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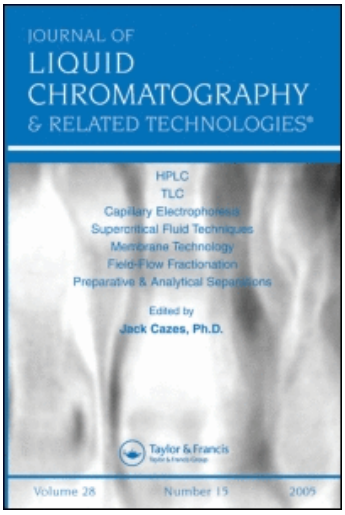


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# A Validated HPLC Method for the Determination of the Neuraminidase Inhibitor, Zanamivir (GG167), in Spiked Human Plasma and in Pharmaceutical Formulations

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# A Validated HPLC Method for the Determination of the Neuraminidase Inhibitor, Zanamivir (GG167), in Spiked Human Plasma and in Pharmaceutical Formulations

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## ABSTRACT

A new, simple, precise, sensitive reverse phase-high performance liquid chromatographic (RP-HPLC) technique has been developed for the determination of zanamivir in spiked human plasma and its pharmaceutical formulations. The method employs acetonitrile and water (50 : 50 v/v) as mobile phase with flow rate of 1.2 mL $min^{-1}$, Supelcocil $C_{18}$ (150 × 4.6 mm i.d: 5 μm particle size) column, loop of 20 μL, and UV detection at 230.0 nm. The internal standard method using indinavir as the internal standard is used. The validation of this technique showed

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that it is linear in a range of 8.0–7500.0 ng mL$^{-1}$ ($r$: 0.9987). The limit of detection and quantitation is 0.15 ng mL$^{-1}$ (0.5%) and 0.90 ng mL$^{-1}$ (1.8%), respectively. Each analysis required no longer than 5 min. Furthermore, the typical excipients included in the pharmaceutical product do not interfere with the selectivity of the method. Finally, the proposed chromatographic method was successfully applied to the quantitative determination of the neuraminidase inhibitor zanamivir (GG167) in spiked human plasma and pharmaceutical formulation.

*Key Words:* Zanamivir; Indinavir; Spiked human plasma; Pharmaceutical formulation; Validation; High performance liquid chromatography.

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## INTRODUCTION

Zanamivir is the first in a new class of drugs known as neuraminidase inhibitors. Neuraminidase is the enzyme which breaks the bond between sialic acid and cell surface proteins, thereby, enabling newly formed virus particles to exit the infected cell to infect neighboring uninfected cells. Inhibition of influenza neuraminidase, thus helps prevent the spread of virus from infected cells within the respiratory tract. The agent shows great promise for preventing influenza infections and for reducing the duration and severity of symptoms in people who begin treatment after they start to feel sick. Zanamivir hobbles a critical viral enzyme, called neuraminidase and, in so doing, markedly reduces proliferation of the virus in the body. Zanamivir is indicated for treatment of both influenza A and B in adults and adolescents (12 yr) who present with symptoms typical of influenza when influenza is circulating in the community. Treatment should begin as soon as possible, within hours after onset of symptoms. Zanamivir is orally administered in dose of 10–20 mg daily, is not protein bound and not liver metabolized nor modified. Zanamivir (5-acetylamino-4-[(aminoiminomethyl)amino]-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enoic acid) is shown in Sch. 1.

A new tablet formulation of zanamivir has been newly developed by GlaxoSmithKline Pharm. Co. For the new product, it is necessary to find an analytical method to determine zanamivir in the pharmaceutical dosage form, since zanamivir and the pharmaceutical dosage form have not been listed in any of the Pharmacopoeias yet. To support the initial studies, a high performance liquid chromatographic method involving pre-column derivatization and fluorescence detection was used.[1] The other techniques with atmospheric pressure chemical ionization[2] and with tandem mass spectrometry[3] were developed and validated. Besides, there are many other compounds belonging to the same class as indinavir, ritonavir, saquinavir,

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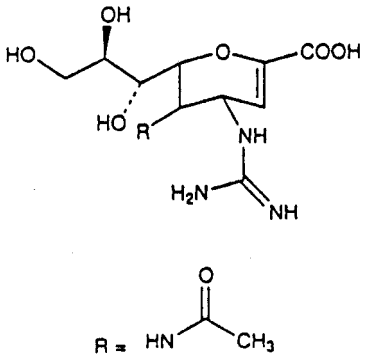


***Scheme 1.*** Chemical formula of zanamivir.

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nelfinavir, zidovudine, and stavudine, and their metabolites in human plasma were described.[4–20]

The present communication, which describes the high performance liquid chromatographic assay procedure exclusively for zanamivir in spiked human plasma and pharmaceutical formulations, is simple, precise, and sensitive. The present method uses a simple mobile phase without the need of buffer, involves no complex procedure to prepare sample solutions, and offers better sensitivity than some of the reported methods.[1–3]

## EXPERIMENTAL

### Materials and Reagents

Zanamivir standard sample and rotadisks (5.0 mg per rotadisk) were provided by GlaxoSmithKline Pharm. Co. (Istanbul, Turkey) and the internal standard (IS), indinavir, was obtained from MSD Pharm. Co. (Istanbul, Turkey). RELENZA® (5.0 mg zanamivir) rotadisks were purchased through a local pharmacy. HPLC-grade acetonitrile was purchased from Merck Co. (Darmstadt, Germany). HPLC-grade water was prepared by using a Milli-Q water purification system from Millipore (Mosheim, France). All other reagents used were of analytical grade, and were used as received. Drug-free human plasma was obtained from the hospital blood bank.

### Instrumentation and Chromatographic Conditions

The HPLC system consisted of a membrane degasser, binary solvent delivery system, a Rheodyne injector equipped with a 20 mL sample loop,

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and a UV/VIS detector (1100 Series, Agilent Technologies, USA). The detection wavelength was at 230.0 nm, and the peak areas were integrated automatically with a Windows NT based LC ChemStation Software.

The chromatographic analysis was performed at ambient temperature with a Supelcocil $C_{18}$ column (150 × 4.6 mm i.d., 5 μm particle size) and a mobile phase composed of acetonitrile : water (50 : 50 v/v). The flow rate was maintained at 1.2 mL $min^{-1}$.

## Preparation of the Standard Solutions and Calibration Curve

Standard stock solutions of 1.0 μg $mL^{-1}$ of zanamivir and indinavir were prepared using a mixture of acetonitrile and water (50 : 50 v/v) in separate volumetric flasks. From this standard stock solution, mixed standard (working standard) solutions were prepared by suitable dilution with the mobile phase to contain 8.0–7500.0 ng $mL^{-1}$ of zanamivir and 7000.0 ng $mL^{-1}$ of indinavir as IS in different 10 mL volumetric flasks. Before being subjected to analysis, all the working standard solutions were filtered through 0.45 μ filter and degassed. A volume of 20 μL of each sample was injected into the column. All measurements were repeated five times for each concentration. The calibration curve was constructed by plotting the peak area ratios of analyte to IS vs. the corresponding drug concentration.

## Spiked Human Plasma Samples

Trichloroacetic acid, perchloric acid, sulfuric acid, ethanol, and acetonitrile were tested for precipitation of human plasma proteins. Acetonitrile was found to be the best precipitant because, when this substance was used in small volumes, the precipitation was successfully completed. Acetonitrile (1.0 mL) was added to human plasma (0.5 mL) containing standard stock solutions of zanamivir and IS. Addition of acetonitrile prevents zanamivir from binding to proteins and coagulates plasma proteins. The mixtures were then vortexed for 10 min. After deproteinization and centrifugation of samples for 15 min at 6000 rpm, the supernatant (0.5 mL) was taken carefully and analyzed as described above. No anticoagulant was used in this proposed method.

## Analysis of Pharmaceutical Formulations

The described method was used to determine zanamivir in pharmaceutical formulations.

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An amount equivalent to the mean mass of one dose was transferred to 100 mL volumetric flasks. The contents were diluted with the mobile phase by stirring for 20 min. The excipient was separated by filtration and the residue was washed three times with the same solvent. After filtration, an appropriate volume of the filtered solution was taken in a 100 mL flask. An appropriate amount of IS was added and diluted to the mark with the mobile phase. Solutions were filtered through 0.45 μm Millipore membrane filters, and injected separately into the chromatographic system. The amount of zanamivir per dose mass was calculated from a linear regression equation.

### Recovery Studies

To keep an additional check on the accuracy of the developed assay method and to study the interference of formulation/additives, analytical recovery experiments were performed by the standard addition method. The known amount of the pure sample solutions were added to the preanalyzed samples of each drug including a constant level of the IS, and the mixtures were analyzed by the proposed method. The percentage recovery was calculated from the total amount of drug found. After five repetitive experiments, the recoveries were calculated.

## RESULTS AND DISCUSSION

### Optimization of the Chromatographic Conditions

Optimum conditions were fixed by varying one parameter at a time, by keeping other parameters constant and observing its effect on the response factor, and also on the peak resolution. The effect of wavelength on the response factor and on the peak resolution was observed over the wavelength range 220.0–240.0 nm. Since the absorbance at 230.0 nm was much higher than that at 220.0 and 240.0 nm, 230.0 nm was chosen as the detection wavelength. Similarly, effect of composition of the mobile phase was studied by changing the composition of acetonitrile and water. The optimum ratio of acetonitrile : water was found to be 50 : 50 v/v. The effect of flow rate was observed by varying the flow rate from 0.8 to 1.5 mL $min^{-1}$. The lower flow rates lead to an increase in retention time and high flow rates lead to a considerable increase in the pressure. Therefore, 1.2 mL $min^{-1}$ was found to be optimal for all measurements. A Supelcocil $C_{18}$ (150 × 4.6 mm i.d., 5 μm particle size) column is recommended because of its demonstrated ruggedness and reproducibility in this assay. Indinavir was applied as an IS,

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neutralizing the error inherent in sample injection, thereby eliminating random errors. A typical chromatogram for zanamivir and indinavir (IS) using Supelcocil $C_{18}$ (150 × 4.6 mm i.d., 5 μm particle size) column is shown in Fig. 1(a). In Fig. 1(a), for the estimation of zanamivir and indinavir, a sharp and symmetrical peak was obtained with good baseline, thus facilitating the accurate measurement of the peak area. The average retention times for zanamivir and indinavir were found to be $3.3 \pm 0.04$ and $2.2 \pm 0.08$ min, respectively. Under the described HPLC parameters, the respective compounds were clearly separated and their corresponding peaks were sharply developed at reasonable retention times.

Linearity and range of the method were determined by analyzing seven different concentrations ($n = 5$) of the mixed standard solutions containing 8.0–7500.0 ng $mL^{-1}$ of zanamivir and 7000.0 ng $mL^{-1}$ of indinavir under the chromatographic conditions mentioned above. The response factor of the standard solutions was calculated. The calibration curve was linear in the range of 8.0–7500.0 ng $mL^{-1}$. The calibration curve equation is $Y = a + bC$, where "$Y$" represents the zanamivir peak area to indinavir (IS) peak area ratio and "$C$" represents zanamivir concentration. Table 1 represents calibration characteristics for the peak area ratio of varying amounts of zanamivir to a constant amount of indinavir (7000.0 ng $mL^{-1}$).

Validation of the procedures for the quantitative assay of the drug were examined via evaluation of the limit of detection (LOD), limit of quantitation (LOQ), repeatability, recovery, specificity, and robustness. The LOD and LOQ were separately determined at a signal-to-noise ratio (S/N) 3 and 10. The LOD and LOQ were experimentally verified by diluting known concentrations of zanamivir until the average responses were approximately three or seven times the standard deviation of the responses for five replicate determinations. The LOD and the LOQ of zanamivir were found to be 0.15 and 0.90 ng $mL^{-1}$, respectively.

Intra-day precision and accuracy of the method were evaluated by assaying freshly prepared solutions in triplicate at concentrations of 8.0, 3750.0, and 7500.0 ng $mL^{-1}$ zanamivir. The RSD ranged from 0.4% to 0.8% (Table 2). Inter-day precision and accuracy of the method calculated from the individual recovery data were evaluated by assaying freshly prepared solutions, in triplicate, for 3 days. The RSD ranged from 0.9% to 1.1% (Table 2). The accuracy results in terms of percentage recoveries are presented in Table 3.

To evaluate method robustness, a few parameters were systematically varied. The parameters included variation of $C_{18}$ columns from different manufacturers and acetonitrile of different lots. Two analytical columns, one (Phenomenex, Partisil 5 ODS $C_{18}$ (150 × 4.6 mm i.d., 5 μm particle size column) and the other (Supelcocil $C_{18}$ (150 × 4.6 mm i.d., 5 μm particle size column), were used during the experiment. The assay results from the two

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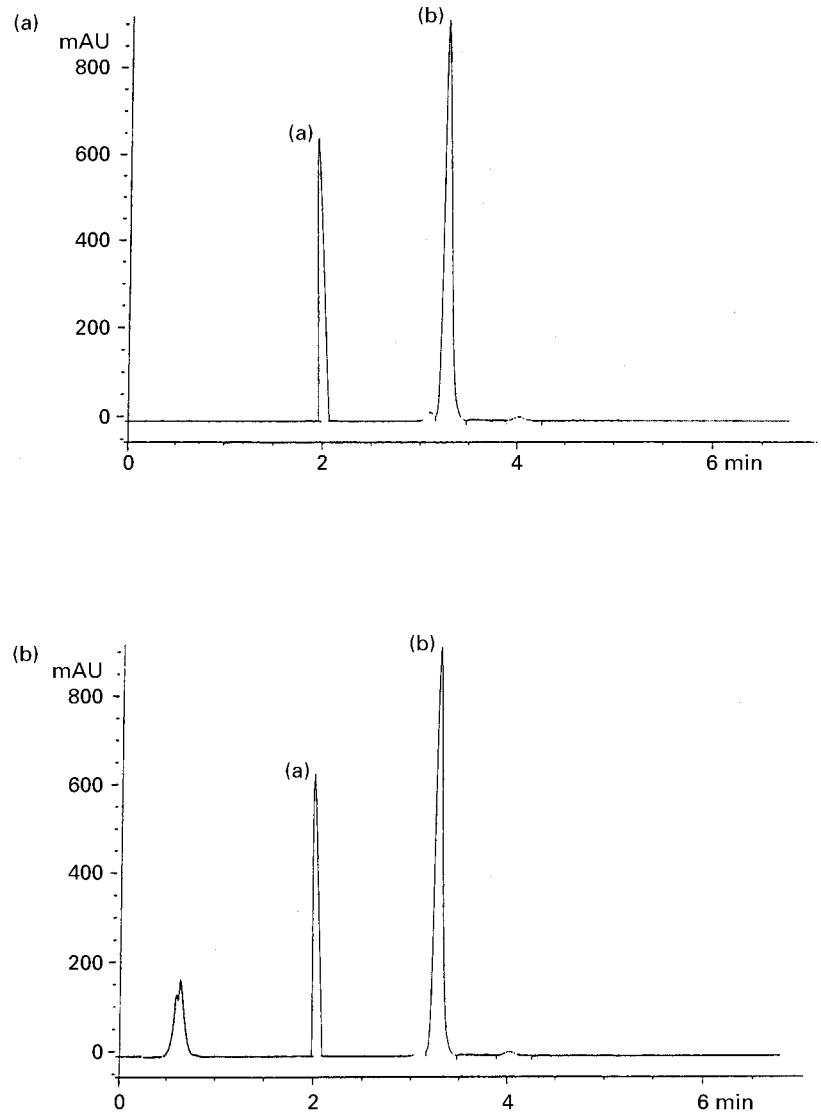


***Figure 1.*** (a) HPLC chromatogram of a 20 μL injection containing a, 7000.0 ng $mL^{-1}$ of indinavir (IS) and b, 7000.0 ng $mL^{-1}$ of zanamivir. (b) HPLC chromatogram of human plasma spiked with a, 7000.0 ng $mL^{-1}$ of indinavir (IS) and b, 7000.0 ng $mL^{-1}$ of zanamivir.

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***Table 1.*** Statistical analysis of calibration curves in the HPLC determination of zanamivir.

| | |
|---|---|
| Parameters | |
| Range (ng mL$^{-1}$) | 8.0–7500.0 |
| Regression equation ($Y$)[a] | |
| Slope ($b$) | 0.1077 |
| Standard deviation on slope ($S_b$) | $2.52 \times 10^{-5}$ |
| Intercept ($a$) | 0.0067 |
| Standard deviation on intercept ($S_a$) | $3.21 \times 10^{-5}$ |
| Standard error of estimation ($S_e$) | $1.03 \times 10^{-5}$ |
| Correlation coefficient ($r$) | 0.9992 |
| Relative standard deviation (%)[b] | 0.3260 |

[a]$Y = a + bC$ where $C$ is concentration in ng mL$^{-1}$ and Y is peak area ratio.
[b]Five replicate samples.

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columns were 98.7% ± 0.71% and 99.7% ± 0.42% (mean ± SD), respectively, indicating that there is no significant difference between the assay results from the two columns. It was also found that acetonitrile of different lots, from the same manufacturer, had no significant influence on the determination. Insignificant differences in peak areas and less variability in retention

***Table 2.*** Intra-day and inter-day assay variations of zanamivir.

| | Mean of concentration (ng mL$^{-1}$) | SD | RSD (%) |
|---|---|---|---|
| Intra-day | | | |
| Day 0 | 8.1 | 0.0188 | 0.5 |
| | 3,752.7 | 0.0202 | 0.6 |
| | 7,498.3 | 0.0195 | 0.5 |
| Day 1 | 8.0 | 0.0315 | 0.6 |
| | 3,750.0 | 0.0318 | 0.6 |
| | 7,500.0 | 0.0321 | 0.4 |
| Day 2 | 8.0 | 0.0215 | 0.5 |
| | 3,750.0 | 0.0213 | 0.4 |
| | 7,500.0 | 0.0228 | 0.8 |
| Inter-day | 8.0 | 0.0364 | 0.9 |
| | 37,500 | 0.0350 | 0.9 |
| | 7,500.0 | 0.0321 | 1.1 |

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***Table 3.*** Accuracy in the assay determination of zanamivir.

| Day | Taken (ng) | Found (ng) | Recovery (%)[a] | RSD (%) |
|---|---|---|---|---|
| 0 | 8.0 | 8.1 | 101.2 | 1.1 |
| | 3,750.0 | 3,747.9 | 99.9 | 0.8 |
| | 7,500.0 | 7,480.5 | 99.7 | 0.8 |
| 1 | 8.0 | 7.9 | 98.8 | 1.5 |
| | 3,750.0 | 3,746.5 | 99.9 | 0.9 |
| | 7,500.0 | 7,490.8 | 99.8 | 0.7 |
| 2 | 8.0 | 7.9 | 98.8 | 0.7 |
| | 3,750.0 | 3,735.1 | 99.6 | 0.9 |
| | 7,500.0 | 7,386.8 | 98.5 | 1.0 |

[a]Percent recovery of zanamivir from the sample against that taken.

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time were observed. The RSD of less than 1.2% for peak areas and retention time were obtained, which indicated that the developed method was capable of producing results with high precision. Specificity of the optimized procedures for the determination of zanamivir was examined in the presence of a placebo. The mean percentage recovery of 3750.0 ng $mL^{-1}$ zanamivir showed no significant placebo interference, thus, the procedures were able to assay zanamivir in the presence of the placebo and, hence, it can be considered specific. Co-administered drugs have been shown not to interfere in the assay. These include ritonavir (retention time, 4.6 min), saquinavir (6.1 min), nelfinavir (5.5 min), perindopril (4.4 min), indomethacin (5.1 min), and piroxicam (5.8 min).

The stability of the zanamivir in solution containing the mobile phase and the IS was determined for the samples stored in a refrigerator and at room temperature. The samples were checked after three successive days of storage and the data were compared with freshly prepared samples. In each case, the RSD values were found to be below 2.2%. This indicates, that the zanamivir is stable in the solution for at least 3 days, and is compatible with IS.

The developed method was applied to the determination of zanamivir in three batches of pharmaceutical formulations, respectively. The results presented in Table 4 are in good agreement with the labeled content. All data represents the average of five determinations. Low values of standard deviation indicate very good reproducibility of the measurement. Figure 1(b) shows the typical chromatogram obtained from the human plasma spiked with zanamivir, indinavir (IS), and which indicates no interferences from the endogenous substances present in the human plasma. Human plasma samples

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***Table 4.*** Results obtained in determination of zanamivir in pharmaceutical formulation.[a]

| Batch | Mean (mg) ± SD[b] |
|---|---|
| 1 | 5.2 ± 0.078 |
| 2 | 4.8 ± 0.075 |
| 3 | 5.2 ± 0.069 |

[a]Relenza® was labeled to contain 5.0 mg zanamivir, per one dose.
[b]Each value is the mean of 10 experiments; SD: standard deviation.

***Table 5.*** Results obtained for zanamivir analysis from human plasma.

| *n* | Added ($ng\,mL^{-1}$) | Found ($ng\,mL^{-1}$) | Recovery (%) |
|---|---|---|---|
| 5 | 8.0 | 7.9 | 98.8 |
| 5 | 3,750.0 | 3,732.6 | 99.5 |
| 5 | 7,500.0 | 7,498.1 | 100.0 |
| | | | X = 99.4 |
| | | | RSD = 1.22% |

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were spiked with zanamivir to achieve final concentrations of 8.0, 3750.0, and 7500.0 $ng\,mL^{-1}$. The determination results and recoveries of known amounts of zanamivir added to human plasma are given in Table 5. The proposed method gives reproducible results, is easy to perform, and is sensitive enough for the determination of zanamivir in human plasma. Care must be taken when analyzing human plasma samples from patients who take indinavir, since this will interfere with the analysis.

## CONCLUSIONS

In summary, a new, simple, and selective HPLC assay was developed and validated for quantitation of zanamivir in spiked human plasma and in pharmaceutical formulations. A large number of pharmaceutical formulations and spiked human plasma can be analyzed within a short time, hence, the proposed method can be used for routine analysis in quality control and development laboratories.

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