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Determination of Zidovudine Triphosphate Intracellular Concentrations in Peripheral Blood Mononuclear Cells from Human Immunodeficiency Virus-Infected Individuals by Tandem Mass Spectrometry

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Nucleoside reverse transcriptase inhibitors (NRTIs) used against the human immunodeficiency virus (HIV) need to be activated intracellularly to their triphosphate moiety to inhibit HIV replication. Intracellular concentrations of these NRTI triphosphates, especially zidovudine triphosphate (ZDV-TP), are relatively low (low numbers of femtomoles per 10^6 cells) in HIV-infected patient peripheral blood mononuclear cells. Recently, several methods have used either high-performance liquid chromatography (HPLC) or solid-phase extraction (SPE) coupled with radioimmunoassay to obtain in vivo measurements of ZDV-TP. The limit of detection (LOD) by these methods ranged from 20 to 200 fmol/10^6 cells. In this report, we describe the development of a method to determine intracellular ZDV-TP concentrations in HIV-infected patients using SPE and HPLC with tandem mass spectrometry for analysis. The LOD by this method is 4.0 fmol/10^6 cells with a linear concentration range of at least 4 orders of magnitude from 4.0 to 10,000 fmol/10^6 cells. In hispanic HIV-infected patients, ZDV-TP was detectable even when the sampling time after drug administration was 15 h. Intracellular ZDV-TP concentrations in these patients ranged from 41 to 193 fmol/10^6 cells. The low LOD obtained with this method will provide the opportunity for further in vivo pharmacokinetic studies of intracellular ZDV-TP in different HIV-infected populations. Furthermore, this methodology could be used to perform simultaneous detection of two or more NRTIs, such as ZDV-TP and lamivudine triphosphate.

Zidovudine (ZDV; 3′-azido-3′-deoxythymidine) was the first drug approved for the treatment of human immunodeficiency virus (HIV) infection. The parent compound (ZDV) is not active against HIV, since it needs to be metabolized in the host cells to 5′-ZDV triphosphate (ZDV-TP), which acts as a competitive inhibitor of HIV reverse transcriptase or is incorporated into the viral genome, terminating DNA chain elongation (7, 9). Recently, several studies have shown that the intracellular concentration of the triphosphate moieties of the nucleoside reverse transcriptase inhibitors (NRTIs) correlated better with the virologic response than did levels of the parent compound in plasma (20, 21). Furthermore, Fletcher et al. demonstrated a strong relationship between intracellular ZDV-TP concentrations and an increase in the percent change in $CD4^+$ cells from the baseline (5).

In recent years, the quantitation of ZDV-TP and other NRTI triphosphates in peripheral blood mononuclear cells (PBMC) has been a high priority in clinical pharmacology. However, one of the major problems in the measurement of intracellular ZDV-TP is the small amounts present in patients (low numbers of femtomoles per 10^6 cells). Thus, specific and sensitive analytical methodologies need to be developed in order to measure these small quantities without drawing large amounts of blood from patients.

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Several approaches have been reported for the determination of ZDV-TP, including a combination of high-performance liquid chromatography (HPLC) and radioimmunoassay (8, 17, 22). This methodology accomplished the separation of ZDV monophosphate (ZDV-MP), ZDV diphosphate (ZDV-DP), and ZDV-TP by strong anion-exchange HPLC. However, the limit of detection (LOD) was only 200 fmol/10^6 cells and the method was also time consuming since it took more than 45 min for each sample to be processed (3, 11). Recently, a variation of this approach was developed in which the separation method was replaced with anion-exchange solid-phase extraction (AX-SPE) (14). This method achieved a LOD of 20 fmol/10^6 cells. However, the method did not use an internal standard in the quantitation process, assuming complete recovery from all of the samples analyzed (14). In addition, the method cannot determine two or more NTRI triphosphates simultaneously.

Several liquid interfaces for mass spectrometry have been used to study nucleosides and nucleotides (1, 4, 6, 12, 16, 25). Electrospray ionization (ESI) is one of the most common interfaces used to obtain structural information and fragmentation patterns of nucleosides (1, 6, 12, 25). ESI-tandem mass spectrometry (MS/MS) provides even better specificity and selectivity when daughter ion experiments are performed in which a specific fragment ion is selected for quantitation analyses (18, 24).

In this report, we describe a method combining an AX-SPE procedure with HPLC-MS/MS to measure intracellular ZDV-TP in PBMC using azidodeoxyuridine (AZdU; 3′-azido-3′-

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deoxyuridine) as the internal standard. The LOD by this methodology is 4.0 fmol/10^6 cells with a linear concentration range of 4 orders of magnitude (4.0 to 10,000 fmol/10^6 cells). This method was successfully applied to measure ZDV-TP in PBMC obtained from HIV-infected patients. Furthermore, this methodology could be used to perform simultaneous detection of two or more NRTIs, such as ZDV-TP and lamivudine triphosphate (3TC-TP).

MATERIALS AND METHODS

Chemicals. ZDV, AZdU, sodium acetate, and acid phosphatase (type XA) were obtained from Sigma Chemical Co., St. Louis, Mo. [^{3}H]ZDV, [^{3}H]ZDV-TP, ZDV-TP, 3TC, and 3TC-TP were purchased from Moravek Biochemicals, Brea, Calif. Potassium chloride, acetonitrile, methanol, and glacial acetic acid (American Chemical Society certified) were obtained from Fisher Scientific, Fair Lawn, N.J. Anion-exchange Sep-Pak plus QMA cartridges were purchased from Waters Co., Milford, Mass. XAD resin was obtained from Serva, Heidelberg, N.Y. RPMI 1640 medium, glutamine, nonessential amino acids, penicillin-streptomycin, and fetal calf serum were obtained from BioWhittaker, Baltimore, Md.

Cell culture and incubation. CEM_{ss} cells, obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, were grown at a density of 0.6×10^6 to 0.8×10^6/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. These cells were incubated for 24 h with ZDV at final concentrations of 5.0 and 10.0 μM. After incubation, the cells were pelleted by centrifugation and then subjected to extraction with 70% methanol (20 μl/10^6 cells). The samples were centrifuged, and the supernatants were collected and stored at −80°C until analysis.

Sample collection from HIV-infected patients. Two tubes of venous blood (16 ml) were sampled in Vacutainer CPT tubes at different times after oral dosing. PBMC were separated from erythrocytes by centrifugation at $1,500 \times g$ for 20 min at room temperature. PBMC were recovered and counted in a Z2 series system (Coulter, Hialeah, Fla.), subjected to extraction with 70% methanol, and stored at −80°C until analysis. All patients provided informed consent, and the Institutional Review Board of the Medical Sciences Campus at the University of Puerto Rico approved the protocol.

Intracellular ZDV-TP isolation. AX-SPE was used to separate ZDV nucleotides as previously described (14). Briefly, the cartridges were preconditioned with 1.0 M KCl and washed with 5 mM KCl. The sample was loaded onto the cartridges, and 11 ml of 74.5 mM KCl was added. The fraction containing ZDV-TP was eluted with 3.3 ml of 1.0 M KCl. Recovery was performed with [^{3}H]ZDV-TP and determined to be >95%. Cleavage of phosphate groups was accomplished by the addition of 2 U of acid phosphatase per ml and incubation for 30 min at 37°C and adjustment of the fraction to pH 4 with sodium acetate. After enzyme digestion, AZdU (internal standard; 100 ng/ml) was added to the extract and recovered simultaneously with ZDV by using an XAD column. The XAD column was preconditioned with water before sample loading. The sample was desalted with 20 ml of water. ZDV and AZdU were eluted with 2 ml of acetonitrile. Desalting and recovery of ZDV and AZdU using XAD columns was >99%. Samples were dried in a CentriVap console (Labconco, Kansas City, Mo.) and reconstituted with 200 μl of the mobile phase prior to HPLC-MS/MS analysis.

HPLC-MS/MS. HPLC analysis was performed on a 1050 system (Hewlett-Packard, San Fernando, Calif.) using a Hypersil C_{18} reversed-phase column (100 by 2.1 mm; 3 μm). The mobile phase consisted of an acetonitrile-methanol mixture (10:30, vol/vol) with 0.25% acetic acid at a flow rate of 0.20 ml/min. The sample volume injected was 20 μl. A Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, United Kingdom) was used for the analysis in the multiple reaction monitoring (MRM) mode. Sample introduction was through ESI in the positive-ion mode. The cone voltage was set between 15 and 20 V, and the source temperature was 120°C. Ions were collisionally activated at a collision energy of 7 eV with the cell pressure set to 7.0×10^1 Pa of argon. MRM data were acquired and analyzed by using MassLynx software (v. 2.0).

Data analysis. Concentrations of analytes were determined by using peak-area ratios for ZDV and AZdU. A calibration curve from ZDV-TP solutions was prepared every time a series of samples were analyzed. Linear regression analyses were performed by using seven ZDV-TP concentrations distributed between 4.0 and 10,000 fmol/10^6 cells. Regression coefficients (r^2) were better than 0.997 for all calibration curves.

RESULTS AND DISCUSSION

The objective of this study was to develop a sensitive and selective method of intracellular ZDV-TP quantitation suitable for the determination of the intracellular pharmacokinetics of this anabolite in ZDV-treated patients. In this method, nucleotides from PBMC were extracted, ZDV-TP was separated from ZDV-MP and ZDV-DP through AX-SPE, and the phosphate groups were removed from ZDV-TP with acid phosphatase. The sample was desalted through an XAD column and quantified by HPLC-MS/MS.

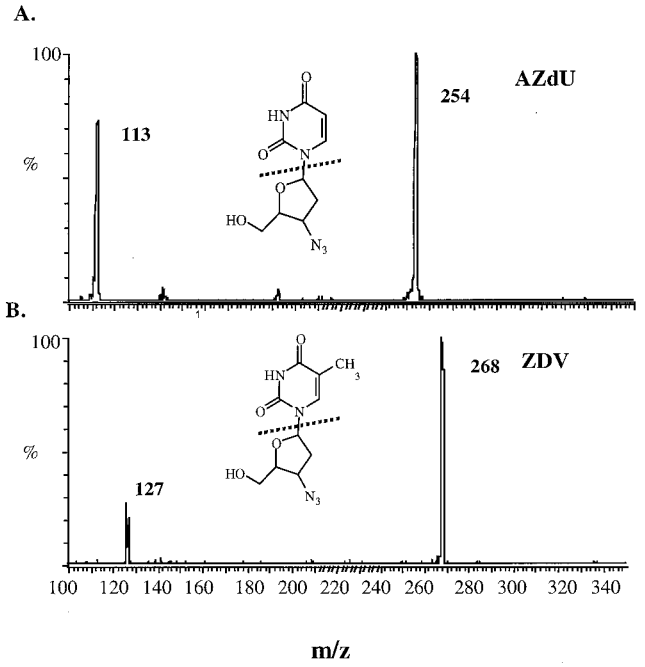

FIG. 1. Electrospray single mass spectra of AZdU (top) and ZDV (bottom). Experimental conditions were as described in Materials and Methods. The protonated molecular ion for AZdU appears at $m/z = 254$, and the fragment ion at $m/z = 113$ is assigned to the protonated base due to the cleavage of the glycosidic bond as illustrated by the dashed line. The protonated molecule of ZDV appears at $m/z = 268$, and the fragment ion at $m/z = 127$ is also due to the cleavage of the glycosidic bond. On the *y* axis is the normalized ion intensity from the fragment ions.

Mass spectrum characterization. Figure 1 shows the ESI mass spectra of ZDV and AZdU, which are the protonated molecules $(M + H)^+$ at $m/z = 268$ for ZDV and $m/z = 254$ for AZdU, the most abundant ions. Furthermore, the fragmentation of both nucleosides occurred through the glycosidic bond producing daughter ions at $m/z = 127$ and $m/z = 113$ for ZDV and AZdU, respectively. The glycosidic bond cleavage is induced by protonation of the base moiety, a general mechanism observed in the fragmentation of nucleosides in the ESI positive-ion mode (12). For the determination of ZDV, MRM only allows the ZDV protonated molecule ($m/z = 268$) to pass through the first quadrupole (MS-1). The daughter ion at $m/z = 127$ is formed in the collision cell and extracted for quantitation in the second quadrupole (MS-2). We used the same procedure for the determination of AZdU (MS-1, $m/z = 254$; MS-2, $m/z = 113$). MRM provides better selectivity and a better signal-to-noise ratio than a single mass spectrometer, enhancing the sensitivity and LOD of the method.

Interference from endogenous nucleotides. We obtained ESI-MS/MS chromatograms from 10^7 CEM_{ss} cells without ZDV with AZdU as the internal standard and ZDV-TP from 10^7 CEM_{ss} cells incubated with 5 μM ZDV for 24 h (data not shown). For the CEM_{ss} cells without ZDV, no appreciable signal appears in the chromatogram. Thus, endogenous nucleotides or other compounds from cell samples do not interfere with the ZDV-TP quantitation process. The chromatogram of AZdU (internal standard) showed a strong signal with a retention time of approximately 2.00 min. A similar chromatogram was obtained for ZDV with a retention time of about

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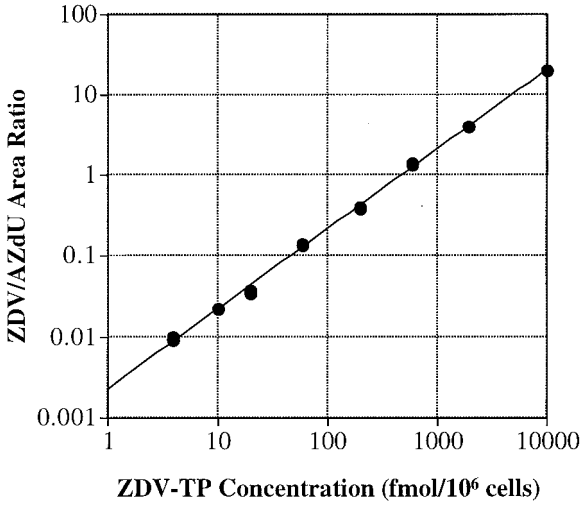

FIG. 2. Calibration standard curve for ZDV-TP constructed from standard solutions (4, 20, 59, 200, 590, 2,000, and 10,000 fmol/10^6 cells) passed through the complete methodology. The data are for two determinations for each concentration. The equation describing the complete range is $y = 0.002x - 0.008$ with a regression coefficient (r^2) of 0.998.

2.20 min. We determined that AZdU did not interfere with the ZDV signal despite the proximity of the retention times. Moreover, the short retention times of both compounds increase the throughput of samples (15 samples/h). This is a critical consideration for the use of this method in large clinical trials.

Standard curve and statistics. Figure 2 shows a typical calibration curve constructed from the ratio between the areas of ZDV and AZdU chromatograms versus ZDV-TP concentration. This calibration curve was obtained by using different ZDV-TP standard concentrations to spike PBMC and passing them through the entire methodology (AX-SPE, enzyme digestion, and XAD column chromatography). The LOD obtained with this methodology is 4.0 fmol/10^6 cells (coefficient of variation [CV], <8%, $n = 3$; accuracy, <10%, $n = 3$), with a linear range of 4.0 to 10,000 fmol/10^6 cells. By using [^{3}H]ZDV-TP, we determined the recovery of ZDV-TP to be >95% for the complete method at different ZDV concentrations ($n = 3$).

It is important to point out the necessity of the internal standard to obtain precise and accurate results. With AZdU, we lowered the CV for the calibration curve from 21 to 3% and the regression coefficient improved from 0.973 to 0.998. Furthermore, the interassay and intra-assay CV improved with AZdU for a ZDV concentration of 590 fmol/10^6 cells from 16 to 3% and for a ZDV concentration of 59 fmol/10^6 cells from 20 to 2%. The accuracy (percent error) of the method with AZdU was 7% for 590 fmol/10^6 cells ($n = 3$) and 8% for 59 fmol/10^6 cells ($n = 3$). Thus, the use of AZdU as the internal standard for the method dramatically improved the precision of the assay and provided an accurate method by which to measure low concentrations of ZDV-TP. To our knowledge, this is the first study that used an internal standard for the quantitation of intracellular ZDV-TP or another NRTI triphosphate in patient samples.

Table 1 shows the interassay variability results for four other concentrations (10, 20, 200, and 1,000 fmol/10^6 cells) used in the validation process. These results confirm the excellent accuracy and precision of the methodology developed. For all of the concentrations studied, the recoveries were >98% and the CVs were <10%. Thus, the methodology can be used to measure intracellular ZDV-TP from ZDV-treated HIV-infected patients.

TABLE 1. Method performance at different ZDV-TP concentrations

No. of expts	ZDV-TP concn (fmol/10^6 cells)		Deviation (%)	CV (%)
	Theoretical	Calculated		
3	10.0	10.8	8.3	4.5
3	20.0	19.7	−1.3	7.4
3	200	197	−1.5	6.9
3	1,000	991	−0.9	2.0

ZDV-MP and ZDV-TP in CEM_{ss} cells. Table 2 shows the concentrations of ZDV-MP and ZDV-TP obtained from the incubation of CEM_{ss} cells in 5.0 and 10.0 μM ZDV for 24 h. As shown in previous studies (13–15, 23), intracellular ZDV-MP concentrations were higher than those of ZDV-TP. This is due to inefficient activity of thymidylate kinase, the enzyme responsible for the conversion of ZDV-MP to ZDV-DP. As part of the same effect, the ZDV-MP concentration almost doubled (from 34.3 to 51.2 pmol/10^6 cells) when the extracellular ZDV concentration increased from 5.0 to 10.0 μM, whereas the ZDV-TP concentration changed insignificantly (from 1.18 to 1.04 pmol/10^6 cells).

Patient samples. The methodology described here was used to measure the intracellular concentrations of ZDV-TP in six samples from Hispanic HIV-infected patients treated with the standard ZDV therapy (300 mg twice a day). Blood samples were drawn at different time points, depending on the availability of the patients in the clinic. Figure 3 shows a typical chromatogram for ZDV obtained from one HIV-infected patient, showing an excellent signal above the background even at this low concentration (41 fmol/10^6 cells). Table 3 shows intracellular ZDV-TP concentrations in HIV-infected patients that ranged between 41 and 193 fmol/10^6 cells. These values are not different from those obtained previously by using other methodologies (2, 10, 13–15). In addition, the interpatient variability in our population is similar to that of other populations (2, 10, 13–15). The highest ZDV-TP concentration was observed in patient 1 (193 fmol/10^6 cells), who was sampled 2 h after drug administration. This ZDV-TP concentration is similar to that reported by Fletcher et al. for patients who had an increase in the percent change of $CD4^+$ cells over the baseline (5). The only other patient who was sampled before 6 h also had ZDV-TP concentrations above 100 fmol/10^6 cells (patient 2; 137 fmol/10^6 cells). All other patients were sampled more than 10 h after drug administration and had measurable intracellular ZDV-TP concentrations.

ZDV-TP and 3TC-TP. Although the aforementioned methodology is a step forward for the quantitation of ZDV-TP in clinical trials, the use of ZDV as monotherapy is no longer the best therapeutic option. Therefore, we need to develop new

TABLE 2. Cellular ZDV-MP and ZDV-TP concentrations[a]

Expt no.	Extracellular ZDV concn (μM)	Concn (pmol/10^6 cells)		ZDV-MP/ZDV-TP ratio
		ZDV-MP	ZDV-TP	
1	5.0	34.08	1.16	29.4
2	5.0	34.52	1.20	28.7
3	10.0	53.04	1.08	49.1
4	10.0	49.40	1.00	49.0

[a] Incubation of CEM_{ss} cells in ZDV at the indicated concentrations was done for 24 h.

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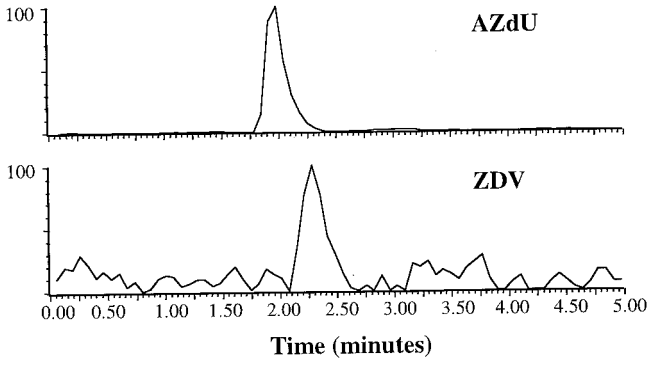

FIG. 3. Chromatograms for AZdU (internal standard) and ZDV obtained from patient 3. The ZDV signal was obtained from an intracellular ZDV-TP sample passed through the method. Chromatographic conditions are as described in Materials and Methods. Retention times for AZdU and ZDV were 2.00 and 2.20 min, respectively. The *y* axis is the normalized ion intensity obtained from daughter ions.

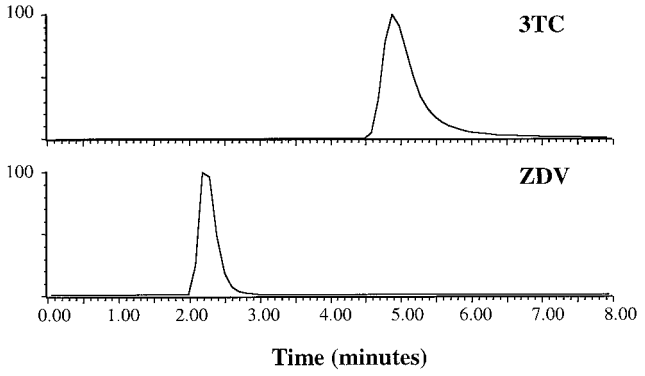

FIG. 4. Chromatograms for 3TC and ZDV obtained from standard 3TC-TP and ZDV-TP solutions used together to spike CEM_{ss} cells. The standard solutions were processed throughout the complete procedure. Retention times for ZDV and 3TC were 2.20 and 5.10 min, respectively. Experimental conditions were the same as for Fig. 3.

methodologies to quantify different NRTI triphosphates simultaneously. Recently, two methods were reported that measure intracellular 3TC-TP concentrations in HIV-infected patients (13, 19). Both methods used AX-SPE to separate 3TC-TP from other nucleotides, and the quantitation process was performed by either radioimmunoassay (13) or HPLC with UV detection (19). However, both methods could not measure ZDV-TP and 3TC-TP simultaneously. Figure 4 shows the chromatograms for the simultaneous determination of ZDV-TP and 3TC-TP in the same sample. ZDV-TP and 3TC-TP standard solutions were used together to spike 10^7 CEM_{ss} cells which were passed through the complete methodology with minor modifications. It is clear from Fig. 4 that ZDV and 3TC can be detected in the same sample. The 3TC-TP quantitation process is still being developed. This approach provides the advantage of allowing two or more NRTI triphosphates to be monitored without the need to obtain additional blood from the patient.

In summary, we described a method for the determination of ZDV-TP in PBMC from HIV-infected patients by using AX-SPE with HPLC-MS/MS. The LOD of the method is 4.0 fmol/10^6 cells, allowing intracellular ZDV-TP quantitation at very low concentrations. This assay can be used for the concurrent determination of ZDV-TP and 3TC-TP in clinical trials to further investigate the relevance and clinical implications of the phosphorylation process of these drugs in different HIV-infected populations and age groups. These results may entice the notion to support individual dose modifications. Clinical studies are in progress to answer these and other questions.

TABLE 3. Intracellular ZDV-TP concentrations of HIV-infected patients[a]

Patient no.	Age (yrs)	Gender[b]	Time (h) between drug administration and blood sampling	Mean intracellular ZDV-TP concn ± SD (fmol/10^6 cells)[c]
1	35	M	2	193 ± 4
2	52	F	4	137 ± 1
3	39	F	11	42 ± 1
4	38	M	11	83 ± 16
5	43	M	12	124 ± 4
6	66	F	15	63 ± 4

[a] Patients were receiving the standard 300-mg ZDV dose twice daily.
[b] M, male; F, female.
[c] Intracellular concentrations are means of two measurements.

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