ELSEVIER

Journal of Chromatography B, 732 (1999) 383–393

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Liquid chromatographic–tandem mass spectrometric method for the determination of the neuraminidase inhibitor zanamivir (GG167) in human serum

G.D. Allen[a], S.T. Brookes[a], A. Barrow[b,*], J.A. Dunn[c], C.M. Grosse[c]

[a]*Department of Mass Spectrometry, Division of Drug Metabolism and Pharmacokinetics, Huntingdon Life Sciences, P.O. Box 2, Huntingdon, Cambridgeshire PE18 6ES, UK*

[b]*Biomet. Division, GlaxoWellcome Research and Development, Park Road, Ware, Hertfordshire SG12 0DP, UK*

[c]*Biomet. Division, GlaxoWellcome Inc., Research Triangle Park, NC 27709, USA*

Received 27 August 1998; received in revised form 23 June 1999; accepted 23 June 1999

Abstract

An LC–MS–MS method for the analysis of the neuraminidase inhibitor, zanamivir, in human serum is described. Zanamivir was extracted from protein precipitated human serum samples using Isolute SCX solid-phase extraction cartridges and analysed using reversed-phase chromatography with TurboIonSpray atmospheric pressure ionisation followed by mass spectrometric detection. The method uses a stable isotope internal standard, is highly specific and sensitive for a compound of this type and has been used for the analysis of human serum and urine samples from clinical studies. The method was extended to the analysis of serum and plasma samples from pre-clinical studies involving the rat, ferret and cell culture media. The method has been shown to be robust and valid over a concentration range of 10–5000 ng/ml using a 0.2-ml sample volume. The main advantages of this method compared to earlier procedures are primarily specificity, sensitivity, ease of sample preparation, small sample volume and short analysis time (ca. 5 min).

Keywords: Solid-phase extraction; LC–MS–MS; Zanamivir; Neuraminidase inhibitor

1. Introduction

The sialic acid analogue zanamivir (5-acetylamino-4-guanidino-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enoic acid) (I) is the first of a new series of selective inhibitor of influenza A and B viral neuraminidases [1]. When administered directly to the human respiratory tract, zanamivir was shown to have potent antiviral effects [2,3]. Zanamivir is currently being developed for the treatment and prophylaxis of influenza A and B. During pre-clinical studies in animals zanamivir was shown to be excreted in the urine as unchanged compound after intravenous dosage [4]. Initial human studies showed that 87% of the compound was excreted unchanged in urine after inhalation administration [5].

To support the initial clinical studies a high-per-

*Corresponding author.

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00306-0

formance liquid chromatography (HPLC) method involving pre-column derivatisation and fluorescence detection was developed and validated over a concentration range of 10–800 ng/ml [6]. As the extended clinical studies required a rapid turnaround for sample analysis it was decided to utilise tandem mass spectrometric (LC–MS–MS) methodologies to overcome some of the problems associated with the initial analytical method such as the need for derivatisation and to enhance the sensitivity. Atmospheric pressure chemical ionisation (APCI) techniques have become widely used in various types of drug disposition work in recent years [7] and it was felt that this type of methodology would provide a more sensitive, rapid, robust and specific analytical procedure. Although this LC–MS–MS method, utilising a stable isotope internal standard (II), was developed primarily for human serum, it was also used for the analysis of zanamivir in human urine, rat serum and plasma and cell culture medium after successful cross validation experiments.

2. Experimental

2.1. Materials and reagents

Zanamivir (I) and its internal standard (I.S.), a ^{13}C, $^{15}N_2$-labelled analogue (II) (both shown in Fig. 1) were supplied by GlaxoWellcome Research and Development (Ware, UK) as reference standards of chemical purity above 98%. Acetonitrile (HPLC grade), methanol (HPLC grade) and acetic acid (Primar) were supplied by Fisher Scientific (Loughborough, UK). Triethylamine was supplied by Aldrich (Gillingham, UK). Blank drug-free human serum was supplied by Charterhouse Clinical Research Unit (London, UK).

2.2. Preparation of stock and working solutions of zanamivir

Zanamivir was weighed out in duplicate and dissolved in deionised water to yield two separate

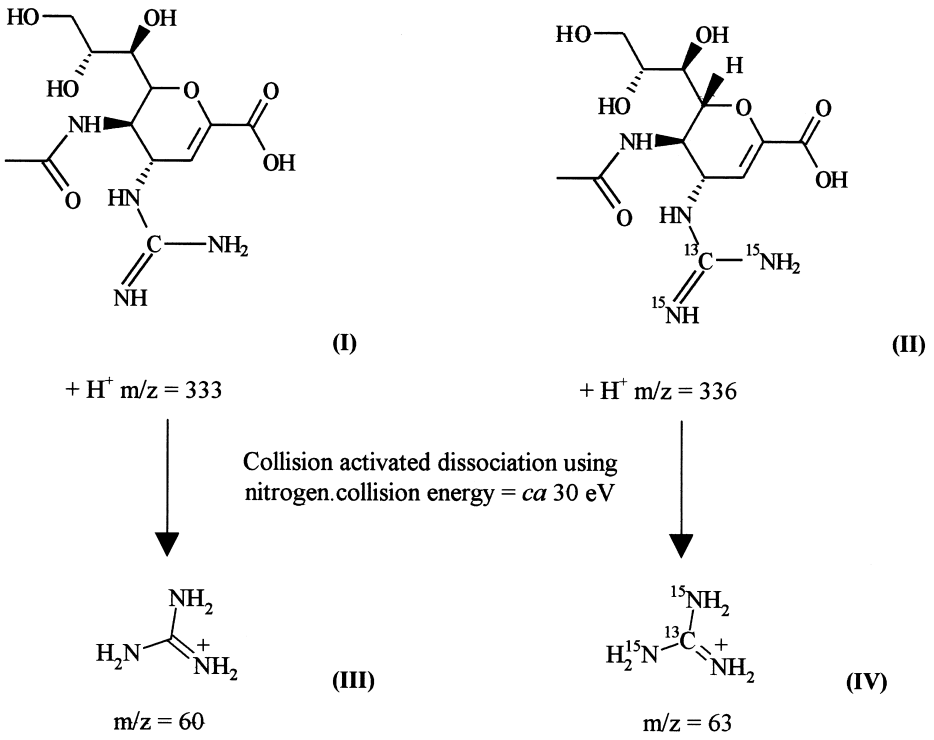

Fig. 1. Structures and proposed fragmentation of zanamivir and internal standard.

stock solutions designated Stock and StockQC at target concentrations of 50 μg/ml and 500 μg/ml, respectively. Working standard solutions were prepared in deionised water by appropriate dilutions of Stock, to yield final target concentrations of zanamivir at 50, 100, 500 and 5000 ng/ml. These solutions were used in the preparation of the calibration standards for each validation batch, and appropriate dilutions of the StockQC were used for the generation of the quality control samples (QCs).

2.3. Preparation of stock and working solutions of internal standard

Internal standard was weighed out and dissolved in deionised water to yield a stock solution designated Stock I.S. at a target concentration of 50 μg/ml. A working standard solution was prepared in deionised water by appropriate dilution of Stock I.S., to yield a final target concentration of internal standard at 5000 ng/ml.

2.4. Preparation of calibration standards and QCs

Drug-free control human serum was spiked with portions of aqueous working standard solutions of zanamivir (derived from Stock) to provide calibration standards in the range 10–5000 ng/ml. Bulk serum QC samples were prepared by spiking drug-free control serum with StockQC solutions to provide samples at target concentrations of zanamivir at 10, 20, 100, 500, 2000 and 5000 ng/ml. Aliquots (0.2 ml) from the QC bulk solutions were transferred into analysis tubes and stored at ca. −20°C until analysis.

2.5. Extraction procedure

An aliquot of human serum (0.2 ml) was spiked with internal standard (5000 ng/ml, 0.1 ml) and mixed with acetonitrile (0.5 ml) and aqueous acetic acid (3%, v/v, 0.1 ml) and allowed to stand at room temperature for 5 min after vortex mixing. The tubes were centrifuged (about 1400 *g*) for 10 min. The supernatant was loaded onto an Isolute SCX cartridge (50 mg/1 ml) which had been primed with methanol (0.5 ml) and aqueous acetic acid (10%, v/v, 0.5 ml). Zanamivir and I.S. were eluted from the cartridge with 10% (v/v) triethylamine in methanol–water (1:1, v/v) (2×0.5 ml) into a clean tube and evaporated to dryness at ca. 60°C under a gentle stream of nitrogen. The residues were dissolved in mobile phase (200 μl) by vortex mixing for about 10 s, transferred into autoinjector vials, the vials capped then centrifuged (about 5000 *g*) for 5 min.

2.6. Chromatography and mass spectrometry

The chromatography system used to analyse the extracted serum samples was a PE series 200 HPLC system, including autosampler and in-line degasser (Perkin-Elmer, Beaconsfield, UK). A Hypersil SAS column (C_1, 5 μm, 10 cm×4.6 mm I.D.) was used for the chromatography, under isocratic conditions, with a mobile phase composed of acetonitrile–water (1:1, v/v) containing 1% (v/v) acetic acid, at a flow-rate of 1 ml/min. Portions of each extract (20 of 200 μl) were injected.

Between the HPLC column and the mass spectrometer (PE Sciex API 300 triple quadrupole, Sciex, Canada) a simple T-splitter was used to maintain a solvent flow of 0.2 ml/min into the TurboIonSpray source (positive ion mode). The positive ions that were detected for zanamivir and I.S. corresponded to multiple reaction monitoring (MRM) transitions of m/z 333→60 and m/z 336→63, respectively. To assist in the production of these product ions for both compounds, nitrogen gas was used in the collision cell at a collision energy of 28 eV.

2.7. Intra-assay precision and bias

A duplicate set of calibration standards prepared from Stock dilutions and replicates of serum (n=6) spiked from StockQC dilutions, were analysed on four separate occasions using the method described in Sections 2.5 and 2.6. The intra-assay variability of the method was determined using the relative standard deviations (RSDs) of the six replicates at each of the six QC concentrations on four separate occasions.

2.8. Inter-assay precision and bias

For each batch (n=4), replicate quality control samples (n=6) were analysed alongside duplicate

calibration standards. The inter-assay variability of the method was determined using the RSDs of the 24 replicates at each of the six QC concentrations for each occasion.

2.9. Specificity

Duplicate samples of drug-free control human serum, individually sourced, were analysed with each batch to determine if any interference from endogenous components was observed.

2.10. Other checks

In order to minimise the possibility of any carry-over effects, samples containing mobile phase only were placed after selected samples known to contain high concentrations of zanamivir, and the individual chromatograms were monitored. A bulk serum QC sample was prepared by spiking drug-free control serum with StockQC to provide samples at a target concentration of zanamivir at 100 000 ng/ml. Aliquots ($n=6$) from the QC bulk sample were diluted with deionised water (1:25 and 1:1000, v/v) to generate target concentrations of zanamivir at 4000 and 100 ng/ml, respectively. The recovery was measured by a comparison of the data for replicate samples of human serum spiked with zanamivir (only) at three concentrations which were extracted and a known concentration of the I.S. added prior to analysis, with serum samples to which a known concentration of the I.S. (only) was added, then extracted, and zanamivir at three concentrations was added prior to analysis.

3. Results and discussion

3.1. LC–MS–MS

During the initial phase of method development it was shown that the compound gave a better response under TurboIonSpray conditions than under heated nebulizer conditions, and that positive ion conditions gave a better signal than negative ion conditions. Using a TurboIonSpray atmospheric pressure ionisation (API) interface, zanamivir and its internal standard showed protonated molecular ions $[M+H]^+$ at m/z 333 (I) and 336 (II), respectively. Fragmentation of these ions using collision activated dissociation (CAD) at a collision energy of ca. 28 eV, resulted in strong product ions for zanamivir (Fig. 2) and its internal standard at m/z 60 (III) and 63 (IV), respectively. The response to these ions was two- to three-times greater than for other ions such as m/z 167, depending on the orifice potential and gas flow-rate. The product ion spectrum and the Q1 spectrum were shown to be the same.

A stable isotope analogue of zanamivir was chosen as the internal standard in preference to a zwitterion analogue as the cold-labelled form should be extracted, eluted, chromatographed and ionised in a manner comparable to zanamivir itself, thus minimising any concentration dependant recovery effects. In addition matrix effects may be large eliminated by the use of a stable isotopically labelled form as an internal standard [8,9].

A chromatographic retention time of about 4 min was selected. Although the existing data suggests that zanamivir is not metabolised to any significant extent [4,5] any more polar metabolites which did extract and co-elute with zanamivir from the SPE columns should be eluted in the solvent front during the chromatographic separation. It was also felt that a longer retention time would help to minimise any matrix effects associated with the analysis of the different biofluids (serum or urine) from the diverse individuals participating in the clinical studies [9]. There was no evidence for ion-suppression effects with extracts of human serum.

3.2. Method validation

The LC–MS–MS method was formally validated in human serum prior to the analysis of samples from clinical studies and cross-validated into other matrices such as human urine, rat plasma, etc.

The calibration lines were constructed by plotting the peak area ratios of zanamivir to internal standard against the actual concentration using $1/y$ weighted least-squares regression. The calibration lines were linear over the range 10–5000 ng/ml and the mean correlation coefficient for the four validation days was calculated as 0.9999. The method displayed good intra- and inter-batch precision, with little bias, over the validated concentration range (Tables 1 and 2). The lower limit of quantification (LLOQ) for this method was 10 ng/ml. At this concentration the bias

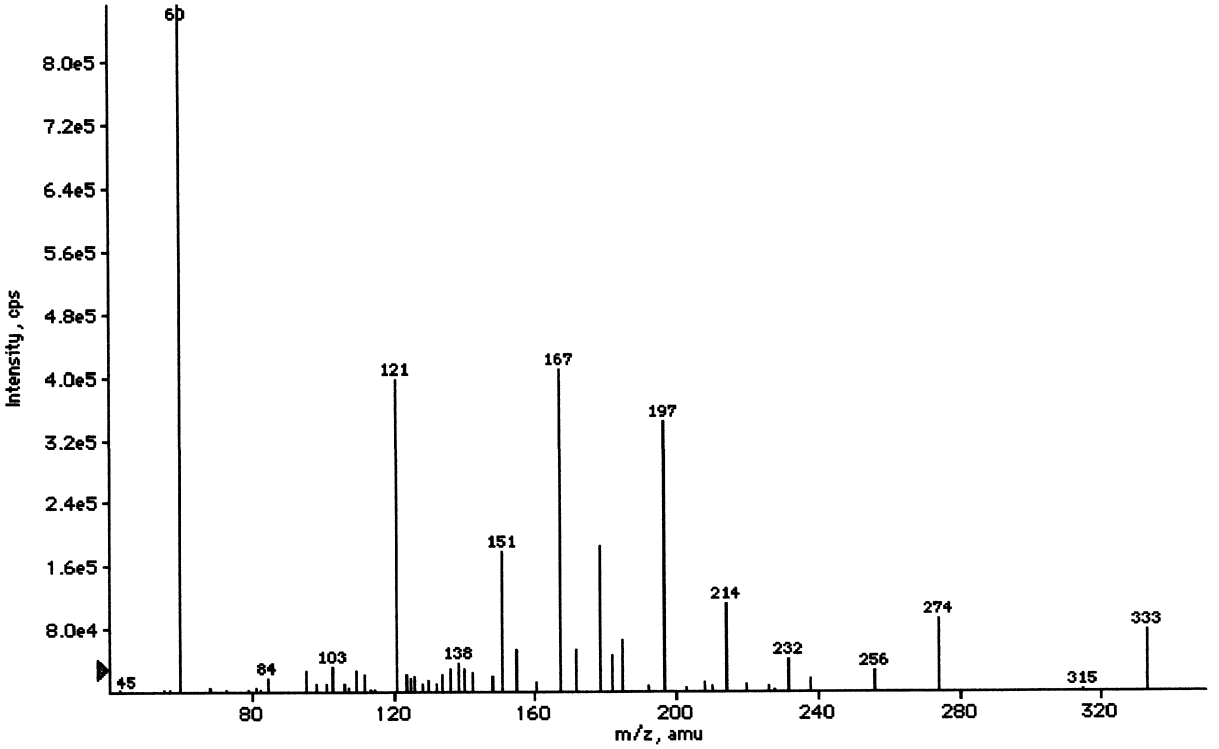

Fig. 2. Product ion mass spectrum of zanamivir.

Table 1
Accuracy and precision data for zanamivir from the pre-study validation: intra-assay variation, $n=6$

Nominal concentration (ng/ml)	Occasion 1 mean ng/ml (RSD, %)	Occasion 2 mean ng/ml (RSD, %)	Occasion 3 mean ng/ml (RSD, %)	Occasion 4 mean ng/ml (RSD, %)
10	10.3 (8.5)	10.1 (7.4)	9.8 (7.4)	10.3 (6.3)
20	20.6 (8.9)[a]	20.7 (7.0)	19.5 (6.1)	19.5 (4.2)
100	99.6 (2.1)	99.0 (1.6)	101.1 (4.0)	103.8 (1.7)
500	507.8 (2.4)	497.5 (0.8)	500.1 (2.7)	507.1 (2.1)
2000	2092.7 (1.4)	2023.2 (2.4)	2070.5 (0.6)	2025.3 (2.9)
5000	5060.9 (2.0)	4998.0 (1.1)	4953.9 (1.3)	4992.0 (2.4)

[a] One replicate was incorrectly spiked ($n=5$).

Table 2
Accuracy and precision data for zanamivir from the pre-study validation: inter-assay variation, $n=24$

Nominal concentration (ng/ml)	Mean±SD (ng/ml)	Bias (%)	RSD (%)
10	10.1±0.7	1.0	6.9
20[a]	20.1±1.4	0.5	7.0
100	100.9±3.1	0.9	3.1
500	503.1±10.9	0.6	2.2
2000	2052.9±49.4	2.6	2.4
5000	5001.2±91.2	0.0	1.8

[a] One replicate was incorrectly spiked ($n=23$).

was −0.4% ($n=24$), the RSD for the inter-batch results was 6.9% and the RSD for the intra-batch results ($n=6$) ranged between 6.3% and 8.5%.

The specificity of the method was assessed by the examination of the MRM chromatograms of blank serum together with pre-dose and post-dose samples of serum from the clinical samples. Control serum showed no endogenous interference in either MRM channel. MRM chromatograms for extracts of serum spiked with known quantities of zanamivir and the internal standard showed only one chromatographic

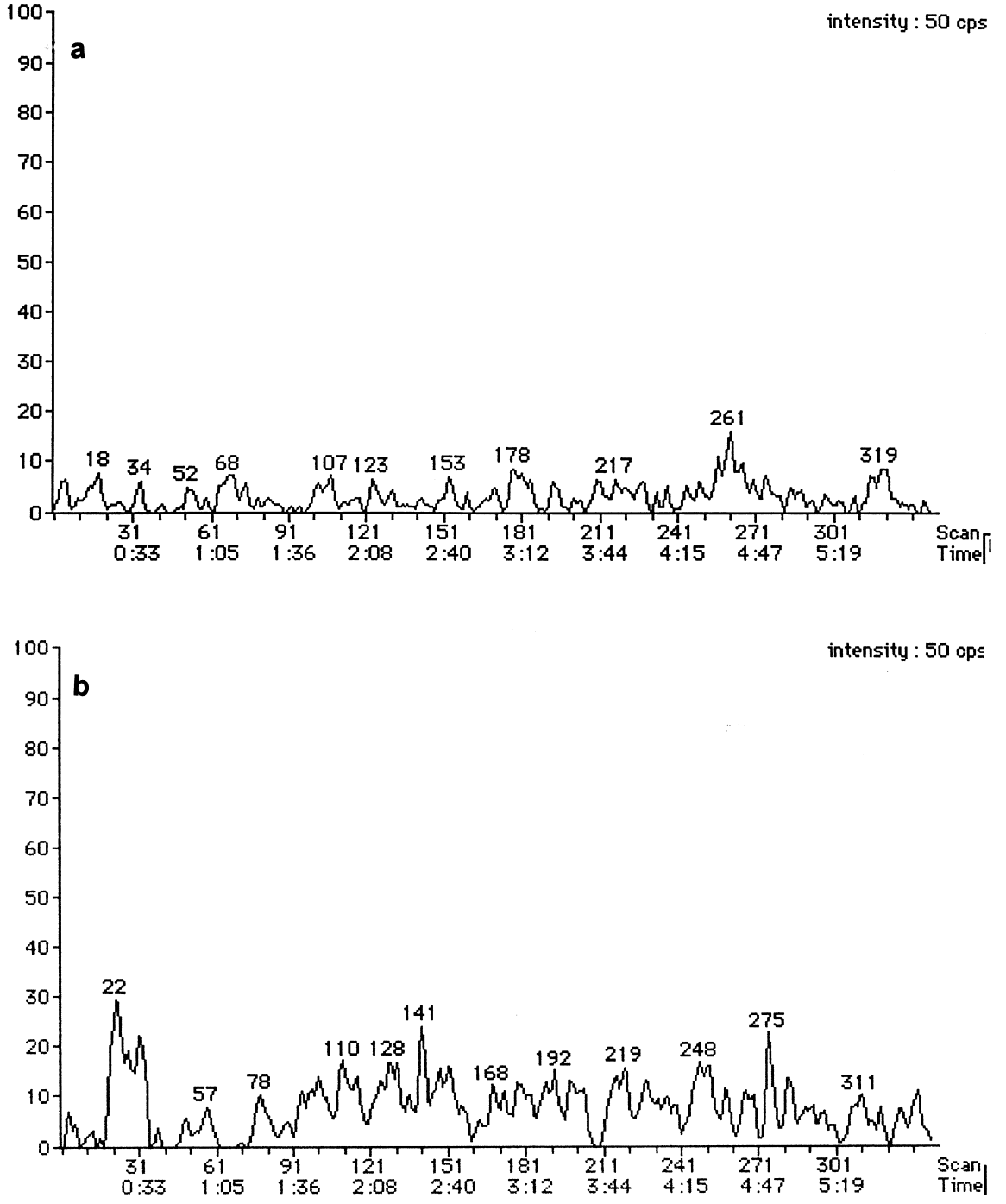

Fig. 3. (a) Representative MRM chromatogram of an extract of control serum. (b) Representative MRM chromatogram of an extract of control serum (I.S.).

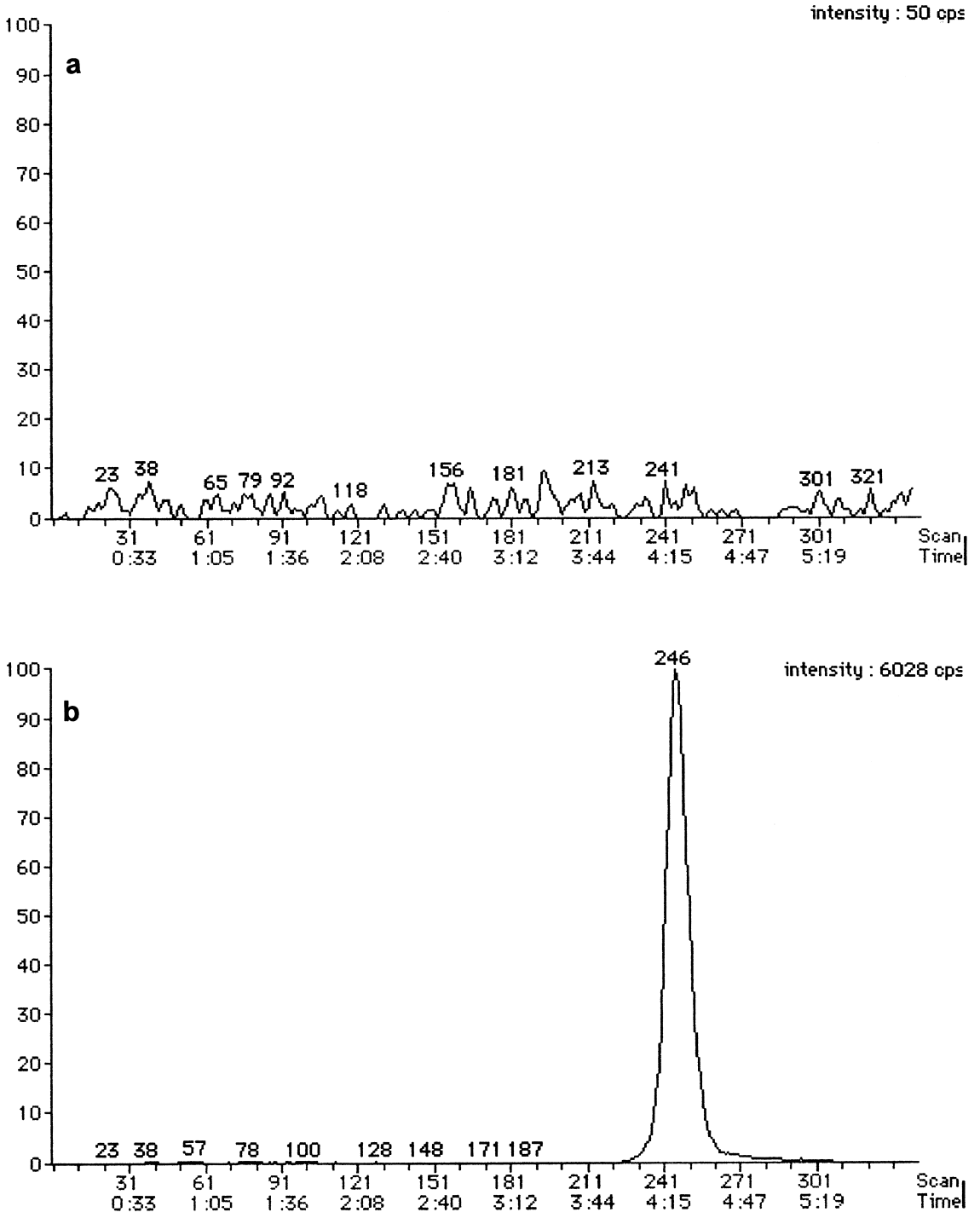

Fig. 4. (a) Representative chromatogram of an extracted zanamivir sample at 0 ng/ml. (b) Representative MRM chromatogram of an extracted zanamivir sample at 0 ng/ml (I.S.).

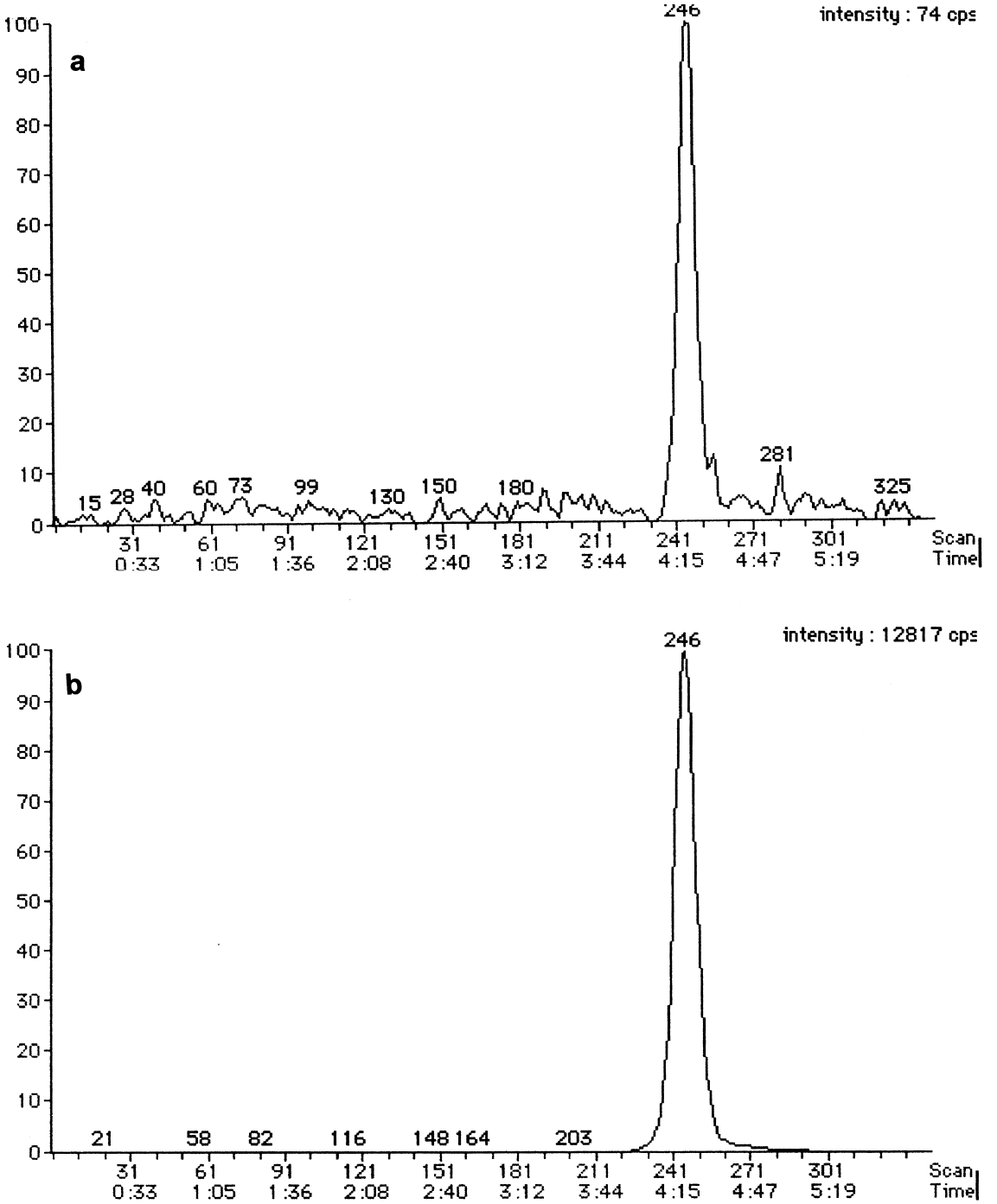

Fig. 5. (a) Representative MRM chromatogram of an extracted zanamivir sample at 10 ng/ml (LLOQ). (b) Representative MRM chromatogram of an extracted zanamivir sample at 10 ng/ml (I.S.).

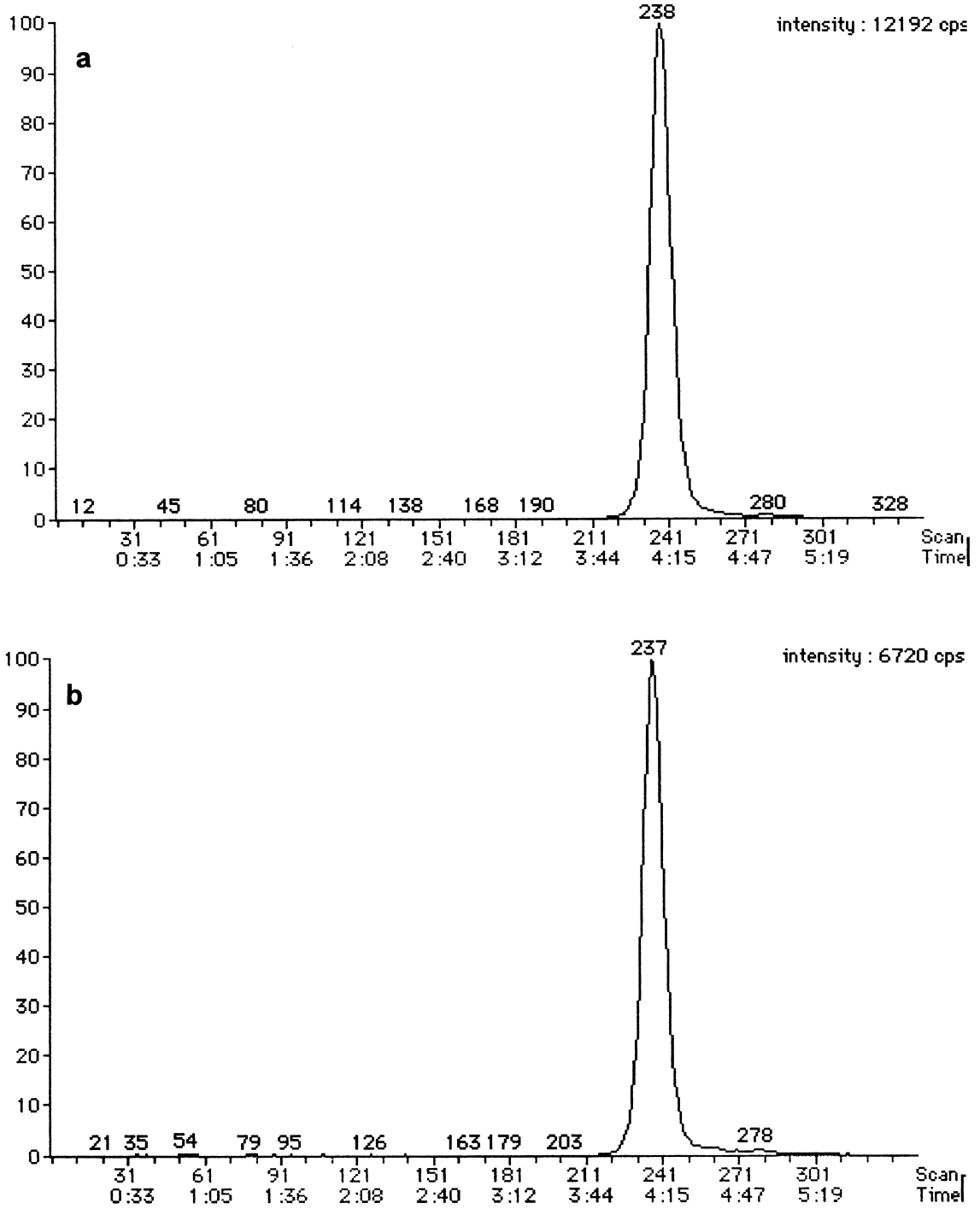

Fig. 6. (a) Representative MRM chromatogram of an extracted zanamivir sample at 5000 ng/m (ULOQ). (b) Representative MRM chromatogram of an extracted zanamivir sample at 5000 ng/ml (I.S.).

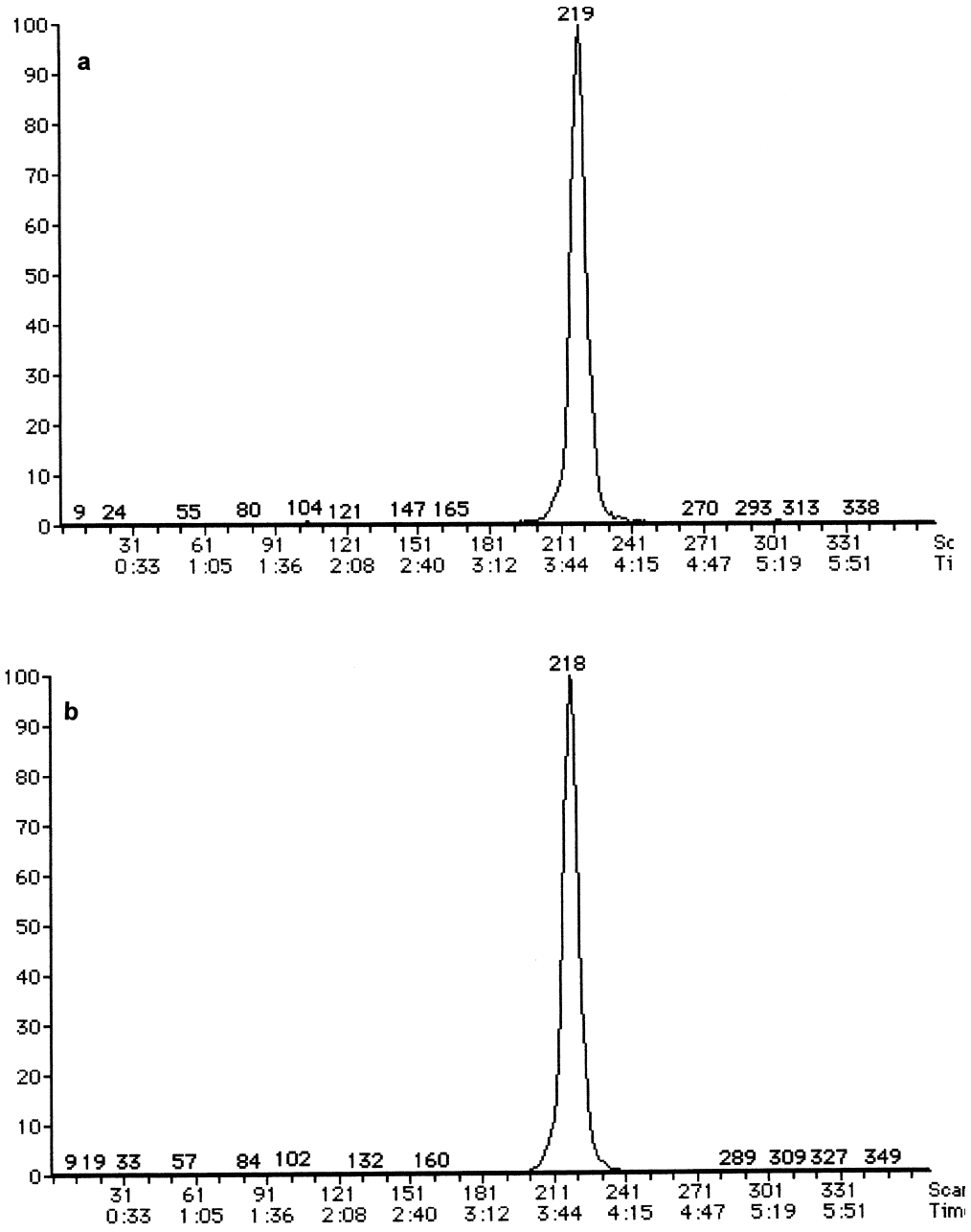

Fig. 7. (a) Representative MRM chromatogram of an extract of a human serum sample at 10 h post-dose (intravenous study with 600 mg infused over 0.5 h). (b) Representative MRM chromatogram of internal standard for an extract of a human serum sample at 10 h post-dose (intravenous study with 600 mg infused over 0.5 h).

peak per channel (Figs. 3–6). The absence of a response in the extract of serum containing 0 ng/ml of zanamivir but spiked with the I.S. (Fig. 4a) confirmed the isotopic purity of the I.S.

The effect of diluting samples containing high concentrations of zanamivir with water were assessed and the observed concentrations were in good agreement with the theoretical spiked concentrations. Assessment at the two dilution factors selected resulted in a mean bias of −5.1% and 5.5% and RSDs of less than 1%, and it was concluded that samples of a concentration greater than 5000 ng/nl could be diluted prior to re-analysis. Autosampler injection carry-over was not observed in any of the validation runs at a level likely to compromise the assay results. The recovery ranged between 25 to 30%.

As work carried out previously to support the fluorescence derivatisation HPLC method [6] had indicated that zanamivir was stable at room temperature, stable in human serum and urine and was stable over three freeze–thaw cycles (Harker, unpublished observations) no additional attempts were made to confirm the stability.

3.3. Method application

The method has subsequently been used to support clinical studies in man following administration of zanamivir after single and repeat intravenous infusions, oral and inhaled administration. Ion chromatograms of a serum sample taken at 10 h after the intravenous infusion of zanamivir are shown in Fig. 7. Duplicate QC samples at three concentrations representing the low, medium and high regions of the calibration range were included in every analytical batch. During one study involving the analysis of 21 batches of samples the RSD for the low, medium and high level QC samples were calculated as 8.3, 8.0 and 6.8%, respectively. The daily calibration data for the nine calibration samples analysed in each batch was assessed and the RSD was shown to range from 8.5 to 20.9%.

The method has also been cross validated in to other biological matrices including human urine, cell culture media, and rat serum and plasma. The cross validation into human urine was carried out by the analysis of replicates ($n=6$) of six validation control samples on one day. The RSD values ranged from 2.1 to 7.0% for these samples.

4. Conclusions

Zanamivir was extracted from protein precipitated human serum samples using Isolute SCX solid-phase extraction cartridges. A LC–MS–MS method for the determination of zanamivir in human serum has been shown to be valid over a concentration range of 10–5000 ng/ml using a 0.2-ml sample volume. The broad range of cross validations into human urine and into biological fluids from animals combined with the large number of samples analysed by this method showed it to be sensitive, robust and ideally suited to the rapid turnaround of both clinical and pre-clinical studies.

References

[1] S.L. Waghorn, K.L. Goa, Drugs 55 (1998) 721.
[2] F.G. Hayden, A.D.M.E. Osterhaus, J.J. Treanor, D.M. Fleming, F.Y. Aoki, K.G. Nicholson, A.M. Bohnen, H.M. Hirst, O. Keene, K. Wightman, New Engl. J. Med. 337 (1997) 874.
[3] The MIST Study Group, Lancet 352 (1998) 1877.
[4] M.J. Daniel, A.J. Harker, R.D.M. Gray, Pre-clinical disposition studies of GG167, a novel anti-influenza agent. Poster presentation at the Options For The Control of Influenza, Third Symposium, Cairns, 1996.
[5] F.G. Hayden, M. Lobo, E.K. Hussey, C.U. Eason, in: L.E. Brown, A.W. Horton, R.G. Webster (Eds.), Options for the Control of Influenza III, Elsevier, Oxford, 1996, p. 719.
[6] R.J. Stubbs, A.J. Harker, J. Chromatogr. B 670 (1995) 279.
[7] E. Brewer, J. Henion, J. Pharm. Sci. 87 (1998) 395.
[8] I. Fu, E.J. Woolf, B.K. Matuszewski, in: 8th International Symposium on Pharmaceutical and Biomedical Analysis, 1997, Abstract M/P-A9.
[9] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 10 (1998) 882.