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Determination of zanamivir in rat and monkey plasma by positive ion hydrophilic interaction chromatography (HILIC)/tandem mass spectrometry

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Abstract

A hydrophilic interaction chromatography (HILIC)/mass spectrometric assay was developed for the determination of zanamivir, a neuraminidase inhibitor used to treat influenza, in rat and monkey plasma. An organic solvent with hydrophilic properties, methanol, was used to precipitate proteins in plasma to assure the highly polar zanamivir of staying in solution. Chromatographic separation was obtained using a HILIC silica column with multiple reaction monitoring turboionspray positive ion detection. The stable label of zanamivir, [$^{13}C_1$ $^{15}N_2$] GR121167C, was used as the internal standard. The assay was validated for the determination of zanamivir in rat and monkey plasma. The lower and upper limits of quantitation were 2 and 10000 ng/mL, using 0.05 mL plasma aliquot, respectively. The signal to noise ratio of a typical 2 ng/mL was ~5:1. The inter-day precision (relative standard deviation) and accuracy (relative error) in rat plasma, derived from the analysis of validation samples at 5 concentrations, ranged from 6 to 10% and −6.5 to 0.2%, respectively. The inter-day precision (relative standard deviation) and accuracy (relative error) in monkey plasma, derived from the analysis of validation samples at five concentrations, ranged from 2 to 8% and −2.3 to 2.1%, respectively. Zanamivir was found to be stable for at least 5 days at approximately −80 °C and at room temperature in plasma. This assay incorporates a simple protein precipitation with methanol and hydrophilic interaction chromatography which is sensitive, accurate, precise, and is being used to support oral formulation and toxicokinetic studies in rat and monkey, respectively.

Keywords: Zanamivir; Neuraminidase inhibitor; LC–MS/MS; Hydrophilic interaction chromatography (HILIC)

1. Introduction

Zanamivir (ZAM, Fig. 1) is a first in class neuraminidase inhibitor used to treat all strains of the influenza virus. Zanamivir has been shown to interact with a group of amino acids in the active site of neuraminidase which blocks its action, preventing release and spread of the newly formed virons [1]. Zanamivir has been shown to be effective in preventing, controlling, or rapidly reducing: illness with fever [2], influenza in family contacts [3], nursing home outbreaks [4], elevated body temperature [5], and viral load [6]. In vitro data suggests that zanamivir is a highly potent inhibitor of several strains of influenza A [7]; however, extremely low bioavailability limits efficacy following an oral dose, thus preventing the development of a conventional oral dosage form, the preferred formulation for children and the elderly [1]. The identification of a novel formulation that enhances the systemic exposure of zanamivir after oral administration would significantly increase its clinical utility and offer alternative treatment options to the public in the event of a pandemic.

There are several reported assays for the determination of ZAM in dog plasma and human serum [8–11]. These methods employ techniques such as SCX solid phase extraction, pre-column fluorescence derivatization, UV detection, and protein precipitation with 10% trichloroacetic acid (TCA) or acetonitrile. Although these techniques were effective for the aforementioned publications, extended preparation times, lack of sensitivity/selectivity, and potential for ZAM precipitation precluded their use in our work, which necessitated a 2 ng/mL limit of detection.

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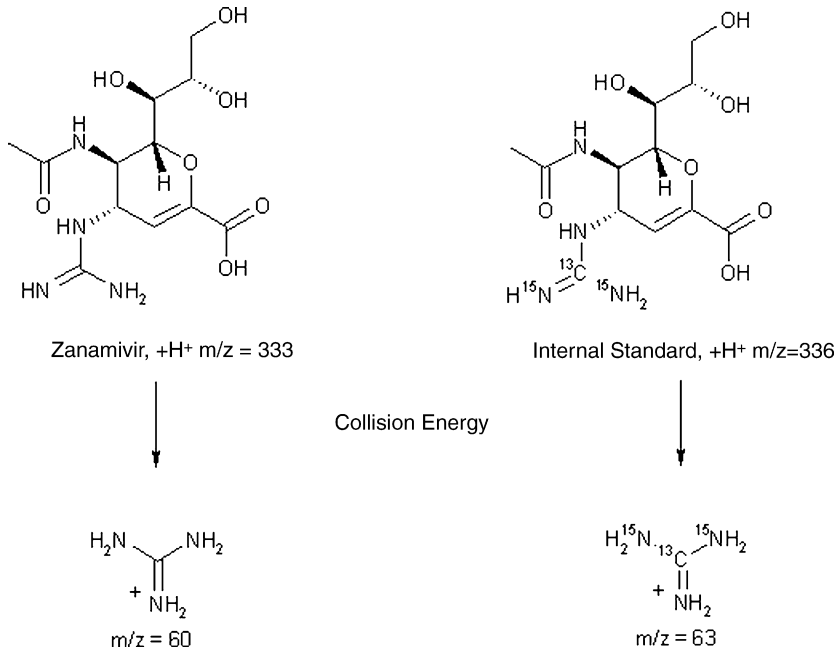

Fig. 1. Chemical structures of zanamivir and internal standard (GR121167C).

This method incorporates hydrophilic interaction chromatography (HILIC) silica, a stationary phase capable of retaining very polar compounds and improving sensitivity over reverse phase columns [12]. To date, there have been no validated methods or sample stability data published on zanamivir in rat and monkey plasma. This method will be used to support formulation and toxicokinetic studies in rat and monkey after oral administration.

To support the distribution and toxicokinetics of ZAM in rat and monkey plasma after oral administration this HILIC–MS–MS method was developed and validated over a concentration range of 2–10000 ng/mL, improving the lower limit of quantitation four fold over published values. The stable label of ZAM, [$^{13}C_1$ $^{15}N_2$] GR121167C, was used as the internal standard (Fig. 1).

2. Experimental

2.1. Chemicals and materials

ZAM and compound GR121167C (internal standard, IS) were obtained from Medicinal Chemistry at GlaxoSmithKline (Research Triangle Park, NC). Rat and monkey plasma was obtained from Bioreclamation (Hicksville, NY). HPLC grade methanol and acetonitrile were obtained from EMD Chemicals (Gibbstown, NJ). Analytical grade ammonium acetate was obtained from Mallinckrodt (Phillipsburg, NJ) and water was from a Millipore Milli-Q system (Bedford, MA).

2.2. Equipment

The HPLC system consisted of the following components: one Hewlett-Packard 1100 G1312A binary pump and one Hewlett-Packard 1100 G1322A degasser. (Palo Alto, CA). The autosampler was a CTC Analytics Leap Technologies HTS PAL (Carrboro, NC). The chromatographic system consisted of Waters Atlantis HILIC silica column 2.1 mm × 50 mm, 3 μm (Milford, MA).

Mass spectrometric detection was performed on an Applied Biosystems/MDS Sciex API4000 triple quadrapole (Foster City, CA) operating in positive turboionspray mode controlled by Analyst (version 1.4.1) software.

2.3. LC–MS/MS conditions

A gradient HPLC method was employed for separation. Mobile phase A consisted of 10 mM ammonium acetate with 1% methanol and mobile phase B consisted of acetonitrile. The gradient profile was as follows (min/%B): 0.0/80, 1.0/40, 2.0/40, 2.1/80, 5.0/80. The flow rate was set at 0.3 mL/min. The autosampler was programmed to inject 10-μL sample aliquots every 5 min.

The API4000 triple quadrapole turboionspray source of the mass spectrometer was operated in positive ion mode, with the curtain, ion source 1, and ion source 2 gasses set at 40, 50, and 65 arbitrary units (AU), respectively. The source temperature was maintained at 600 °C and the source parameters were optimized for ZAM and GR121167C (IS) in multiple reaction monitoring (MRM) mode. In MRM mode, ZAM was monitored at the transition 333 → 60 and IS was monitored at the transition 336 → 63. The product ions were generated for both compounds with collision energy of 37 eV.

2.4. Preparation of standards and quality control samples

All stock and working solutions were prepared in Milli-Q water. Stock solutions of ZAM (separate weighing for calibration standards and QC's) and GR121167C (IS) were prepared at a concentration of 0.5 mg/mL. GR121167C (IS) was diluted

to a working IS solution of 500 ng/mL in MeOH. ZAM stock solutions were further diluted to obtain working solutions with concentrations of 200/20/2 μg/mL. Water was spiked with appropriate volumes of working solutions to provide stock calibration standard (20, 50, 100, 200, 500, 1000, 5000, 10000, 50000, 100000 ng/mL) and stock quality control (20, 400, 20000, 80000, 100000 ng/mL) concentrations. Each stock calibration standard and stock quality control was diluted 1:10 in rat or monkey plasma (10 μL stock to 90 μL plasma) for calibration standard and QC samples. All solutions were stored at 4 °C.

2.5. Sample preparation

Calibration standards and quality control samples were prepared on the day of the analysis as mentioned above.

All plasma samples (50 μL) were extracted by protein precipitation with MeOH containing 500 ng/mL IS (150 μL) and vortexed for 2 min. The total volume of sample obtained was 200 μL. Samples were then centrifuged at 3600 rpm for 15 min. The supernatant (120 μL) was transferred to a 96 well plate and 10 μL was injected onto the LC–MS/MS system.

2.6. Assay validation procedures

Validation samples were prepared and analyzed to evaluate the intra-day and inter-day accuracy and precision of the analytical method in rat and monkey plasma. The acceptance criteria for accuracy and precision are within 15%. Analyst (version 1.4.1) software (MDS Sciex, Toronto, Ontario, Canada) was used for all quantitation and integration. All calculations for validation statistics were done using Microsoft Excel 2002 (Seattle, WA).

2.6.1. Assay specificity

The specificity of the assay was determined by comparing chromatograms of six different batches of blank rat and monkey plasma with the corresponding lower limit of quantitation (LLOQ, 2 ng/mL) spiked plasma. Each plasma sample was prepared as mentioned in the Sample Preparation Section 2.5 to ensure no interference of ZAM and IS from plasma.

2.6.2. Linearity

Calibration standards in duplicate ranging from 2 to 10000 ng/mL for ZAM were analyzed in three separate runs. Ratios of analyte verses IS were calculated for each point and standard curves were constructed by least square linear regression analysis using a weighting factor of $1/x^2$, in which x is the concentration in ng/mL.

2.6.3. Precision and accuracy

The precision and accuracy of the method were determined by analysis of quality control samples as five replicates at concentrations (10000, 8000, 2000, 40, and 2 ng/mL). They were analyzed along with two sets of standard samples on each of 3 days using the same instrument. Average intra-day precision was defined as the average relative standard deviation of the five replicates and inter-day precision as the relative standard deviation of the overall measured concentrations from the 3 days ($n = 15$).

2.6.4. Extraction efficiency

The extraction efficiency was determined by comparing the peak areas of blank rat or monkey plasma samples spiked before extraction with blank rat or monkey plasma spiked after extraction at three different concentrations levels (8000, 2000, 40 ng/mL). All samples were analyzed in triplicate.

2.6.5. Stability

The stability of ZAM in plasma was examined at ambient temperature and −80 °C at various concentrations out to 5 days. The samples under ambient temperature were analyzed once every 24 h. The samples under −80 °C were allowed to thaw without assistance at room temperature, an aliquot was extracted for analysis, and the remaining sample refrozen for 24 h under the same conditions. The freeze-thaw cycles were repeated for 5 consecutive days. The analytes were considered stable in plasma when 85–115% of the initial concentration was found. The stability of stock and working solutions (kept at 4 °C) were determined by comparing peak areas of stored solutions with freshly prepared solutions on a weekly basis. Stability of stock and working solutions were accepted when 95–105% of the initial concentration was found.

3. Results and discussion

3.1. LC–MS/MS conditions

ZAM, being a zwitterion, showed non-retention and poor peak shape on reversed phase columns in our lab (data not shown). Columns designed for aqueous conditions require a highly aqueous mobile phase to elute the analyte of interest which is not ideal for desolvation and can decrease sensitivity [13]. HILIC separates compounds by passing a hydrophobic mobile phase across a neutral hydrophilic stationary phase, causing solutes to elute in order of increasing hydrophilicity – the opposite of reverse phase chromatography [14]. HILIC, being the analytical column of choice for our validation studies, constitutes highly volatile mobile phases (>80% organic) for retention, selectivity, and sensitivity, therefore, ideal for enhanced compound ionization by electrospray mass spectrometry [12]. Data reported by Grumbach et al. [12] shows the added capabilities of HILIC in relation to reversed phase chromatography in the areas of selectivity, sensitivity, and retention. Grumbach reports the added selectivity of HILIC with polar molecules using morphine and its glucuronide metabolite where the more polar glucuronide retains later than the less polar morphine. Grumbach also shows the added sensitivity of HILIC using salbutamol (100 ng/mL) and bamethan (50 ng/mL). Using a reversed phase Atlantis dC18 column these compounds were not detected, but on Atlantis HILIC the peak areas were 19,567 and 110,085, respectively. Grumbach also reports the added retention features of HILIC using the highly polar compound allantoin, which is not retained on Atlantis dC18 but has a retention time of 2.2 min on the Atlantis HILIC. Since ZAM contains several ionizable nitrogen's and is highly polar, positive ion electrospray with HILIC separation was chosen for LC–MS.

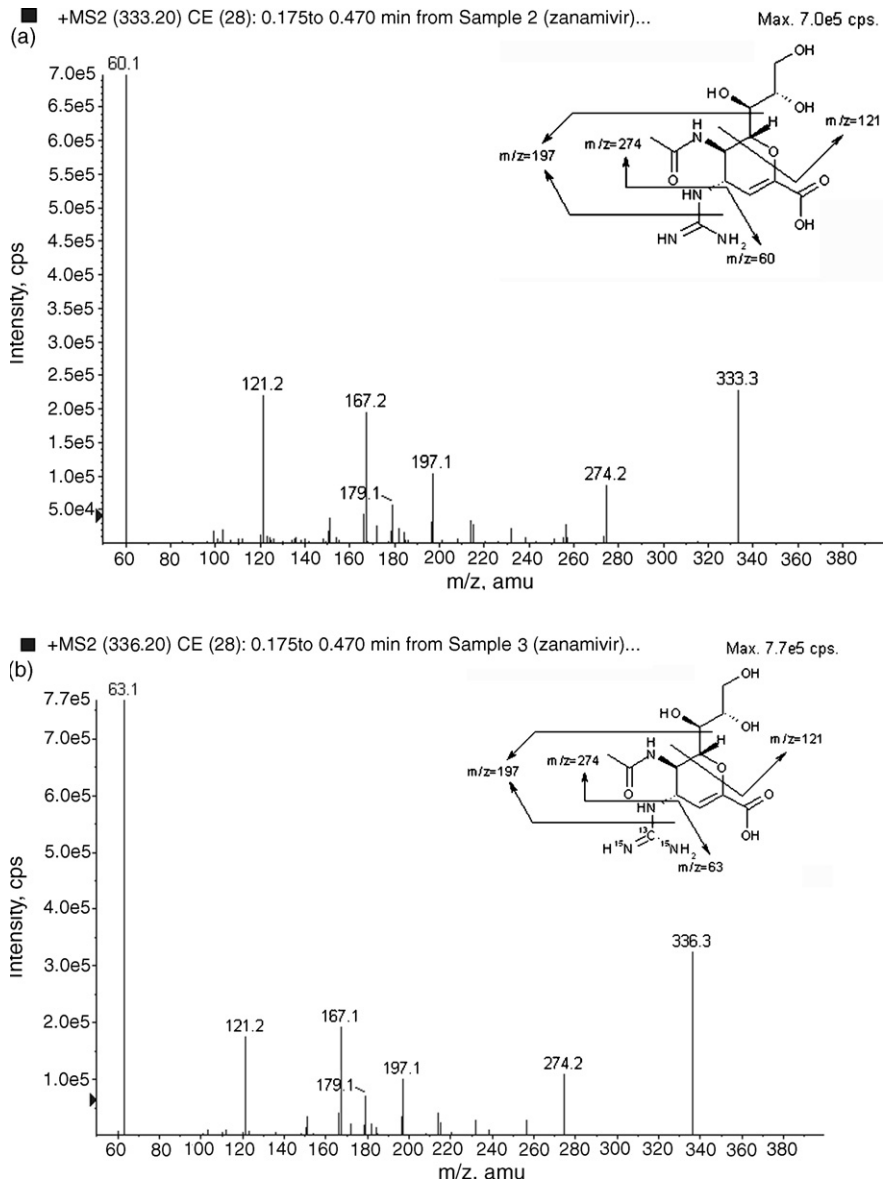

Fig. 2. Product ion mass spectra of zanamivir (a) and internal standard (b, GR121167C) showing the fragmentation of each.

The product ion mass spectra and fragmentation pattern, of ZAM and IS are shown in Fig. 2. The MRM transitions for ZAM (333 → 60) and IS (336 → 63) were chosen as these transitions provided the base ion peaks for the tandem mass spectrometric experiments (Fig. 2.). They were also chosen for best selectivity because the monitored fragment is where the stable label is located. Other fragment options in the MRM experiment were 274, and 121 (Fig. 2.), but these did not give the sensitivity and selectivity needed for the assay.

3.2. Assay validation

3.2.1. Assay specificity

The specificity of the assay was demonstrated by the absence of endogenous substances, in drug free plasma, that could interfere with the quantitation of ZAM at the LLOQ and IS. Protein precipitation is the simplest approach for removing the majority of the protein matrix [15–17]. Protein precipitation has the advantage in that it can be used generically for a large number of compounds. One disadvantage is that the sample is not as clean as other extraction methods. However, MS/MS has high selectivity in the MRM mode making protein precipitation an acceptable method for discovery PK studies [15,16]. Using a 3:1 methanol to plasma protein precipitation, the separation power of the HILIC column, and the selectivity of tandem mass spectrometry minimized potential interferences. Ion chromatograms of a 2 ng/mL spiked standard and IS with its associated blank sample are presented in Fig. 3. The endogenous peak in the blank at MRM transition 333 → 60 did not interfere with the integration of ZAM.

3.2.2. Linearity

The assay for ZAM was linear over a concentration range from 2 to 10000 ng/mL. Correlation coefficients ($r2$) ranged

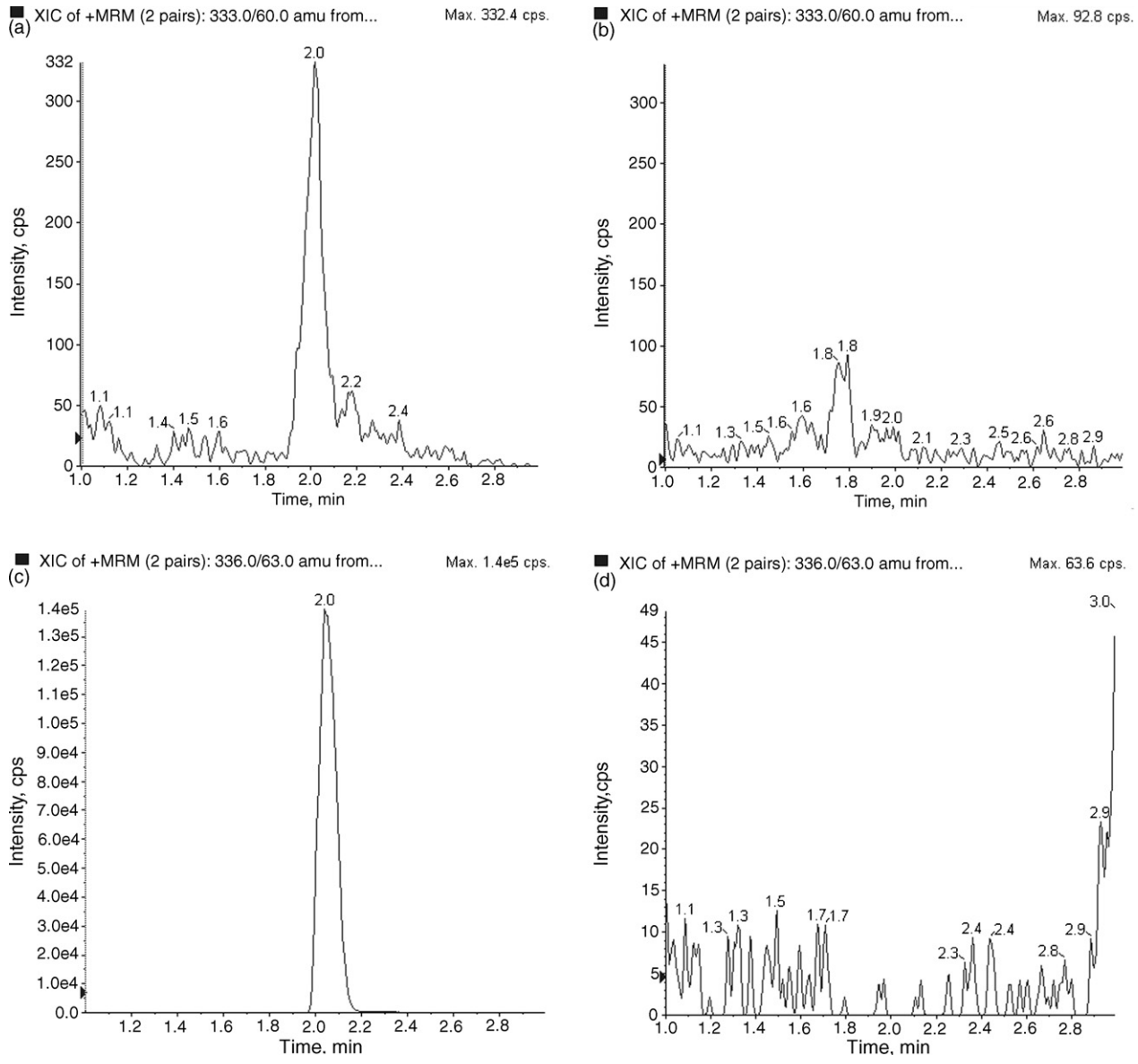

Fig. 3. Representative ion chromatograms of a 2 ng/mL zanamivir standard (a, MRM 333 → 60), zanamivir blank (b, MRM 333 → 60), 500 ng/mL IS (c, MRM 336 → 63), and IS blank (d, MRM 336 → 63) extracted from rat plasma.

from 0.9948 to 0.9991 and 0.9974 to 0.9990 in rat and monkey plasma, respectively. The calibration curves did not exhibit non-linearity within the chosen range as seen by the inter-day slope and *y*-intercept values, 0.000721 and 0.00161 for rat and 0.000729 and 0.000631 for monkey, respectively.

3.2.3. Precision and accuracy

The data for the intra-day assay precision and accuracy for rat and monkey, determined by analyzing five replicates (10000, 8000, 2000, 40, and 2 ng/mL) on each of three days, are reported in Tables 1 and 2, respectively. The data for the inter-day assay precision and accuracy for rat and monkey, determined by analyzing five replicates (10000, 8000, 2000, 40, and 2 ng/mL) on each of 3 days, are reported in Table 3. The accuracy of the method was determined by calculating the percent relative error (%RE), and the precision was determined by calculating the percent relative standard deviation (%RSD). In rat plasma, the inter-day precision (%RSD) ranged from 6 to 10, and the inter-day accuracy (%RE) ranged from −6.5 to 0.2. In monkey plasma, the inter-day precision (%RSD) ranged from 2 to 8, and the inter-day accuracy (%RE) ranged from −2.3 to 2.1, over the five concentrations evaluated. The results demonstrate that the values are within an acceptable range and the method is accurate and precise.

3.2.4. Extraction efficiency

Methanol was chosen as the extraction solvent due to its greater polar properties over acetonitrile and TCA. The extraction efficiency of ZAM and the internal standard (GR121167C) was >90%.

3.2.5. Stability

Stock and working solutions of ZAM and IS were found to be stable for at least 92 days when prepared in water (ZAM) or

Table 1
Intra-day validation statistics for zanamivir in rat plasma

Day	Parameter	Validation sample level (ng/mL)				
		2	40	2000	8000	10000
1	Average (ng/mL)	1.87	38.68	1894.00	7642.00	8790.00
	SD (ng/mL)	0.19	2.95	25.10	402.02	423.50
	Accuracy (%RE)	−6.50	−3.30	−5.30	−4.48	−12.10
	Precision (%RSD)	10.16	7.63	1.33	5.26	4.82
	n	5	5	5	5	5
2	Average (ng/mL)	2.10	41.46	1990.00	7744.00	9288.00
	SD (ng/mL)	0.24	3.61	79.69	347.75	375.39
	Accuracy (%RE)	5.00	3.65	−0.50	−3.20	−7.12
	Precision (%RSD)	11.43	8.71	4.00	4.49	4.04
	n	5	5	5	5	5
3	Average (ng/mL)	1.98	40.08	1940.00	7898.00	9976.00
	SD (ng/mL)	0.09	2.04	200.00	663.23	475.43
	Accuracy (%RE)	−1.00	0.20	−3.00	−1.28	−0.24
	Precision (%RSD)	4.55	5.09	10.31	8.40	4.77
	n	5	5	5	5	5

Table 2
Intra-day validation statistics for zanamivir in monkey plasma

Day	Parameter	Validation sample level (ng/mL)				
		2	40	2000	8000	10000
1	Average (ng/mL)	2.00	38.50	2051.83	7894.35	10230.17
	SD (ng/mL)	0.11	1.16	37.78	99.08	399.18
	Accuracy (%RE)	0.00	−3.75	2.59	−1.32	2.30
	Precision (%RSD)	5.50	3.01	1.84	1.26	3.90
	n	5	5	5	5	5
2	Average (ng/mL)	2.00	40.05	2002.90	7778.60	10251.54
	SD (ng/mL)	0.21	3.95	26.87	409.97	181.12
	Accuracy (%RE)	0.00	0.12	0.15	−2.77	2.52
	Precision (%RSD)	10.50	9.86	1.34	5.27	1.77
	n	5	5	5	5	5
3	Average (ng/mL)	2.00	39.64	2045.23	7773.07	10143.82
	SD (ng/mL)	0.18	0.90	41.35	504.14	602.38
	Accuracy (%RE)	0.00	−0.90	2.26	−2.84	1.44
	Precision (%RSD)	9.00	2.27	2.02	6.49	5.94
	n	5	5	5	5	5

Table 3
Inter-day validation statistics for zanamivir in rat and monkey plasma

Species	Parameter	Validation sample level (ng/mL)				
		2	40	2000	8000	10000
Rat	Average (ng/mL)	1.98	40.07	1941.33	7761.33	9351.33
	SD (ng/mL)	0.20	2.96	122.76	467.19	639.85
	Accuracy (%RE)	−1.00	0.17	−2.93	−2.98	−6.49
	Precision (%RSD)	10.10	7.39	6.32	6.02	6.84
	n	15	15	15	15	15
Monkey	Average (ng/mL)	2.00	39.39	2033.32	7815.34	10208.51
	SD (ng/mL)	0.16	2.35	40.07	356.08	401.12
	Accuracy (%RE)	0.00	−1.53	1.67	−2.31	2.09
	Precision (%RSD)	8.00	5.97	1.97	4.56	3.93
	n	15	15	15	15	15

methanol (IS) and stored at 4 °C. Also, ZAM was found to be stable at ambient temperature and −80 °C (in rat and monkey plasma) for at least 5 days.

4. Conclusion

An LC–MS/MS assay for the quantitation of ZAM in rat and monkey plasma has been developed and validated. The assay incorporates a simple protein precipitation with methanol and hydrophilic interaction chromatography which is sensitive, accurate, precise, and has demonstrated usefulness in the analysis of rat and monkey plasma samples. No significant interferences caused by endogenous compounds were observed. The method will be used to support oral formulation and toxicokinetic studies in rat and monkey, respectively.

References

[1] J.L. McKimm-Breschkin, Treat Respir. Med. 4 (2) (2005) 107.
[2] A.S. Monto, D.P. Robinson, M.L. Herlocher, J.M. Hinson Jr., M.J. Elliott, A. Crisp, JAMA 282 (1999) 31.
[3] F.G. Hayden, L.V. Gubareva, A.S. Monto, T.C. Klein, M.J. Elliot, J.M. Hammond, S.J. Sharp, M.J. Ossi, N. Engl. J. Med. 343 (2000) 1282.
[4] L. Lee, M. Loeb, A. Phillips, J. Nesbitt, K. Smith, M. Fearon, M.A. McArthur, T. Mazzulli, Y. Li, A. McGeer, Infect. Control Hosp. Epidemiol. 21 (2000) 700.
[5] G.E. Vogel, Med. Microbiol. Immunol. (Berl.) 191 (2002) 161.
[6] T. Puhakka, H. Lehti, R. Vainionpaa, V. Jormanainen, M. Pulkkinen, S. Sharp, C. Kerr, M. Dempsey, C. Ring, C. Ward, M. Tisdale, Scand. J. Infect. Dis. 35 (2003) 52.
[7] E.A. Govorkova, I.A. Leneva, O.G. Goloubeva, K. Bush, R.G. Webster, Antimicrob. Agents Chemo. 45 (2001) 2723.
[8] R.J. Stubbs, A.J. Harker, J. Chromatogr. B 670 (1995) 279.
[9] G.D. Allen, S.T. Brookes, A. Barrow, J.A. Dunn, C.M. Grosse, J. Chromatogr. B 732 (1999) 383.
[10] N. Erk, J. Liq. Chromatogr. Related Technol. 27 (10) (2004) 1541.
[11] V.M. Johnson, Internal GSK Audit Report Confidential, 2000.
[12] E.S. Grumbach, D.M. Wagrowski-Diehl, J.R. Mazzeo, B. Alden, P.C. Iraneta, LCGC North Am. 22 (10) (2004) 1010.
[13] W. Naidong, W. Shou, Y.L. Chen, X. Jiang, J. Chromatogr. B 754 (2001) 387.
[14] A.L. Alpert, J. Chromatogr. 499 (1990) 177.
[15] W.A. Korfmacher, K.A. Cox, M.S. Bryant, J. Veals, K. Ng, C.C. Lin, Drug Discov. Today 2 (1997) 532.
[16] W.A. Korfmacher, in: W.A. Korfmacher (Ed.), Using Mass Spectrometry for Drug Metabolism Studies, CRC Press, Boca Raton, 2005, pp. 1–34.
[17] D.T. Rossi, M. Sinz, Mass Spectrometry in Drug Discovery, Marcel Dekker Inc., New York, 2002.