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Relevance of a combined UV and single mass spectrometry detection for the determination of tenofovir in human plasma by HPLC in therapeutic drug monitoring

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Abstract

A sensitive high-performance liquid chromatography method coupled to UV and single mass spectrometry (MS) detection was developed for the determination of tenofovir in human plasma. A solid phase extraction procedure (Bond-Elut® C18 Varian cartridges) provided high extraction efficiency (91% for tenofovir and 68.8% for the internal standard, 3-methylcytidine). An atlantis®-dC-18 analytical column is used with an isocratic mode elution of a mixture (pH 2.5) of ammonium acetate/methanol (98.5:1.5, v/v). Detection was performed at 260 nm and by using the ion at *m/z* 288. The signals from both detectors were validated over the range of 10–1000 ng mL^{-1} and were found to be linear, accurate and precise. At the lowest limit of quantification, 10 ng mL^{-1} for UV and 5 ng mL^{-1} for MS, the average coefficient of variation was 6.9 and 3.9%, respectively. To investigate the potential of the validated method for clinical studies, more than 170 samples from HIV-infected adult patients were then analyzed with this assay. A good correlation was observed between the results obtained with both detectors. However, in several cases discordant results were observed between UV and MS detections. Therefore, tenofovir can sometimes suffer from interferences using either UV or single MS detection. We concluded that the double detection allows to obtain a more specific quantification of tenofovir. The present assay is sound and can be used for therapeutic drug monitoring allowing a higher reliability of the results which are transmitted to the medical team.

Keywords: Tenofovir (TNF); Mass spectrometry; UV; HPLC

1. Introduction

Tenofovir (TNF) is a nucleotide analogue commonly used in HIV-therapy. TNF is converted from pro-drug tenofovir disoproxyl fumarate (Viread®) by diester hydrolysis involving serum and tissue esterases [1]. Then, phosphorylations by cellular enzymes lead to TNF diphosphate, the active form of the drug. Viread® is administered once daily due to the long intracellular half life of TNF diphosphate [2]. A sensitive analytical technique for the quantification of TNF in plasma is needed for the therapeutic drug monitoring. This allows verifying drug compliance in order to prevent the emergence of resistance mutations and may be useful to prevent nephrotoxic events in patients with renal failure.

Several methods for the determination of TNF based on high-performance liquid chromatography using ion-pairing reagent or phosphate buffer in mobile phase with UV or fluorometric detection was previously described [3–6]. Recently, different methods based on liquid chromatography coupled with tandem mass spectrometry were also proposed [7–9]. These assays used expensive and impressive performance apparatus generally dedicated to pharmacokinetics studies or research environment.

The aim of the present work was to develop a simple and reliable procedure to determine TNF in human plasma with liquid chromatography coupled with a double detection UV and single mass spectrometry. LC-UV apparatus remain the most common apparatus for drugs analysis in hospital laboratories, but the single mass spectrometry detector is becoming more and

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more available. For this purpose, both detection modes were fully validated. Then, more than one hundred-seventy patient samples were analyzed with LC-UV–MS coupled detection. We pointed out that in several cases discordant results were observed between UV and MS detections. Therefore, tenofovir measurement can sometimes suffer from interferences using either UV or single MS detection. Finally, our goal was to focus on the relevance of using the combination of UV and single MS detections for the quantification of TNF in therapeutic drug monitoring (TDM).

2. Materials and methods

2.1. Chemicals

Tenofovir (TNF) monohydrate powder (purity 99.0%) was kindly supplied by Gilead™ (Foster city, USA). Anhydrous ammonium acetate, formic acid, trifluoroacetic acid (TFA) and 3-methylcytidine methosulfate (purity 98%), used as internal standard (I.S.), were from Sigma–Aldrich (Saint Quentin Fallavier, France). The chemical structures of TNF and the I.S. are presented in Fig. 1. Lichrosolv® gradient grade methanol and acetonitrile for HPLC were obtained from Merck (Darmstadt, Germany). Deionized water was used throughout the study (Milli-Q plus-185®, Millipore, USA). Drug-free normal human plasma was provided by the regional blood bank (EFS Rhône-Alpes, France).

2.2. Instrumentation and chromatographic conditions

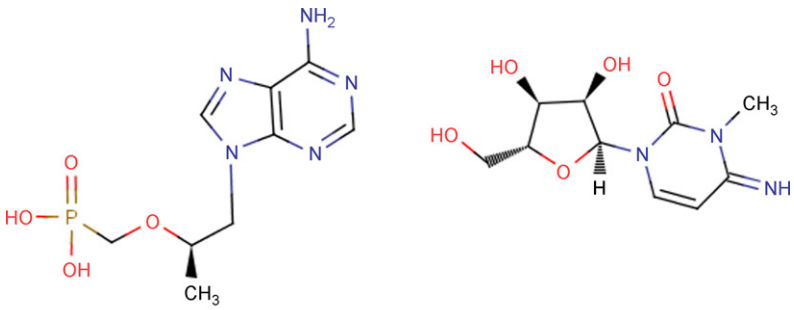
The separation was achieved on an analytical column Atlantis© dC-18 column (150 mm × 3.0 mm I.D.; 3 μm particle size; Waters™, France). The mobile phase, a mixture of ammonium acetate buffer and methanol (98.5:1.5, v/v) was eluted at 0.6 mL min^{-1}. The buffer was prepared as follows: ammonium acetate solution (5 mM) was adjusted at pH 2.5 with formic acid (purity 98%). These isocratic conditions were applied for 8 min; the column was maintained at 30 °C. After each run, the column was washed by a mixture of ammonium acetate buffer and acetonitrile (30/70, v/v) for 5 min in order to elute co-prescribed drugs as well as endogenous compounds with closed chemical structures to TNF. The column was then equilibrated for 10 min with the analytical mobile phase before next sample injection.

The HPLC material is all Model-1100© from Agilent (Palo Alto, USA) and consisted of a multisolvent delivery module with quaternary pump, connected on-line to a degasser. Autosampler was used to perform injection. Detection was achieved using photodiode array detector (DAD) and a single quadrupole mass spectrometer (MS); the eluent from DAD was injected directly to the electrospray ionization (ESI) interface of the MS. MS was operated in positive ion mode. Nitrogen was used as gas to assist the nebulization and desolvation. Optimization of the LC–MS conditions was carried out by varying them in flow injection analysis (FIA) of the compounds. The optimized parameters of the interface were: vaporizer temperature 250 °C, nebulizer gas pressure 60 bar, drying gas flow rate 12 L min^{-1}, fragmentor 100 V and capillary voltage 4000 V.

For UV detection, wavelength was monitored from 200 to 400 nm in order to obtain the full spectrum of TNF and I.S. Both molecules were quantified at 260 nm. Concerning MS detection, the TNF protonated molecules $[M+H]^+ = m/z$ 288 was used as the quantification ion and the ion at $[M+H]^+ = m/z$ 176 (obtained after cleavage of phosphate group) as qualifier ion. The ion ratio 288/176 is equal to 5/1. In case of this ratio being different at ±20%, the quantification was performed with the ion at m/z 176. The detection of the I.S. was made with the protonated molecules $[M+H]^+$ at m/z 258. Data acquisition started 2.0 min after the injection of the sample and was performed in the selected ion monitoring mode with 92 ms dwell time per channel.

2.3. Standard preparation

Standard stock solutions of TNF and I.S. were prepared in distilled water at 100 and 250 μg mL^{-1}, respectively. These solutions were aliquoted and stored at −20 °C until use. It was checked whether the solutions are stable for at least 10 months in these conditions were. For calibration curves, standard solutions were made daily by further dilution of stock solutions with water. One milliliter of blank plasma was spiked with the appropriately diluted standard solutions to final concentration of 10, 50, 100, 500 and 1000 ng mL^{-1}. An extra standard at 5 ng mL^{-1} was analyzed by MS detection. Blank plasma samples were also prepared. Quality control (QC) samples were prepared at final tenofovir concentrations of 10, 100, 400 and 750 ng mL^{-1} by spiking 1 mL of drug-free human plasma with appropriate



Tenofovir **3-methylcytidine**

Fig. 1. Chemical structure of tenofovir and 3-methylcytidine (internal standard).

amounts of diluted stock solution in water. After mixing, the QC samples were then stored in propylene tubes at −20 °C. The interference from concomitant therapeutic agents currently found in the plasma of HIV-infected patients was also investigated. The selectivity was tested with 30 plasma samples from HIV patients not treated with tenofovir but treated with several antiretroviral drugs. All samples were subjected to the sample preparation procedure described below.

2.4. Extraction procedure

Solid phase extraction procedure (SPE) was derived from Sentenac et al. [3] with slight modifications. To 1 mL of sample 20 μL of I.S. solution was added. The tubes were mixed and samples were placed on Varian©Bond Elut C-18 SPE cartridges (500 mg, 3 mL, Interchim) previously conditionned with 3 ml of methanol and 3 ml of 0.6% TFA (v/v in water). The following steps were performed: 1 mL of plasma sample was added, cartridge rinsed twice with 2 mL 0.6% TFA, eluted with 2.5 mL of methanol and evaporated at 40 °C under a gentle stream of nitrogen. The dried extracts were redisolved in 100 μL of water, mixed, then centrifuged (12,000 × *g*, 5 min) in order to eliminate insoluble residue. Finally, 2 μL was injected into the chromatographic system.

2.5. Validation procedures

2.5.1. Calibration curves and validation procedure

For both detection modes, calibration curves were constructed by plotting the peak height ratio (TNF/I.S.) as a function of plasma concentration of TNF. The linearity was tested using an analysis of variance. The significance of the slope and the validity of the linear calibration curves were tested using Fisher Snedecor'S *F*-test ($p < 0.05$). Data were fitted by weighted (1/concentration) linear least-squares regression analysis.

A total of 10 calibration curves were generated during the validation process using freshly prepared calibration samples. Six of these runs included QC samples. The lower and the upper limits of quantification were chosen as the concentration of the lowest and the highest calibration standard, respectively. It was verified that the percent deviation from the nominal concentration (mean accuracy) and the R.S.D. had to be within the range 20% and less than 20%, respectively, for the lower limit of quantification (LLOQ). Accuracy was calculated as the mean relative percent deviation of the back-calculated from the nominal concentration (difference between found concentration and nominal concentration, expressed in percent). Within-day and between-day precisions were expressed as the coefficient of variation or relative standard deviation (R.S.D.% = 100 × S.D./mean) of each calculated concentration. The limit of detection (LOD) was defined as the simple concentration resulting in a peak area of three times the standard deviation of the noise level.

The extraction recovery of TNF was determined at five concentrations (10, 50, 75, 200, 500 and 1000 ng mL^{-1}), and of the I.S. at 5000 ng mL^{-1}, by comparing the peak height of the plasma samples obtained from the SPE extraction to the peak height obtained by direct injection of the same amount of the drug in water.

2.5.2. Short-term and long-term stability of TNF

The stability of tenofovir in human plasma was studied for two concentrations (75 and 400 ng mL^{-1}) in all experiments and results were compared to day (D) 0 values. Stability at room temperature and at +4 °C was assessed in duplicate at D 1, D 2 and D 3. Long-term stability at −20 °C over 4 months and stability over three freeze–thaw cycles were also studied. At last, the stability of the drugs was verified in the autosampler (room temperature) over 24 and 48 h after the final reconstitution of extracted samples.

2.6. Analysis of patient samples

More than 170 samples from HIV-infected patients were analyzed. Patients were orally treated once daily with Viread® (tablet of 300 mg of tenofovir disoproxil fumarate) or with Truvada® (tablet of 300 mg of tenofovir disoproxil fumarate and 200 mg of emtricitabine). Blood samples were collected in glass tubes containing EDTA; then samples were centrifuged for 10 min at 4000 × *g* and plasmas were immediately frozen (−20 °C) until assay. For the analysis of patient samples, QC samples at 75 and 400 ng mL^{-1} were included in all batches.

3. Results and discussion

3.1. Chromatographic conditions

Figs. 2 and 3 display the chromatograms from blank, plasma and patient sample with UV and MS detection. In both cases, TNF and 3-methylcytidine (I.S.) are well resolved and adequately separated from other peaks. The retention time was 4.6 ± 0.1 and 2.7 ± 0.1 min for TNF and I.S., respectively. HPLC separation was carried out on an Atlantis™-dC-18 column since this type of column provides an adequate retention for polar compounds as TNF ($\log P = -1.7$) and is compatible with aqueous mobile phase. Furthermore, this column exhibits a long life-time with the analytical conditions described in the present paper.

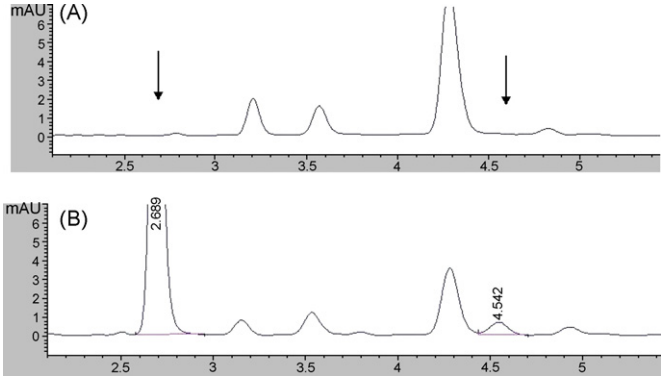


Fig. 2. Chromatograms with UV detection (260 nm) for blank drug-free human plasma (A) and human plasma extracts at 56 ng mL^{-1} (B). Arrows indicate the retention time of internal standard (I.S., 2.7 min) and tenofovir (TNF, 4.6 min).

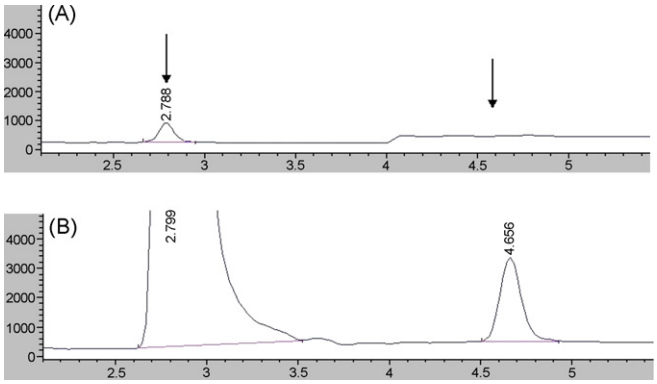


Fig. 3. Chromatograms with MS detection (ion *m/z* 288) for blank drug-free human plasma (A) and human plasma extracts at 51 ng mL^{-1} (B). Arrows indicate the retention time of internal standard (I.S., 2.7 min) and tenofovir (TNF, 4.6 min). (Data acquisition started 2.0 min after the injection of the sample.)

Potential interferences from endogenous substances or potential co-medication drugs were investigated by the analysis of 30 multitreated HIV-infected patients without TNF treatment. Drugs present in samples were protease inhibitors (nelfinavir, indinavir, saquinavir, ritonavir, lopinavir, amprenavir, atazanavir, tipranavir), reverse transcriptase inhibitors (zidovudine, lamivudine, abacavir, didanosine, stavudine, nevirapine, efavirenz, emtricitabine, delavirdine), or HIV-associated infections drugs (adefovir, acyclovir, gancyclovir, valacyclovir). From these samples no interferences were observed. In fact, among all anti-HIV agents, only reverse transcriptase inhibitors could interfere with TNF and I.S. chromatography because of their polar nature and that protease inhibitors and non nucleoside reverse transcriptase inhibitors need much higher solvent concentration in the mobile phase to be eluted (30% minimum). Among nucleoside reverse transcriptase inhibitors, only lamivudine and didanosine have shown retention time close to those of TNF and I.S., the others elute after 10 min (Fig. 4).

3.2. Performances of the assays

3.2.1. Calibration curves and limit of quantification

The dynamic range for calibration curves was set from TNF concentrations described in different clinical studies [3,4,6,9]. The calibration curves were plots of peak height ratios of TNF/I.S. versus the TNF concentration. The concentration of the standards ranged from 10 to 4000 ng mL^{-1} and from 5 to 1000 ng mL^{-1} for UV and MS detection, respectively. In both cases, the weighting factor (1/concentration) was found to provide the best fit and a linear least-square regression was used. The average determination coefficient was better than 0.999 for each detection mode. The LLOQ was determined as 5 and 10 ng mL^{-1} for MS and UV detection, respectively. This was the lowest concentration that could be measured with a precision within 20% and accuracy between 80 and 120%. These LOQs were in the same range as those previously described with UV or spectrofluorimetric detection [3,4]. The LOD was equal to 3 and 2 ng mL^{-1} for UV and MS detection, respectively.

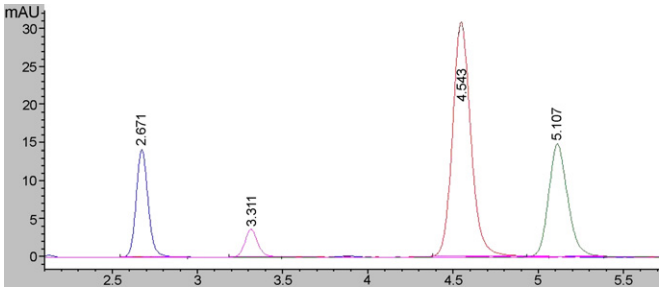


Fig. 4. Chromatogram using UV detection (260 nm). 3-Methylcytidine methosulfate (2.671 min) (internal standard), didanosine (3.311 min), tenofovir (4.543 min) and lamivudine (5.107 min).

Table 1
Inter-day validation of the linearity of the determination of tenofovir with LC-UV assay

TNF concentration (ng mL^{-1})			
Spiked	Found (mean ± S.D., 10 days)	Precision (%) (between-run)	Accuracy (%)
10	10.9 ± 0.8	6.9	109.4
50	52.1 ± 3.0	5.8	104.2
200	190 ± 9	4.8	95.2
500	518 ± 27	5.2	103.5
1000	992 ± 38	3.9	99.2

Data are from 10 calibration curves.

3.2.2. UV detection assay

The concentrations back-calculated from the equation of the regression analysis were less than 10% which presents an acceptable limit of variance (within 20% for both precision and accuracy) (Table 1). The within-run and between-run variability (precision), expressed as the percentage relative standard deviations, was always less than 15%, whereas the mean predicted concentration (accuracy) was within 10% of the nominal value (Table 2).

3.2.3. MS detection assay

The concentrations back-calculated from the equation of the regression analysis were less than 10% for all the concentrations tested (Table 3). The within-run and between-run variability (precision), expressed as the percentage relative standard deviations, was always less than 5%, whereas the mean predicted

Table 2
Assessment of accuracy and precision of tenofovir with LC-UV assay

Spiked (ng mL^{-1})	Found (ng mL^{-1})	Precision (%)	Accuracy (%)
Within-day (n = 6)			
10	9.8	5.9	98
100	102	4.0	102
400	374	3.5	94
750	774	3.3	103
Between-day (n = 6)			
10	11.2	8.6	112
100	101	2.7	101
400	374	5.1	94
750	785	2.7	105

Table 3
Inter-day validation of the linearity of determination of tenofovir with LC–MS (ion *m/z* 288) assay

TNF Concentration (ng mL^{-1})			
Spiked	Found (mean ± S.D., 10 days)	Precision (%) (between-run)	Accuracy (%)
5	5.3 ± 0.2	3.9	106.0
10	10.5 ± 0.8	8.0	104.6
50	51.8 ± 3.1	6.0	103.6
200	198 ± 10	5.0	99.3
500	493 ± 25	5.1	98.7
1000	995 ± 34	3.4	99.5

Data are from 10 calibration curves.

concentration (accuracy) was within 10% of the nominal value (Table 4). This quantification was performed by using the ion at *m/z* 288. The lower abundance of ion at *m/z* 176 allows only a quantification of TNF from 50 to 1000 ng mL^{-1}. In this range, the precision and the accuracy are similar than those obtained for the ion at *m/z* 288 (data not shown).

The lack of matrix effect was tested as follows. Ten different plasmas from patients untreated with TNF were extracted with SPE procedure. Then, each extract was spiked with TNF at a final concentration of 75 ng mL^{-1}. No significative difference was observed (mean plasma concentration 75.8 ± 5.1 ng mL^{-1}) with unextracted pure solution at 75 ng mL^{-1}.

3.2.4. Extraction

Extraction coefficient was calculated from different concentrations (10, 50, 75, 200, 500 and 1000 ng mL^{-1}). The extraction efficiency of TNF was concentration independent and averaged 90.6 ± 7.5%. The extraction coefficient of 3-MCT was 68.8% for a concentration of 5000 ng mL^{-1} in the plasma. The present extraction procedure derived from that described by Sentenac et al. [3] which obtained 63.7% of extraction efficiency. In our experiment we did not acidify the plasma prior to introduction into the cartridges and the SPE columns were eluted with 2 mL of methanol. In these conditions we achieved a high level of extraction recovery and good sample clean up which is demonstrated by the corresponding clear chromatogram layout. We also tested lower eluent volume (0.5 and 1 mL), but unsatisfactory extraction recovery was provided. Our results are in agreement with two studies recently reporting on SPE procedure for TNF extraction from plasma. Bezy et al. [9] indicated an extraction efficiency of 71% with an Oasis™ HLB cartridge only conditioned with methanol and water. Rezk et al. [6] described an extraction procedure with a Varian™ Bond-Elut® C18 cartridge conditioned with methanol and ammonium acetate (pH5) and obtained an extraction recovery equal to 98.6%.

Table 4
Assessment of accuracy and precision of tenofovir with LC–MS (ion *m/z* 288) assay

Spiked (ng mL^{-1})	Found (ng mL^{-1})	Precision (%)	Accuracy (%)
Within-day (n = 6)			
10	10.5	9.7	105
100	101	6.8	101
400	416	3.4	104
750	800	3.1	107
Between-day (n = 6)			
10	11.1	6.9	111
100	98	5.3	98
400	416	1.9	104
750	788	2.3	105

3.3. Stability of tenofovir

Tenofovir was stable under all conditions tested. All assayed concentrations were within 10% deviation from the reference concentration found at day 0 of each stability study. Spiked plasmas are stable at −20 °C for at least 4 months and after three freeze–thaw cycles. The samples were also stable for at least three days at +4 °C and at room temperature. The processed extracts were stable in the autosampler (at room temperature) for at least 48 h. These data are in agreement with several other studies indicating that no significant degradation occurred for TNF during storage and analytical process [4–6].

3.4. Analysis from patient samples and correlation between UV and MS detection

The usefulness and applicability of the present methods were then tested on 170 human plasma samples with UV and mass detectors coupled on-line. Within each batch, QC samples at 75 and 400 ng mL^{-1} were analyzed. The results (mean ± S.D.) were (n = 20) 76 ± 6 and 72 ± 6, 411 ± 26 and 415 ± 27 ng mL^{-1} for UV and MS, respectively. These data were compared statistically by Student's test, and no significative difference was found ($p < 0.05$).

The plasma concentration of all the patients was measured at steady state and at various times after the intake of Viread®. The tenofovir residual concentrations were quantified in the plasma of 100 HIV-infected patients. The mean residual concentration calculated was 73.7 ± 32.6 ng mL^{-1} (min–max: 15–155). The concentrations ranged from 115 to 621 ng mL^{-1} within the first 3 h following administration (n = 50). The concentrations at 12 h (±2 h) after administration ranged from 60 to 220 ng mL^{-1} (n = 50). These results are in agreement with those reported by other authors [10–12]. The lowest residual concentration was 15 ng mL^{-1}, showing that the methodology developed has suitable sensitivity to be applied in therapeutic drug monitoring. This allows quantification of low TNF plasma concentrations necessary for testing compliance of these long-term treated chronic patients. Fig. 5 illustrates the correlation between UV and MS detections. A good correlation was observed between the tenofovir concentration values obtained with UV and MS detection. The double detection allows for each clinical sample to verify the lack of interfering compound with TNF. This is performed using the UV spectrum (from 200 to 400 nm) obtained with DAD, and the measure of *m/z* ion ratio 288/176 (expected ratio to 5/1). If conditions for both parameters are met, results from UV and MS detections are within a range of 20%. However, in some cases (indicated by arrows on Fig. 5) results from

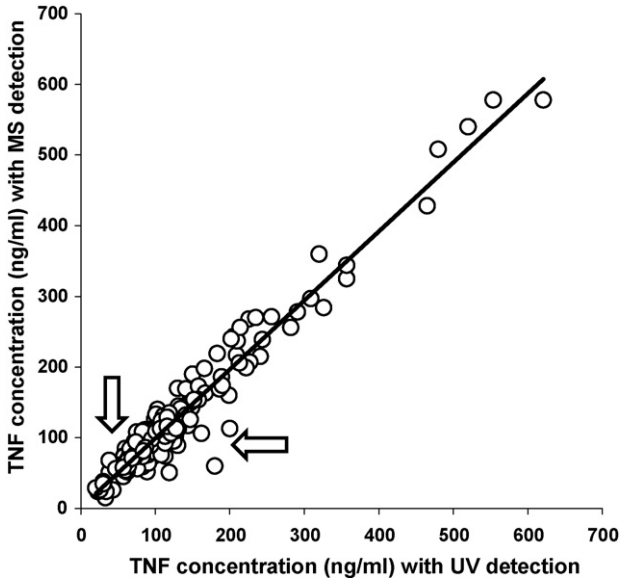


Fig. 5. Correlation from clinical samples analyzed with double detection UV and MS. Data were obtained from 170 patient samples. Equation corresponds to: $y = 0.98x + 1.77$, and correlation coefficient is equal to 0.97. Results indicated by arrows are discussed in the text.

UV and MS detection were not in accordance, which means a difference over 20% between data. The thorough study of these runs enabled us to distinguish two situations. In the first instance, the UV spectrum from the chromatograpic peak of TNF may not be superimposable on UV spectrum of pure TNF solution indicating the presence of interference from plasma sample. On the contrary, the *m*/*z* ion ratio 288/176 was kept at a ratio of 5/1 as expected. In this case the MS data (ion *m*/*z* 288) were used for the quantification of TNF. In the second instance, the UV spectrum from the chromatograpic peak of TNF was not modified but the ion ratio 288/176 was different at ±20% from the expected value of 5/1. In this case, we observed that the quantification performed at 260 nm was in agreement with the result obtained using the ion at *m*/*z* 176 but not with the ion at *m*/*z* 288. These cases indicate that it is most relevant to carry out analysis with both detections in order to minimize the risk of misquantification of TNF in clinical samples. Finally, this approach ensures a higher reliability of the results which are transmitted to the medical team.

4. Conclusion

Determination of TNF plasmatic concentration allows to verify the dose adaptation for patients who exhibit a low renal function, might be useful to evaluate a drug–drug interaction and to appreciate observance for this long-term treatment of HIV patients. In this purpose, an LC-UV–MS assay for TNF in human plasma has been developed and validated with respect to linearity, precision and accuracy, and analysis of real samples was demonstrated. The assay results obtained by double detection are in good agreement. The present assay enabled us to quantify TNF in all our clinical samples which would not have been the case had we only used one detection mode (UV or single MS). These methods can be used for the routine determination of TNF in multitreated HIV-infected patients with or without renal dysfunction and is applicable to test treatment compliance.

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