

Development of an LC–MS Method for Measuring TNF in Human Vaginal Tissue

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Abstract

A sensitive, accurate, and precise liquid chromatography–mass spectrometry assay for the determination of tenofovir (TNF) in human vaginal tissue was developed and validated. After homogenization of the tissue, solid-phase extraction on Varian Bond Elut-C₁₈ column was used for sample clean up. Chromatographic separation of TNF and the internal standard (tolbutamide) was achieved with a Varian Polaris 3C₁₈-A reversed-phase analytical column (150 mm × 2 mm). A gradient method using 0.1% formic acid in water and 0.1% formic acid in acetonitrile was employed. Detection of TNF and tolbutamide was achieved by electrospray ionization mass spectrometry in the positive ion mode using 288.05 and 271.00 *m/z*, respectively. Linear TNF calibration curves were obtained between 1–1,000 ng/mL with a correlation coefficient (*r*²) greater than 0.999. Intra- and inter-day accuracy for TNF ranged from 89.7% and 109.4% and from 97.3% and 104.9%, and precision ranged from 1.3% and 10.9% and 2.6% and 9.0%, respectively. This is the first validated method developed to quantitate TNF in human tissues.

Introduction

Tenofovir (TNF) is the first nucleotide reverse transcriptase inhibitor approved by the U.S. Food and Drug Administration for treatment of human immunodeficiency virus (HIV)-1 and is used in combination with other antiviral medications to treat HIV infected patients (1). TNF is a monophosphonate adenosine analogue (Figure 1) that is diphosphorylated to its active moiety, TNF diphosphate (TNF-DP), within the cell where it functions as a competitive inhibitor of reverse transcriptase and an HIV-DNA chain terminator.

Currently, a major effort is underway to develop pre-exposure prophylaxis strategies (PrEP) against HIV infection, including oral and topical dosing of antiretroviral agents. One approach currently in clinical study is the use of TNF, both with the oral dosing formulation (REF) and as a 1% w/w topical gel (2). To understand the efficacy or failure of these approaches, it is critical to understand the pharmacokinetics of TNF at the site of infection (namely, mucosal tissues).

Several high-performance liquid chromatography (HPLC) or

HPLC–tandem mass spectrometry (MS–MS) methods have been developed and published to quantify TNF in plasma (3–10). Yet, there is no known efficient method which can quantitate TNF in vaginal tissue. It is believed that most of TNF will exist as diphosphate form in the tissue because intracellular phosphorylation occurs very quickly. Therefore, we have developed a highly sensitive, accurate, and precise liquid chromatography–mass spectrometry (LC–MS) method to measure the concentration of total TNF after enzymatic hydrolysis using phosphatase in this biological matrix.

Experimental

Chemicals

TNF was obtained from the NIH AIDS Research & Reference Reagent Program. Tolbutamide was purchased from Sigma (St. Louis, MO). TNF-DP was kindly obtained from Gilead (Foster, California). The structures of TNF and tolbutamide are shown in Figure 1. Formic acid, LC–MS-grade acetonitrile, methanol, and ammonium acetate were purchased from Fisher Scientific (Fairlawn, NJ). Phosphatase for enzymatic hydrolysis of TNF-DP was purchased from Sigma. All other reagents were HPLC-grade. Purified compressed nitrogen gas was purchased from National Welders Supply (Charlotte, NC).

Human vaginal tissue

Blank human vaginal tissue was obtained from the National Development and Research Institute (NDRI) (New York, NY). In addition, six fresh vaginal tissue samples were obtained from surgical waste at University of North Carolina Hospitals. All patients gave written informed consent prior to donation. The tissue collection protocol was approved by the institutional review board at the University of North Carolina at Chapel Hill. The tissues were stored at –70°C in a freezer before analysis.

Instruments

The Shimadzu LC–MS consisted of Shimadzu LC–MS SIL-HTc autosampler, Shimadzu LC–MS LC-20AD solvent delivery system, Shimadzu LC–MS 2010A MS Detector, and Shimadzu LC–MS solution software (Tokyo, Japan). The analytical column was a Varian Polaris 3C₁₈-A (2 mm × 150 mm, Palo

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alto, CA) protected by a Phenomenex Polar RP guard cartridge (Torrance, CA). A Fastprep-24 Homogenizer and Lysing Matrix A tubes for homogenization of tissue were purchased from MP Biomedicals (Solon, Ohio).

Preparation of TNF, quality control, and internal standard solutions

Stock solutions of TNF and TNF-DP (as TNF) were prepared at a concentration of 1 mg/mL. For TNF, 5.3 mg of TNF monohydrate (molecular weight 305.23 g) powder was accurately weighed and dissolved in 3.0 mL of HPLC-grade water alkalized with 50 μ L of 5.0 M sodium hydroxide solution, and the volume was adjusted to 5.0 mL using a 5.0-mL volumetric flask. The TNF-DP stock solution was prepared by diluting 84.5 mM TNF-DP solution from Gilead with HPLC-grade water to make 1 mg/mL solution. Further dilution with HPLC water was used to prepare an intermediate stock solution for TNF and TNF-DP at a concentration of 100 μ g/mL. This intermediate TNF stock solution was used to prepare seven working standard solutions (1, 5, 10, 50, 100, 500, and 1000 ng/mL) in tissue homogenate. Tissue quality control (QC) samples were prepared from a separately prepared intermediate 100 μ g/mL stock to create concentrations of 3, 150, and 750 ng/mL in tissue homogenate for both TNF and TNF-DP.

An internal standard stock solution was prepared by accurately weighing 5 mg of tolbutamide and dissolving this in a volumetric flask with HPLC-grade water to achieve a final concentration of 1.0 mg/mL. The internal standard (IS) working solution was prepared by diluting an aliquot from the stock solution in 150 mM ammonium acetate buffer (pH adjusted to 5.0 using 33% acetic acid) to a final concentration of 4.0 μ g/mL.

Homogenization of tissues

For calibrator and QC preparation, drug-free vaginal tissue was homogenized in a Lysing matrix "A" tube using a Fastprep 24 homogenizer at a speed of 4 M/s for 60 s until the mixture formed a homogeneous solution. Then, 200 μ L of vaginal tissue homogenate (20 mg of tissue) was mixed with 100 μ L of working standard or QC solution, 50 μ L of water and 50 μ L of the IS working solution.

For clinical sample preparation, 20 mg of tissue, 50 μ L of IS working solution and 330 μ L of water were homogenized in a Lysing Matrix tube A using the Fastprep 24 homogenizer at a speed of 4 M/s for 60 s until the mixture formed a homogeneous solution.

Enzymatic hydrolysis of TNF-DP in tissue

In tissue cells, phosphorylation occurs rapidly and most of TNF exists as a phosphorylated form in the cell. To measure the total TNF concentration in tissue, an enzymatic hydrolysis process using phosphatase is an essential step (9). An aliquot (20 μ L) of homogenate was used for another assay to measure protein content, and 50 μ L of phosphatase solution (26 units/mL acid phosphatase in 50 mM ammonium acetate pH 4.0) was added to the remaining homogenate and incubated at 37°C for 1 h in water bath. After incubation, the homogenate was centrifuged at 13,200 rpm for 10 min.

Solid phase extraction procedure (6)

Solid-phase extraction columns (1.0 mL, 200 mg BOND ELUT-C₁₈ Varian, Harbor City, CA) were placed on a vacuum elution manifold (20-SPE System, Waters, Milford, MA). The cartridges were rinsed with 500 μ L of methanol, and then conditioned with 500 μ L of 150 mM ammonium acetate (pH 5.0). After loading 200 μ L of 150 mM ammonium acetate (pH 5.0) to the cartridge, 360 μ L of centrifuged blank, calibrator, QC, or patient tissue homogenate was added to the SPE column. Tissue homogenates were allowed to pass through the column bed with minimal suction (1–3 mmHg). The column was further washed with 900 μ L of 100 mM ammonium acetate buffer (pH 7.0) and then suctioned to dryness for 2 min using 10–15 mmHg of vacuum. The retained drugs were then eluted with 500 μ L of methanol. The eluent solution was evaporated to dryness under a gentle nitrogen stream at 45°C, and the residue was reconstituted with 50 μ L of reconstitution solution (3% acetonitrile–1% acetic acid in HPLC water). The resulting solutions were sonicated for 30 s, carefully vortexed for 30 s, and centrifuged at 13,200 rpm for 10 min. The supernatants were transferred to 200- μ L HPLC autosampler vials (Microvials, Agilent), and 5 μ L was injected onto the column.

Chromatography and LC–MS conditions

Chromatographic separation was achieved with gradient elution on a Varian Polaris 3C₁₈-A analytical column, (3.0 μ m particle size) protected by a Polar RP guard cartridge. Two mobile phases were used: Mobile phase A was composed of 0.1% formic acid in HPLC water, measured pH 2.5. Mobile phase B was composed of 0.1 % formic acid in acetonitrile. A linear gradient was programmed from 3% to 90% mobile phase B over 5.5 min with holding until 7.5 min and from 90% to 3% mobile phase B between 7.5 min and 7.7 min with holding until 13.0 min. The analysis was performed at room temperature with a gradient mobile phase flow rate of 0.2 mL/min over the 13 min run time. No carryover was found when the TNF concentrations were within the range of the calibrators. The detector settings were: ESI with the stainless steel spray needle, positive polarity ionization; detector voltage, 1.5 kV; Curved Desolvation Line (CDL) temperature, 250°C; Heat block temperature, 200°C; nebulizing gas flow, 1.5 L/min; drying gas on. Two different mass-to-charge ratios in positive ion mode were used to monitor the intensity during elution of the analytes; 288.05 *m/z*-TNF, 271.00 *m/z*-tolbutamide (IS). The positive ion mode was chosen for this assay, as an initial study indicated a more sensitive response than with the negative ion mode. The low pH of mobile phase was necessary for adequate analyte retention.

Validation of the method (11,12)

Validation of the analytical method included evaluation of calibration curve performance, recovery, intra- and inter-day accuracy, precision of the method, stability of the analytes at various test conditions (determined by TNF and TNF-DP QC performance), evaluation of specificity and matrix effect, and comparing differences between fresh and frozen tissue.

Inter- and intra-day accuracy and precision for the assay were characterized by QC performance (four concentrations for

TNF and three concentrations for TNF-DP) on three separate days using three replicates each day. Concentrations for TNF QCs included 1 ng/mL (equivalent to the lower limit of quantification of the assay), 3 ng/mL, 150 ng/mL, and 750 ng/mL. Precision was quantified as the coefficient of variation (CV %) within a single run (intra-day) and between different runs (inter-day). Accuracy was quantified as the percentage of deviation between nominal and measured concentrations. The lower limit of quantification was determined as a signal-to-noise ratio of 5:1 with CVs of accuracy and precision of less than 20% at this concentration.

The stability of TNF and TNF-DP in tissue was also investigated using the medium (150 ng/mL) and high (750 ng/mL) QCs in triplicate. Short-term stability testing was performed by leaving stock solutions at room temperature for 6 h and spiked tissue samples at room temperature for 24 h prior to extraction. Long term stability testing was performed by leaving stock solutions and spiked tissue samples at -70°C for two months. Stabilities of TNF and TNF-DP during sample handling were evaluated by subjecting samples to three freeze-thaw cycles. Finally, testing was performed to verify stabilities of TNF and TNF-DP when held in an autosampler for 72 h.

Specificity was tested using tissues from six different women. Matrix effects were assessed by calculating the response ratio between analytes, with and without matrix, and by calculating the % differences between fresh and frozen tissue at one standard concentration of 500 ng/mL.

Analysis of clinical samples

We examined the applicability of the method by analyzing tissue collected from HIV-negative subjects after a single dose of 1% TNF vaginal gel.

Results

Linearity

The peak area ratios of the calibration standards to the internal standard were proportional to the concentration of each drug in tissue over the range tested. The linear range for TNF was 1–1,000 ng/mL, and the regression coefficients (r^2) were all greater than 0.999. Calibration parameters of slope and intercept were calculated.

Selectivity

Figure 2 shows a representative chromatogram of blank tissue (A); the approximate retention times of TNF and IS were 3.7 min and 7.5 min, respectively. No other peak was interfered with the peak of TNF or IS in this method.

Accuracy and precision

The intra- and inter-day accuracy and precision (CV %) of TNF and TNF-DP are listed in Table I. The accuracy of TNF ranged from 89.7 to 109.4% with a mean of 99.1%. Throughout the concentration range of the control samples, the mean intra- and inter-assay precision for TNF remained less than 10.9%. All the accuracies and precisions of the analytes were

within the acceptable range according to bioanalytical method validation guidelines (11).

The limit of quantification

The low limit of quantification for TNF was 1 ng/mL. Accuracy and precision at the lower limit of quantification were less than 10.9% for TNF and, therefore, within the acceptable range (11).

Recovery

The absolute recovery of TNF from tissue extracted using SPE was calculated by comparing peak areas of extracted QC samples to peak areas of QC sample concentrations spiked into blank tissue after the extraction was performed. This method was used to account for matrix effects with the MS detection. This extraction method resulted in a clean baseline (Figure 2A). Table II shows the recovery results and a mean recovery of 74.1% using three QC samples for TNF and 70.0% for IS.

Stability

Drug stability in biological fluids is a function of the sample storage conditions, the chemical properties of the drug, the matrix, and the container system (11). The stability of TNF and TNF-DP were evaluated under conditions reflecting situations likely to be encountered during actual sample handling and analysis. TNF and TNF-DP were found to be stable in stock solutions during sample collection and handling, after short and long-term storage, after three freeze-thaw cycles, and during the analytical process. Table III demonstrates that all results in the stability testing of TNF and TNF-DP were within the acceptance range of $\pm 15\%$ deviation from the nominal concentration.

Matrix effect

The approach used to assess possible matrix differences among different tissues was evaluated by measuring TNF response with and without matrix as previously described (12). Mean (CV) matrix effect ($n = 5$) for TNF was 83.1% (6.5%). Although ionization of TNF was suppressed with the

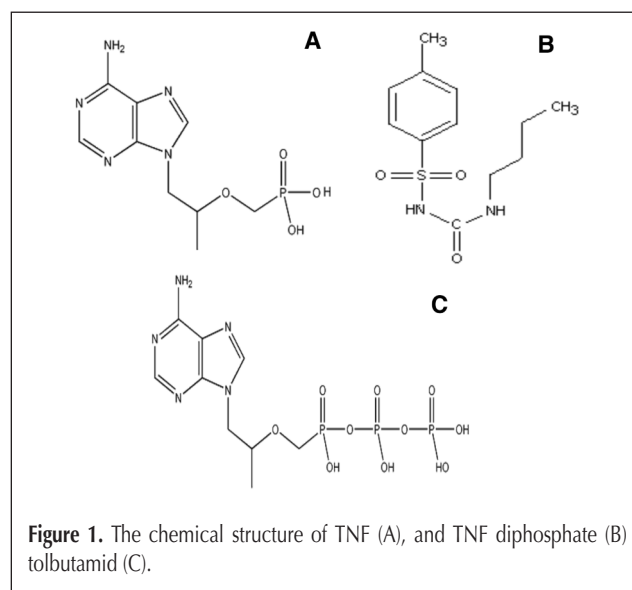


Figure 1. The chemical structure of TNF (A), and TNF diphosphate (B) tolbutamid (C).

tissue matrix, the matrix effect was consistent in the five different tissue samples tested.

Application of the method

Figure 2B–2C show representative chromatograms from clinical samples 2 and 24 h after administration of TNF gel preparation. No other interfering peaks were found.

Discussion

This paper details the validation of a most accurate, sensitive, and selective method for measuring TNF in human tissue using LC–MS. Although several methods for measuring TNF in blood plasma using HPLC or LC–MS–MS have been reported (3–10), this method is the first to measure TNF in tissue samples at a low limit of quantification (1 ng/mL).

We have previously published a validated method of measuring TNF in blood plasma using LC–UV (6). The solid-phase extraction procedure utilized in this plasma method was also found to be optimal for MS detection of tissue concentrations.

This method required careful optimization of the MS conditions, including ionization mode and the LC conditions (column and mobile phase composition and flow). Because TNF can accept a proton from an acidic mobile phase, positive detection mode with a mobile phase containing 0.1% formic acid was chosen. With optimization of the gradient conditions, and the addition of a long re-equilibration time, TNF's retention time remained consistent. An acidic reconstitution solution was used to further enhance ionization. To retain TNF, we tested three different columns; Atlantis C₁₈ (Waters), Zorbax C₁₈ (Agilent), and Polaris 3C₁₈-A (Varian). With the exception of the Varian Polaris 3C₁₈-A column, all columns resulted in an unacceptable early elution of TNF. The Varian Polaris 3C₁₈-A column eluted TNF at approximately 3.5 min with excellent peak shape.

Tolbutamide was chosen as IS due to its similar behavior to TNF when subjected to the solid-phase extraction procedure and its good resolution from TNF on the chromatogram. The amount of

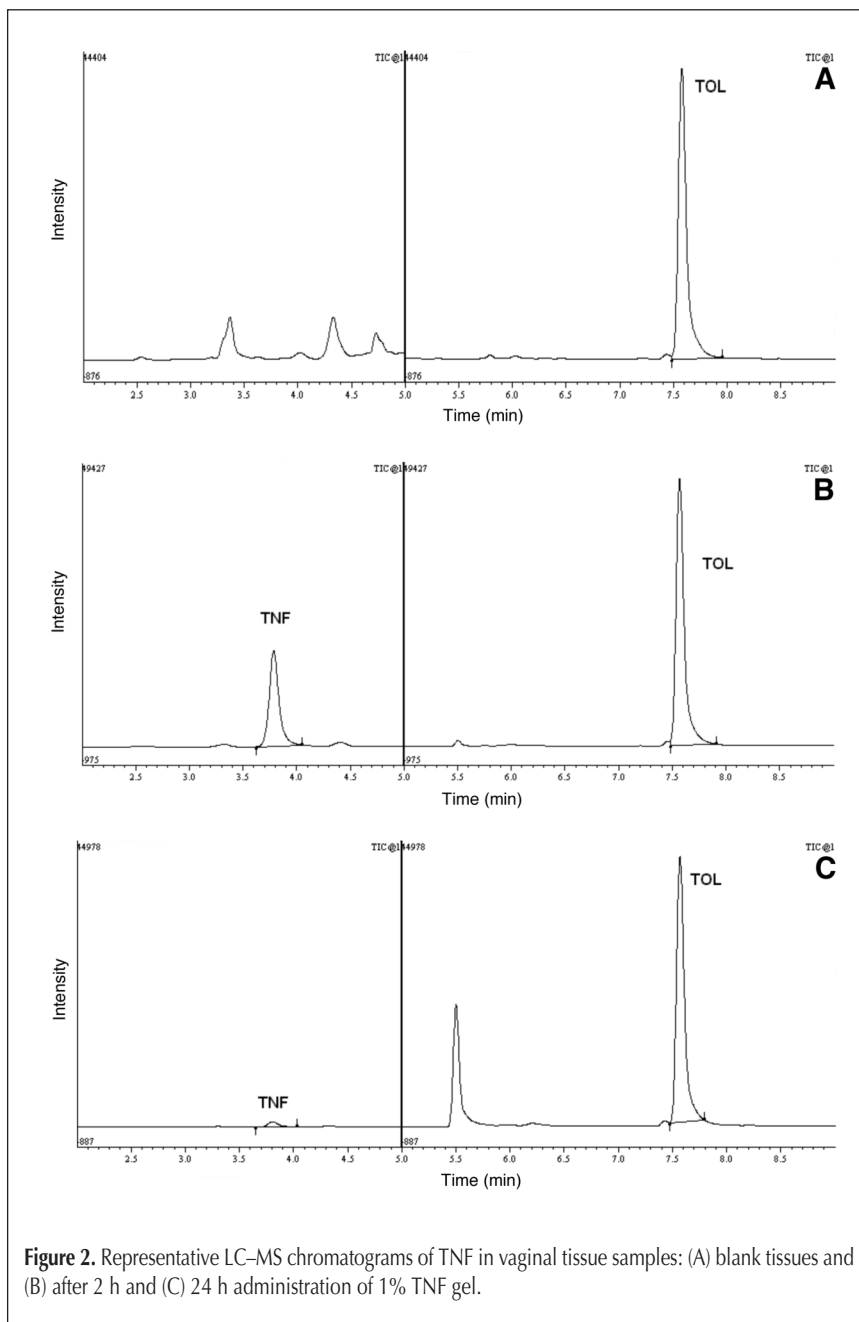


Figure 2. Representative LC–MS chromatograms of TNF in vaginal tissue samples: (A) blank tissues and (B) after 2 h and (C) 24 h administration of 1% TNF gel.

Table I. Accuracy and Precision for the Determination of TNF and TNF-DP in Human Tissue

| Nominal Conc. (ng/mL) | Intra-day | | | Inter-day | | |
|----------------------------|-----------------------------|--------------|------------------|-----------------------------|--------------|------------------|
| | Measured TNF Conc. (ng/mL)* | Accuracy (%) | Precision CV (%) | Measured TNF Conc. (ng/mL)* | Accuracy (%) | Precision CV (%) |
| 1 (TNF) | 1.0 ± 0.1 | 100.7 ± 11.0 | 10.9 | 1.0 ± 0.0 | 97.3 ± 3.1 | 3.1 |
| 3 (TNF) | 2.7 ± 0.0 | 89.7 ± 1.1 | 1.3 | 3.0 ± 0.3 | 101.1 ± 9.1 | 9.0 |
| 150 (TNF) | 150.6 ± 8.0 | 100.4 ± 5.3 | 5.3 | 151.5 ± 3.9 | 101.0 ± 2.6 | 2.6 |
| 750 (TNF) | 820.3 ± 58.9 | 109.4 ± 7.9 | 7.2 | 786.6 ± 39.2 | 104.9 ± 5.2 | 5.0 |
| 3 (TNF-DP [†]) | 3.0 ± 0.1 | 98.6 ± 3.7 | 3.7 | 3.0 ± 0.1 | 99.3 ± 1.8 | 1.8 |
| 150 (TNF-DP [†]) | 133.3 ± 5.5 | 88.9 ± 3.7 | 4.1 | 136.6 ± 6.6 | 91.1 ± 4.4 | 4.8 |
| 750 (TNF-DP [†]) | 807.3 ± 31.5 | 107.6 ± 4.2 | 3.9 | 728.2 ± 54.3 | 97.1 ± 7.2 | 7.5 |

* Data are mean values ± SD (n = 3). † as TNF base.

| Test of Recovery | Low QC [†] | Medium QC [‡] | High QC [§] |
|------------------|---------------------|------------------------|----------------------|
| Recovery (%) | 74.4 ± 2.6 | 70.0 ± 5.4 | 78.0 ± 0.8 |

* Data are mean values ± SD (n = 3). † Low QC is 3 ng/mL.
 ‡ Medium QC is 150 ng/mL. § High QC is 750 ng/mL.

| Test of stability | Stability of TNF (%) | | Stability of TNF-DP (%) | |
|-------------------------|------------------------|----------------------|-------------------------|----------------------|
| | Medium QC [†] | High QC [‡] | Medium QC [†] | High QC [‡] |
| Freeze/Thaw | 98.2 ± 5.4 | 90.2 ± 1.8 | 95.3 ± 5.3 | 99.2 ± 3.6 |
| Short-term [§] | 102.6 ± 0.8 | 101.0 ± 1.4 | 88.5 ± 1.5 | 98.5 ± 1.6 |
| Long-term** | 106.6 ± 7.9 | 99.09 ± 2.4 | 104.49 ± 2.9 | 102.0 ± 2.5 |
| Post-preparative | 92.3 ± 8.1 | 86.2 ± 5.2 | 98.4 ± 6.8 | 81.8 ± 1.4 |
| Stock solution | 103.6 ± 4.9 | 100.4 ± 0.8 | | |

*Data are mean values ± SD (n = 3). † Medium QC is 150 ng/mL.
 ‡ High QC is 750 ng/mL. § Short-term stability was tested after 24 h at room temperature.
 ** Long-term stability was tested after two months at -70°C.

tolbutamide was selected based on recovery and reproducibility in the assay. Tolbutamide was added to tissues before homogenization to compensate for any loss of sample during this process.

Complete homogenization of tissues was critical to extracting all intracellular TNF and obtaining accurate and precise protein concentrations. Homogenization conditions were optimized for speed and duration of the cycle, in addition to the type of homogenization tubes. Complete homogenization required approximately ten cycles of a 60 s run. Five min of rest was provided between runs to prevent overheating of the homogenate.

Phosphorylation of TNF occurs rapidly in cells, and in vitro data suggest that most of the intracellular drug exists as the diphosphate form (9). Due to the small biopsy sample sizes, this method was designed to measure total TNF drug concentration (extracellular + intracellular) in tissues using enzymatic hydrolysis with phosphatase. The conditions for enzymatic hydrolysis were optimized for the amount of phosphatase, incubation time, and temperature. The optimized condition yielded greater than 98% cleavage of TNF diphosphate.

This method has been fully validated. Intra- and inter-day accuracy and precision at LLOQ, low, medium, and high concentration were acceptable and within 15% of nominal (Table I). TNF was acceptably stable under all the storage conditions tested. The mean extraction efficiency of TNF was 74.1%, and the mean matrix effect was 83.1%, and consistent between the different tissue samples evaluated. Assay specificity was evaluated in six different tissues. The responses of TNF from fresh and frozen tissues were compared in six different tissues, and the maximum difference was 8.3%. Calibration curves were found linear over the range of 1–1000 ng/mL ($r^2 > 0.998$).

Conclusion

A SPE extraction method followed by an automated and optimized LC–MS method has been developed for the analysis of total TNF in human vaginal tissue. This method is sensitive, accurate, and precise and will assist in the drug development process. This approach can be used to assess the pharmacokinetics of TNF in human vaginal tissue.

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References

- D.J. Porshe. TNF disoproxil fumarate (Viread). *J. Association of Nurses in AIDS care. J. Assoc. Nurses AIDS care* **13**(3): 100–102 (2002)
- K.H. Mayer. Safety and tolerability of TNF vaginal gel in abstinent and sexually active HIV-infected and uninfected women. *AIDS* **20**(4): 543–551 (2006).
- V. Jullien, J.M. Treluyer, G. Pons, and E. Rey. Determination of TNF in human plasma by high-performance liquid chromatography with spectrofluorimetric detection. *J. Chromatogr. B* **785**: 377–381 (2003).
- R.W. Sparidans, K.M.L. Crommemtuyn, J.H.M. Schellens, and J.H. Beijnen. Liquid chromatographic assay for the antiviral nucleotide analogue TNF in plasma using derivatization with chloroacetaldehyde. *J. Chromatogr. B* **791**: 227–233 (2003).
- S. Sentenac, C. Fernandez, A. Thuillier, P. Lechat, and G. Aymard. Sensitive determination of TNF in human plasma samples using reversed-phase liquid chromatography. *J. Chromatogr. B* **793**: 317–324 (2003).
- N.L. Rezk, R.D. Crutchley, and A.D.M. Kashuba. Simultaneous quantification of emtricitabine and TNF in human plasma using high-performance liquid chromatography after solid phase extraction. *J. Chromatogr. B* **822**: 201–208 (2005).
- V. Bezy, P. Morin, P. Couerbe, G. Leleu, and L. Agrofoglio. Simultaneous analysis of several antiretroviral nucleosides in rat-plasma by high-performance liquid chromatography with UV using acetic acid/hydroxylamine buffer Test of this new volatile medium-pH for HPLC–ESI-MS/MS. *J. Chromatogr. B* **821**: 132–143 (2005).
- T. Delahunty, L. Bushman, and C.V. Fletcher. Sensitive assay for determining plasma TNF concentrations by LC/MS/MS. *J. Chromatogr. B* **830**: 6–12 (2006).
- T. King, L. Bushman, J. Kiser, P.L. Anderson, M. Ray, T. Delahunty, and C.V. Fletcher. Liquid chromatography–tandem mass spectrometric determination of TNF-diphosphate in human peripheral blood mononuclear cells. *J. Chromatogr. B* **843**: 147–156 (2006)
- M.L. Barkil, M.C. Gagnieu, and J. Guittion. Relevance of a combined UV and single mass spectrometry detection for the determination of TNF in human plasma by HPLC in therapeutic drug monitoring. *J. Chromatogr. B* **854**: 192–197 (2007).
- Food and Drug Administration, Center for Drug Evaluation and Research, Guidance for Industry, Bioanalytical Method Validation (2001).
- S. Bansal and A. Destefano. Key elements of bioanalytical method validation for small molecules. *The AAPS Journal* **9**: 109–114 (2007).