SCX (100 mm × 4.6 mm, 5 μm) chromatography columns were obtained from Keystone Scientific (Bellefonte, PA). A Waters model 486 UV absorbance detector (Millipore, Milford, MA) was set to a wavelength of 238 nm for all analyses. Data were acquired via an HP35900A A/D interface and converter (Hewlett-Packard, Palo Alto, CA) to an HP 1000 model A900 computer (Hewlett-Packard, Palo Alto, CA). All chromatography was analyzed using an HP3350 Laboratory Automation System (LAS; rev.E.00.00)

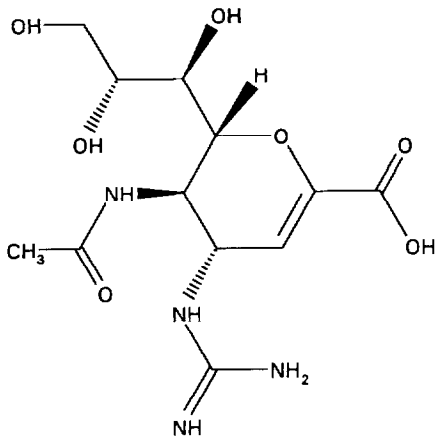

Fig. 1. Structure of GG167.

(Hewlett-Packard, Palo Alto, CA). Chromatograms which required reintegration were processed through automatic or manual definition using the HP19471A Advanced Graphic Chromatogram Processor (LPLOT; rev.E.00.00 (Hewlett-Packard, Palo Alto, CA)). Linear regression calculations were made using Post-Run Analysis of Bioanalytical Samples software (PRANBAS; rev.02.03.00) (Glaxo Inc, Research Triangle Park, NC). Linear regression analysis with $1/x$ weighting was used to derive the external standard curves from analyte area counts.

2.2. Chemicals

GG167 (GR121167X) was synthesized by Glaxo Group Research (Ware, Hertfordshire, UK). Potassium phosphate was from Aldrich Chemical (Milwaukee, WI). Acetonitrile was high purity grade from Burdick & Jackson (Muskegon, MI). Deionized water was prepared by a Milli-Q system from Millipore (Marlborough, MA). Drug-free control urine was obtained from willing, healthy volunteers. Sparge gas was helium, high purity grade from National Specialty Gases (Durham, NC).

2.3. Preparation of mobile phase

Mobile phase 1 was prepared in the following manner: 5.44 g of potassium phosphate (KH_2PO_4) was dissolved with 1800 ml of deionized water, the pH was adjusted to 2.5 with concentrated phosphoric acid, and the volume adjusted to 2 l with deionized water. The phosphate buffer (1080 ml) was mixed with acetonitrile (4920 ml) then filtered (0.45 μm) before use. Final composition was 20 mM phosphate (pH 2.5):ACN (18:82, v/v).

Mobile phase 2 was prepared in the following manner: 9.52 g of potassium phosphate (KH_2PO_4) was dissolved with 1800 ml of deionized water, the pH was adjusted to 2.5 with concentrated phosphoric acid, and the volume adjusted to 2 l with deionized water. The phosphate buffer (2000 ml) was mixed with acetonitrile (2000 ml) then filtered (0.45 μm) before use. Final composition was 35 mM phosphate (pH 2.5):ACN (50:50, v/v).

2.4. Preparation of GG167 urine calibration standards

A 1.0 mg ml^{-1} stock solution was made by dissolving 5.0 mg of GG167 in 5.0 ml of drug-free control urine. Calibration standard samples were prepared on the day of analysis from the 1.0 mg ml^{-1} urine stock by diluting with drug-free control urine. The concentrations used for the standard curve were 0.3, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 μg ml^{-1}.

2.5. Preparation of GG167 quality control (QC) samples

Quality control sample sets (A and B) were prepared in bulk (25 ml) by two chemists from independently prepared 1.0 mg ml^{-1} urine stock solutions. QC set A was prepared by chemist 1 and B was prepared by chemist 2. The concentrations of each QC set were 0.75, 1.5, 2.5, 25.0 and 75.0 μg ml^{-1}. All QC samples were separated into 400 μl aliquots and stored at $\leqslant -30$ °C until the day of analysis.

2.6. Method description

The system used the six-port valves to direct the sample flow between two LC columns (schematically represented in Fig. 2). Urine (7 μl) was injected directly onto Column 1 (Nucleosil-Diol, 250 mm × 4.6 mm, 7 μm). Column 1 was eluted with mobile phase 1 (20 mM phosphate (pH 2.5):ACN (18:82, v/v)) at 2.0 ml min^{-1} using Pump 1. The GG167 peak was "heart-cut" to Column 2 (Spherisorb-SCX, 100 mm × 4.6 mm, 5 μm) and eluted with mobile phase 2 (35 mM phosphate (pH 2.5):ACN (50:50, v/v)), at 1.5 ml min^{-1} using Pump 2. Final chromatography was performed on Column 2 (Spherisorb-SCX, 100 mm × 4.6 mm, 5 μm). GG167 was eluted in a run time of 16–18 min (total run time = 20 min). Detection was by UV absorbance at $\lambda = 238$ nm.

Four analytical runs generated on four separate days were included in the validation. Analytical runs consisted of calibration standards (0.3, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 μg ml^{-1}) in dupli-

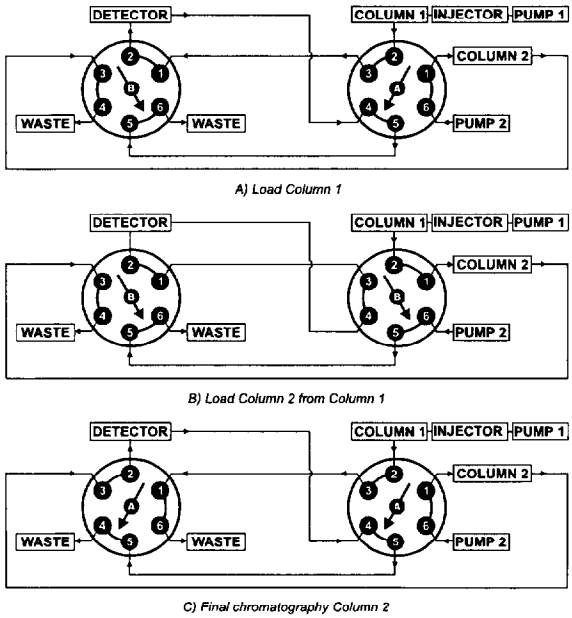

Fig. 2. Schematic of configuration of the column switching valves during the stages of operation. (A) Initial chromatography is performed using a Nucleosil-Diol column (7 μm, 250 mm × 4.6 mm) eluted with 20 mM phosphate buffer (pH 2.5):acetonitrile (18:82, v/v) at 2.0 ml min^{-1}. (B) GG167 is "heart-cut" to a Spherisorb-SCX column (5 μm, 100 mm × 4.6 mm) and eluted with 35 mM phosphate buffer (pH 2.5):acetonitrile (50:50, v/v) at 1.5 ml min^{-1}. (C) Final chromatography is performed using a Spherisorb-SCX column (5 μm, 100 mm × 4.6 mm) and eluted with 35 mM phosphate buffer (pH 2.5):acetonitrile (50:50, v/v) at 1.5 ml min^{-1}. UV detection occurs during the loading of column 1 (A) and during the final chromatography of column 2 (C), but not during elution of the tandem columns (B).

cate and two sets of quality control samples (0.75, 1.5, 2.5, 25.0, and 75.0 μg ml^{-1}) in duplicate. Additional urine samples were included in each analytical run. Analytical runs consisted of ⩽96 total samples. One set of calibration standards was analyzed at the beginning of a run and a second set at the end of a run. Quality control samples and stability samples were interspersed throughout the analytical run to monitor method performance.

The stability of GG167 in urine was determined. A total of six samples of each concentration were exposed at each of the following conditions: (a) room temperature (RT) for 14 days (using 1, 10, and 100 μg ml^{-1}); (b) +4 °C for 41 days (using 0.75, 25.0, and 75.0 μg ml^{-1}); and (c) freeze–thaw (two-cycles, using 0.75, 25.0, and 75.0 μg ml^{-1}). The samples were analyzed after exposure to the different temperatures and time periods. The interpolated concentrations of the GG167 peaks were compared to the corresponding control samples for each set. Stability was expressed as a percent difference from the control.

The recovery of GG167 from urine during the course of the column switching was considered to be 100%. Undiluted urine (7 μl) was injected directly onto the chromatography column without loss from any sample preparation steps.

3. Results

3.1. Chromatography

Separation of GG167 from the endogenous constituents of urine was performed using a Nucleosil-Diol column, as column 1 with a mobile phase of 20 mM phosphate buffer pH 2.5:acetonitrile (18:82, v/v) at 2.0 ml min^{-1} from pump 1. The effluent from the Nucleosil-Diol column was monitored by UV at $\lambda = 238$ nm. The run time for elution of GG167 on the Nucleosil-Diol column had been determined to be approximately 13.0–13.5 min by monitoring the chromatography of a pure reference sample of GG167 without column switching. One of the six-port valves was programmed to allow the effluent from the Nucleosil-Diol column (column 1), containing GG167, to pass to the Spherisorb-SCX column (column 2) prior to this predetermined run time (12 min). The effluent from the tandem columns was allowed to pass to waste as GG167 was loaded ("heart-cut") from the Diol to the SCX column. After GG167 had been transferred (14.5 min) to the SCX column, both six-port valves were switched to allow the SCX column to be eluted with a mobile phase of 35 mM phosphate buffer (pH 2.5):acetonitrile (50:50, v/v) at 1.5 ml min^{-1} from pump 2 for final separation and the effluent monitored by UV at $\lambda = 238$ nm. GG167 was completely baseline-resolved with a signal (peak area) for the 0.3 μg ml^{-1} standard of 79 times ($n = 8$) the signal

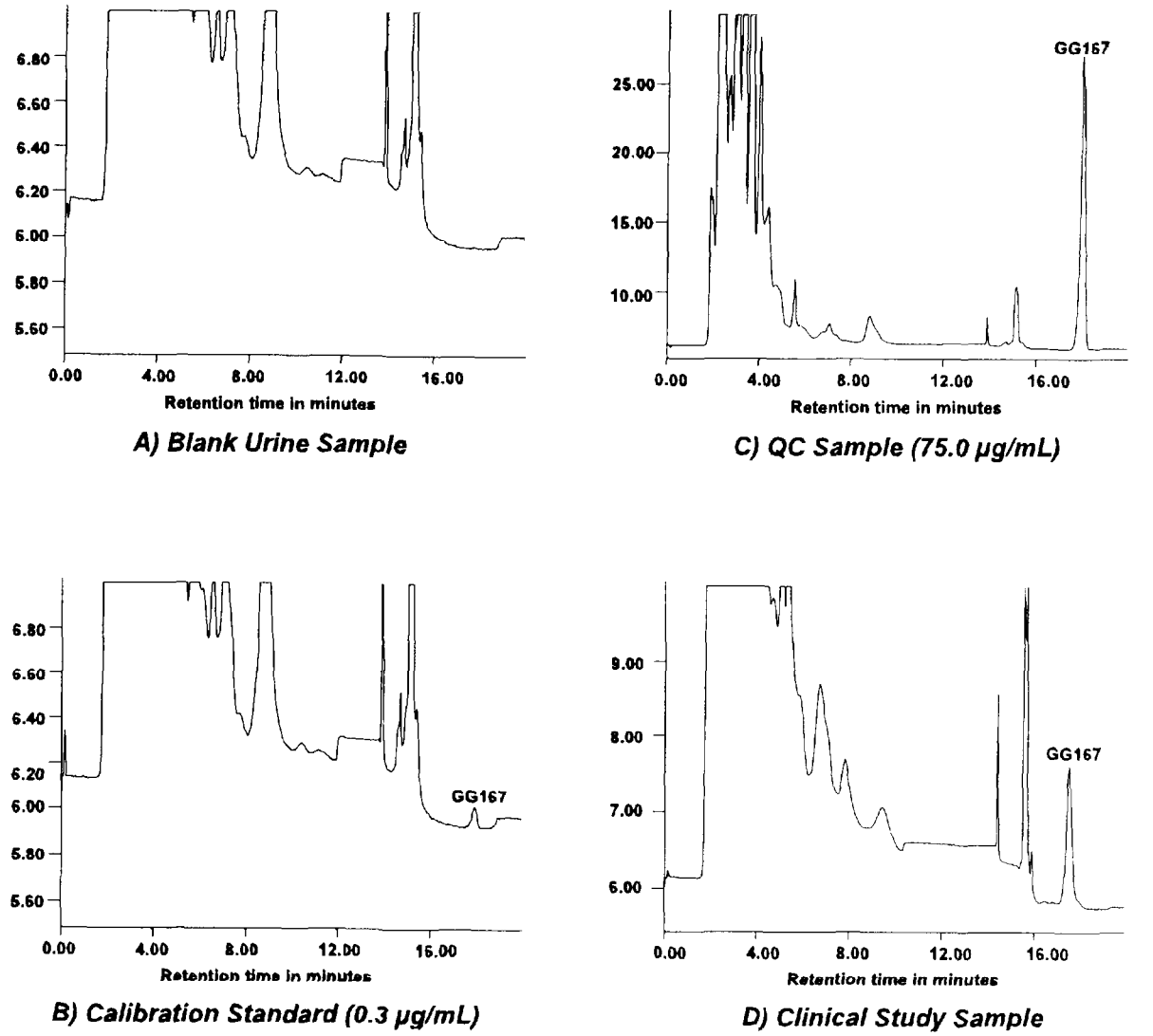

Fig. 3. Representative chromatography (A) Separation of drug-free control urine. (B) Separation of 0.3 µg ml^{-1} of GG167 in control urine. (C) Separation of 75.0 µg ml^{-1} of GG167 in control urine. (D) Separation of GG167 from clinical study patient urine. Sample volume was 7 µl. Full chromatographic conditions are described in the text. Column switching from column 1 (Diol) to column 2 (SCX) occurred at 12 min. Chromatography from 0–12 min is the elution of column 1 (Diol) at 2 ml min^{-1} with mobile phase 1. Chromatography from 12–14.5 min is the elution of column 1 (Diol) in series with column 2 (SCX) with mobile phase 1. Chromatography from 14.5 min to end of run is elution of column 2 (SCX) at 1.5 ml min^{-1} with mobile phase 2. UV detection occurs during the loading of column 1 (Diol) and during the final chromatography of column 2 (SCX), but not during elution of the tandem columns.

of a drug-free, blank urine sample as determined during the validation exercise.

3.2. Precision and accuracy

Peak area responses were measured for the quantitation of GG167. Representative chromatography is shown in Fig. 3(a–d). The 0.3 µg ml^{-1} GG167 peak was well resolved from all endogenous peaks. As noted above, the GG167 peak for a 0.3 µg ml^{-1} sample had an area 79 times ($n = 8$) the area of blank urine

Table 1
Standard curve samples

Run number	Concentration (μg ml^{-1})						
	0.3	0.5	1.0	5.0	10.0	50.0	100.0
1	0.28	0.53	1.00	5.03	10.48	49.27	104.20
	0.31	0.49	0.99	4.88	9.92	48.08	98.13
2	0.32	0.57	0.90	5.30	9.63	49.13	102.10
	0.30	0.55	0.86	4.87	9.69	48.19	101.10
3	0.25	0.53	1.13	4.82	9.87	47.96	97.55
	0.24	0.55	1.06	–[a]	10.95	51.53	102.20
4	0.30	0.45	0.95	4.94	10.13	49.76	102.40
	0.32	0.53	1.00	4.97	10.20	49.16	103.10
Mean	0.29	0.52	0.99	4.97	10.11	49.14	101.35
SD	0.032	0.038	0.085	0.160	0.439	1.167	2.343
% RSD	11.0	7.3	8.6	3.2	4.3	2.4	2.3
n	8	8	8	7	8	8	8
% Nominal	96.6	105.0	98.5	99.5	101.1	98.3	101.3

[a] Outside acceptance range, not included in calculations.

(baseline noise at the runtime of GG167). The retention time for GG167 was approximately 17 min. A weighted linear regression ($1/x$) was used for the calibration and quantitation. Daily calibration data demonstrated acceptable accuracy with all calibrators yielding interpolated concentrations at >96% of the expected nominal concentration (Table 1). The calibrator concentration groups had % RSD ranging from 2.3 to 11.0 over the concentration span. All standard curves for each of the four validation runs exceeded the target correlation coefficient requirement for linearity of 0.99 with the lowest r value being 0.9994. Table 2 shows the slope, intercept and correlation coefficients for each analytical run in the validation.

Table 2
Calibration curve regression ($1/x$ weighting)

Run number	Slope	Intercept	r
1	7508.6	−196.5	0.9995
2	7584.6	458.8	0.9996
3	7976.9	116.5	0.9994
4	7652.8	492.4	0.9998

All QC samples yielded interpolated concentrations within 10% of the expected values at each level. All QC samples demonstrated acceptable precision at all concentration levels with % RSD values ranging from 9.7 at the lowest QC concentration (0.75 μg ml^{-1}) to 4.2 at the highest QC concentration (75.0 μg ml^{-1}) (Table 3).

3.3. Stability

GG167 was stable under exposure conditions potentially encountered during the conduct of clinical analysis. GG167 was shown to be stable in urine with <10% difference in the interpolated concentrations versus control over the range of temperature and time exposures (Tables 4–6). In all cases, the interpolated concentrations at each concentration level and for each exposure condition were within 90% of nominal.

3.4. Specificity

Prior to validation, drug-free control urine samples were screened for possible interferences at the elution time of GG167. Urine samples from six normal, healthy, donors and six predose clinical

Table 3
Assay precision for Quality Control concentrations[a]

Run number	Chemist	QC Concentration (μg ml^{-1})				
		0.75	1.5	2.5	25.0	75.0
1	A	0.79	1.54	2.50	21.88[b]	72.98
		0.80	1.44	2.39	22.25[b]	78.71
	B	0.77	1.50	2.57	25.05	75.62
		0.82	1.44	2.33	24.62	73.49
2	A	0.80	1.29[b]	2.40	24.36	73.56
		0.70	1.36	2.40	24.06	71.70
	B	0.76	1.36	2.14[b]	22.78	73.82
		0.64	1.39	2.28	22.94	73.56
3	A	0.68	1.49	2.21	21.98[b]	66.61
		0.81	1.32[b]	2.51	22.58	77.55
	B	0.62	1.52	2.37	22.94	77.79
		0.73	1.37	2.34	22.60	70.07
4	A	0.68	1.53	2.48	23.72	73.69
		0.83	1.32[b]	2.20[b]	22.54	71.39
	B	0.67	1.57	2.50	24.09	73.25
		0.84	1.37	2.26	22.25[b]	70.96
Mean		0.75	1.42	2.37	23.16	73.42
SD		0.072	0.089	0.126	1.003	3.057
% RSD		9.7	6.3	5.3	4.3	4.2
n		16	16	16	16	16
% Nominal		99.4	95.0	94.7	92.7	97.9

[a] Quality control (QC) samples were analyzed in each of four validation runs. QC sample sets (A and B) were prepared by two chemists from independently prepared 1.0 mg ml^{-1} GG167-urine stock solutions and stored frozen until analyzed.
[b] Outside acceptance range, included in calculations.

study subjects were analyzed by the method. Chromatography at the elution time of GG167 was free of any interferences which could be misinterpreted as drug-based.

4. Discussion

The use of column switching as automated sample pretreatment for GG167 is effective. Traditional reverse phase approaches did not allow sufficient retention of GG167 from endogenous material. Separation of GG167 on various C_1, Diol, and SCX supports without column switching did not achieve the resolving power necessary for the method to support clinical sample analysis. Ultimately, separation on a Nucleosil-Diol column yielded good retention of GG167 with the majority of endogenous peaks eluting before the GG167 peak. By "heart cutting" the GG167 peak region from the Nucleosil-Diol column to the Spherisorb-SCX column, the GG167 peak became well separated from other endogenous interferences. It is believed that the separation of GG167 on the Nucleosil-Diol column proceeds largely by a polar–non-polar interaction mechanism. Increasing organic strength (acetonitrile or other more non-polar solvents) serves to increase retention. To a smaller degree, pH does have an effect, with retention decreasing somewhat as pH increases. Separation of GG167 on the Spherisorb-SCX column, under the mobile phase conditions presented here, proceeds by a mechanism which is effected to a large degree by buffer strength, pH, and organic strength. Initially, the GG167 peak region is introduced to the Spherisorb-SCX column from the Nucleosil-Diol column, where it is briefly concentrated at the head of the column

during the transition from mobile phase 1 to mobile phase 2. This concentration effect was evidenced by a decrease in peak widths ($\approx 50\%$) of GG167 when chromatographed using the column switching combination described above, compared with GG167 chromatographed on Nucleosil-Diol alone. The peaks were narrower and more symmetrical using the column switching routine, even though the run time using the column switching was 25–35% longer. The Spherisorb-SCX column rapidly re-equilibrated to mobile phase 2 and discrete separation was achieved.

The effect of pH on the retention of GG167 was important on both the Diol and SCX columns. At lower pH levels, GG167 becomes protonated, and was more retained by active sites on both supports. The ionic strength of both mobile phase 1 and mobile phase 2 was kept as high as possible to keep the relative pH low to enhance retention. Ionic strengths sufficient to achieve an adequate degree of buffering in the presence of high acetonitrile concentrations are however not without problems. The solubility of potassium phosphate changes in these mobile phases as a function not only of acetonitrile, but also as a function of temperature and system pressure. Initial efforts with mobile phase 1 used potassium phosphate concentrations of 25 mM. Such concentrations behaved well at room temperature or higher, but fine precipitates began forming under pressure in the pumps and the autosampler, and eventually were laid down on the inlet frit of the column causing system high

Table 4
GG167 stability in urine (room temperature)[a]

Conditions	Concentration ($\mu g\ ml^{-1}$)		
	1	10	100
Control	1.01	9.87	97.07
	1.00	9.91	101.60
Mean	1.01	9.89	99.34
% of nominal	101.0	98.9	99.3
14 Days	0.96	9.31	96.08
Room temperature	0.92	9.61	97.94
with light exposure	0.98	9.62	98.05
	0.95	9.65	97.51
	0.89	9.83	98.95
	1.00	9.84	99.40
Mean	0.95	9.64	97.99
SD	0.040	0.193	1.166
% RSD	4.2	2.0	1.2
n	6	6	6
% of nominal	95	96	98
% Difference from control	−5.9	−2.5	−1.4

[a] Human urine spiked with various concentrations of GG167 was analyzed after exposure to the conditions indicated. The peak areas of GG167 from the exposed samples were compared with the GG167 peak areas from corresponding control samples. Stability is expressed as the percent difference as compared to stability control samples (day 0). A percent difference of $\pm 10\%$ was considered as acceptable stability.

Table 5
GG167 stability in urine (41 Days at 4 °C)[a]

Conditions	Concentration ($\mu g\ ml^{-1}$)		
	0.75	25	75
Control	0.69	23.33	70.03
	0.71	23.71	67.47
Mean	0.70	23.52	68.75
% of nominal	93.3	94.1	91.7
41 Days at	0.68	24.17	71.93
4 °C	0.77	24.83	73.36
	0.80	24.86	73.45
	0.75	24.82	70.87
	0.74	24.86	73.64
	0.77	24.97	73.42
Mean	0.75	24.75	72.78
SD	0.041	0.290	1.123
% RSD	5.4	1.2	1.5
n	6	6	6
% of nominal	100	99	97
% Difference from control	7.1	5.2	5.9

[a] Human urine spiked with various concentrations of GG167 was analyzed after exposure to the conditions indicated. The peak areas of GG167 from the exposed samples were compared with the GG167 peak areas from corresponding control samples. Stability is expressed as the percent difference as compared to stability control samples (day 0). A percent difference of $\pm 10\%$ was considered as acceptable stability.

pressure shut-downs. Wide fluctuations in the ambient room temperature below 18 °C gave rise to similar precipitate formation and the eventual system high pressure shut-down. The optimal concentration of phosphate buffer for mobile phase 1 was determined to be 20 mM, as this concentration maintained pH sufficiently and did not lead to precipitation as described. Greater latitude was available in the choice of buffer concentration in mobile phase 2 as the acetonitrile concentration was less. Initial efforts with mobile phase 2 also used potassium phosphate concentrations of 25 mM; however, peak shape and size tended to vary from run to run. Chromatography of GG167 on the Spherisorb-SCX column was dramatically improved in terms of peak shape, reproducibility, and peak area using higher concentrations of phosphate buffer. The optimal concentration of phosphate buffer for mobile phase 2 was determined to be 35 mM, as this concentration kept the pH low enough to facilitate retention on the cation exchange support as well as yielding enhanced UV absorbance. In additional studies with GG167 an enhancement of $\geqslant 100\%$ in peak height and $\geqslant 33\%$ in peak area was observed at a true pH 2.0 when compared to a true pH 4.0. Although greater enhancement of UV absorbance was noted at a pH of 2.0, a pH of 2.5 was chosen to extend the life of the Diol column.

Table 6
GG167 stability in urine (freeze–thaw (two cycles))[a]

Conditions	Concentration ($\mu g\ ml^{-1}$)		
	0.75	25	75
Control	0.69	23.33	70.03
	0.71	23.71	67.47
Mean	0.70	23.52	68.75
% of nominal	93.3	94.1	91.7
Freeze–thaw	0.62	24.37	70.67
(two cycles)	0.69	24.33	70.52
	0.67	24.05	70.70
	0.74	23.85	69.19
	0.73	23.97	70.62
	0.73	24.02	70.60
Mean	0.70	24.10	70.38
SD	0.046	0.207	0.588
% RSD	6.7	0.9	0.8
n	6	6	6
% of nominal	93	96	94
% Difference from control	0.0	2.5	2.4

[a] Human urine spiked with various concentrations of GG167 was analyzed after exposure to the conditions indicated. The peak areas of GG167 from the exposed samples were compared with the GG167 peak areas from corresponding control samples. Stability is expressed as the percent difference as compared to stability control samples (day 0). A percent difference of $\pm 10\%$ was considered as acceptable stability.

The injection volume of urine was limited as a function of the high organic strength of mobile phase 1 (82% ACN (v/v)). Attempts were made to inject larger urine volumes; however, extreme peak deformations resulted. When chromatographed using only the Nucleosil-Diol column with mobile phase 1, injection of 20 µl resulted in near-baseline-resolved peak splitting. Fig. 4 demonstrates the effect of increased injection volume of an aqueous sample onto a high organic strength mobile phase system. Although peak area response was linear for the split peaks, it was impossible to negotiate the sample through the column switching system with reliability. Matrix matching of samples with mobile phase 1 was attempted in an effort to improve sensitivity with increased sample size; however, the advantages of increased organic material in the sample were offset by the time required to perform the dilution, the decrease in concentration, and occasionally by separation of the sample into two discrete phases. The injection volume was kept as low as possible to inhibit deformation of peak shapes due to the high organic concentration of mobile phase 1.

An area of objection for this approach to the analysis of GG167 in urine is the volume of mobile phase used. The use of stop flow, flow reduction, or recycling of mobile phases with the columns during the off cycle periods could make more efficient use of solvents. Smaller diameter columns could potentially increase sensitivity, and would most certainly decrease the amount of solvent waste by the system, although similar difficulties associated with injection volume as noted above could limit the use of the microbore diameters.

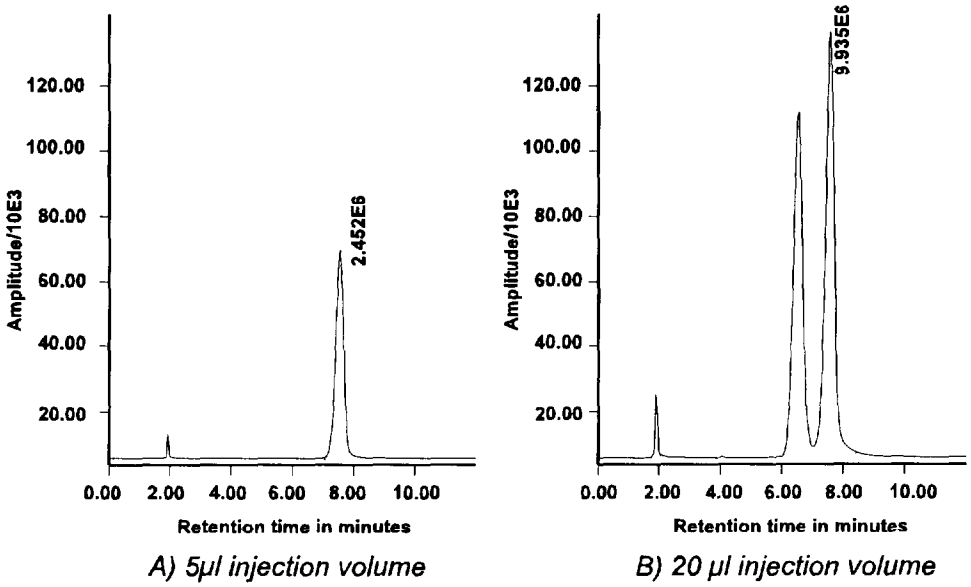

Fig. 4. Effect of increased injection volume of aqueous sample on peak shape. (A) Injection of 5 µl sample of 1000 µg ml^{-1} GG167 in 1% acetic acid. (B) Injection of 20 µl sample of 1000 µg ml^{-1} GG167 in 1% acetic acid. Chromatography performed using a Nucleosil-Diol column (7 µm, 250 mm × 4.6 mm) without column swtiching, eluted with 25 mM phosphate buffer (pH 2.5):acetonitrile (18:82, v/v) at 2.0 ml min^{-1}, and UV detection at $\lambda = 238$ nm. Peak area increased linearly from $\approx 2.5 \times 10^6$ with a single peak for the 5 µl injection to $\approx 10.0 \times 10^6$ with a split peak for the 20 µl injection. The effect was independent of concentration of GG167.

Further exploration of alternative chromatography columns may reveal solid phase supports which would improve the analysis of GG167. It is thought that perhaps a true mixed bed containing both polar and cation exchange supports could allow single column isocratic separation. It should be pointed out that the use of a two column, "heart-cut", column switching system can afford faster method development than searching through the vast array of different single column systems.

4.1. Clinical study utility

This automated method has been subsequently used in the analysis of GG167 in urine samples from phase-1 single center studies. Urine GG167 concentrations were analyzed to determine the systematic absorption of GG167 from intranasal administration for the treatment and prevention of influenza infection in healthy volunteers experimentally inoculated with influenza A virus. GG167 concentrations in urine samples, collected over a 3 h period after dosing, ranged from <0.3–20 µg ml^{-1}, 0–39% of the dose, which is consistent with previously reported pharmacokinetics in uninfected healthy subjects [17].

5. Conclusion

An assay for the determination of GG167 in human urine has been validated which uses direct on-column injection and column switching. The method is linear over the concentration range 0.3–100 µg ml^{-1}. The assay has been shown to be rugged and sensitive enough to support clinical trials. This method has been used to analyze GG167 in urine samples from subjects administered intranasal GG167 in clinical studies.

The utility of column switching in automated analysis of compounds in biological fluids is quite powerful. Column switching coupled with a reassessment of the utility of polar support chemistries and ion exchange support chemistries affords considerable resolving power for the separation of highly polar compounds in complex biological matrices.

Acknowledgments

The authors gratefully acknowledge the assistance of Dianne S. Calvert for her efforts with the graphics design of the valve Figure and presentation of Tables and Figures.

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