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Simultaneous determination of zidovudine and nevirapine in human plasma by RP-LC

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Abstract

A simple method for the simultaneous determination of zidovudine and nevirapine in human plasma by reversed-phase liquid chromatography with UV detection at 265 nm was developed. A solid–liquid extraction procedure with internal standard was applied to the samples prior to analysis. The system requires a Zorbax SB-C18 column, 250 × 4.6 mm I.D. and a mobile phase composed of potassium dihydrogen phosphate (10 mM; pH 6.5)-acetonitrile (83:17, v/v). Peak-areas are linear; correlation coefficients are better than 0.999; both inter- and intra-day accuracy and precision are lower than 15%. Extraction recoveries are higher than 90% for both zidovudine and nevirapine. The method proposed was employed to determine the levels of the two retroviral drugs in plasma from HIV infected human subjects.

Keywords: Zidovudine; Nevirapine; Reversed-phase chromatography; UV detection

1. Introduction

The introduction of highly effective combination regimens of antiretroviral drugs has led in recent years to substantial improvements in morbidity and mortality [1,2].

At present all the compounds used for the treatment of HIV infections belong to one of the following classes: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs).

During the past few years, increasing interest has been shown in the possible role of monitoring antiretroviral drugs pharmacokinetics in the field of HIV infection [3].

NRTI Zidovudine (AZT), the first antiviral drug approved for the treatment of HIV infection, is anabolized intracellularly to its 5′-triphosphate which acts as a potent inhibitor of HIV reverse transcriptase and a terminator of growing proviral DNA [4].

Progress in the field of antiretroviral therapy for human immunodeficiency virus type 1 infec-

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tion has led to the end of the AZT monotherapy era [5].

Nevirapine (NVP), a NNRTI, is a potent and selective non competitive inhibitor of the reverse transcriptase. In completed clinical trials, NVP has demonstrated antiretroviral activity both in monotherapy and in combination with nucleoside analogues including AZT [6] or the combination AZT-didanosine [7]. Consequently, simultaneous monitoring of both drugs could allow an adjustment of doses to work out an optimal therapy for HIV infected patients.

Numerous individual methods employing either high performance liquid chromatography with UV detection [8–16] or immunoassay [17–20] or mass spectroscopy [21] have been reported for AZT and NVP.

Only two methods based on UV detection have been published for the simultaneous determination of AZT and NVP [10,12]. One of these [12] has been developed for the simultaneous determination of numerous antiretroviral agents and consequently is very sophisticated, requiring three pumps and two switch valves.

In the other method described by Moyer et al. [10] serum samples are prepared prior to LC quantitation by liquid–liquid extraction, but the recovery of NVP is low (67.4%) and some possible interferences from other co-administered drugs are reported.

Although many clinical research laboratories have the facility of LC–MS analyses, UV detection is still used for relatively simple bioanalytical assays. Consequently, the aim of this study was to develop a relatively simple method, based on UV detection, for the separation and simultaneous quantitation of AZT and NVP in plasma from HIV positive patients.

The application of solid-phase extraction (SPE) of analytes from the biological matrix allows either higher recoveries or the elimination of some possible interferences from other co-administrated drugs.

Moreover, being feasible with standard laboratory equipment, this method could be routinely applied in clinical research allowing to save time and decrease costs compared with other existing methods.

2. Experimental

2.1. Chemicals and reagents

Zidovudine (AZT) and 3-isobutyl-1-methylxanthine (internal standard) were purchased from Sigma (St. Louis, MO); nevirapine (NVP) was kindly provided by Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). Dimethylsulfoxide was obtained from Baker (J.T. Baker Chemicals, Deventer, Holland). Acetonitrile (Carlo Erba reagenti, Rodano, MI, Italy) and methanol (Lab-Scan Analytical Science, Dublin, Ireland) were HPLC-grade. Water was bidistilled and all the other chemicals were of analytical reagent grade.

2.2. Chromatographic system and conditions

The HPLC system consisted of a series 200 LC pump (Perkin Elmer, Norwalk, CT), a pulse dumper LP-21 (SSI, State College, PA), a Rheodyne model 7125 injection valve with a 20 μl sample loop (Rheodyne, Berkely, CA). A Perkin-Elmer model 235 photodiode array detector set at 265 nm was used. For data collection and calculation a Nelson model 1020 workstation (Perkin-Elmer) was utilized.

The chromatographic analysis was performed at ambient temperature onto a 5 μm Zorbax SB-C18 column, 250×4.6 mm I.D. (Hewlett Packard, USA) and a mobile phase composed of potassium dihydrogen phosphate (pH 6.5; 10 mM)–acetonitrile (83:17, v/v). The mobile phase was filtered through 0.45 μm filters (Alltech, Sedriano, Milan, Italy) before use. The flow-rate was maintained at 1 ml/min.

2.3. Standard solutions

Standard stock solutions of AZT and NVP containing 1 mg/ml were prepared in methanol and dimethylsulfoxide, respectively. Standard working solutions of 0.05, 0.1, 0.5, 1, 2 and 5 μg/ml AZT and 0.15, 0.5, 1, 2, 5 and 10 μg/ml NVP were prepared by adding the appropriate amount of standard stock solutions to human blank plasma. Internal standard stock solution was prepared by accurately weighing 10 mg of the

compound and dissolving it into 10 ml of methanol. The solution was diluted with mobile phase to yield a concentration of 2 μg/ml (internal standard working solution).

Stock and working solutions were stored at − 20 °C.

2.4. Patient samples

Plasma samples of ten different HIV-1 infected patients treated with AZT or NVP were analyzed. Antiretroviral information and drug therapy for these patients are listed in Table 1. Samples were heated at 60 °C for 1 h to inactivate HIV and then stored at − 20 °C up to the time of analysis.

2.5. Standard and sample extracts

Both standard working solutions and patient samples (200 μl) were combined with 200 μl of internal standard working solution. The solutions obtained were then transferred to Waters Oasis HLB 1 cc (30 mg) extraction columns, which had been preconditioned with 2 ml of methanol followed by 2 ml of distilled water. The cartridges were washed with 1 ml of potassium phosphate buffer (pH 6.8; 10 mM) and 1 ml of a mixture of potassium phosphate buffer (pH 6.8; 10 mM)–methanol (95:5, v/v). The analytes were eluted with 2 ml of methanol. The extracts obtained were evaporated to dryness under helium at 30 °C and reconstituted with 100 μl of mobile phase (standard and sample extracts). An appropriate fixed amount (20 μl) was directly injected into HPLC system.

3. Results and discussion

The LC method proposed provides a simple procedure for the simultaneous determination of AZT and NVP in human plasma. Fig. 1A shows the chromatogram of a pure standard mixture.

The UV spectrum of NVP shows two maxima (235 and 290 nm) and the AZT has a maximum at about 270 nm. A detection wavelength of 265 nm was chosen to detect both the substances with a good sensitivity.

AZT is a hydrophilic compound poorly, if not at all, extractable in organic solvents, while NVP

Table 1
Antiretroviral regimens and drugs plasma concentration of HIV-1 infected patients

Patients	Viremia	CD4	Antiretroviral	Plasma concentration (μg/ml ± SD)	
				AZT	NVP
1(1)	<80	746	Zidovudine 300 mg^{bid} Nevirapine 200 mg^{bid}	0.08 ± 0.01	7.47 ± 0.55
2(1)	<80	569	Zidovudine 300 mg^{bid} Nevirapine 200 mg^{tid}	0.43 ± 0.10	2.81 ± 0.23
3(2)	21000	732	Nevirapine 200 mg^{bid}	–	6.24 ± 0.54
4(3)	<80	334	Nevirapine 200 mg^{bid}	–	6.90 ± 1.04
5(1)	<80	429	Zidovudine 300 mg^{bid} Nevirapine 200 mg^{bid}	0.11 ± 0.01	4.87 ± 0.15
6(1)	<80	392	Zidovudine 300 mg^{bid} Nevirapine 200 mg^{bid}	0.82 ± 0.02	6.54 ± 0.32
7(1)	<80	416	Zidovudine 300 mg^{bid} Nevirapine 200 mg^{bid}	0.54 ± 0.01	6.71 ± 0.01
8(1)	<80	764	Zidovudine 300 mg^{bid} Nevirapine 200 mg^{tid}	0.05 ± 0.02	3.23 ± 0.21
9(4)	620	160	Nevirapine 200 mg^{bid}	–	5.33 ± 0.27
10(4)	<80	389	Nevirapine 200 mg^{bid}	–	4.06 ± 0.17

bid = Twice a day; tid = three times a day. In combination with: (1)Lamivudine; (2)Lamivudine, Stavudine and Nelfinavir; (3)Lamivudine and Stavudine; (4)Stavudine and Didanosine.

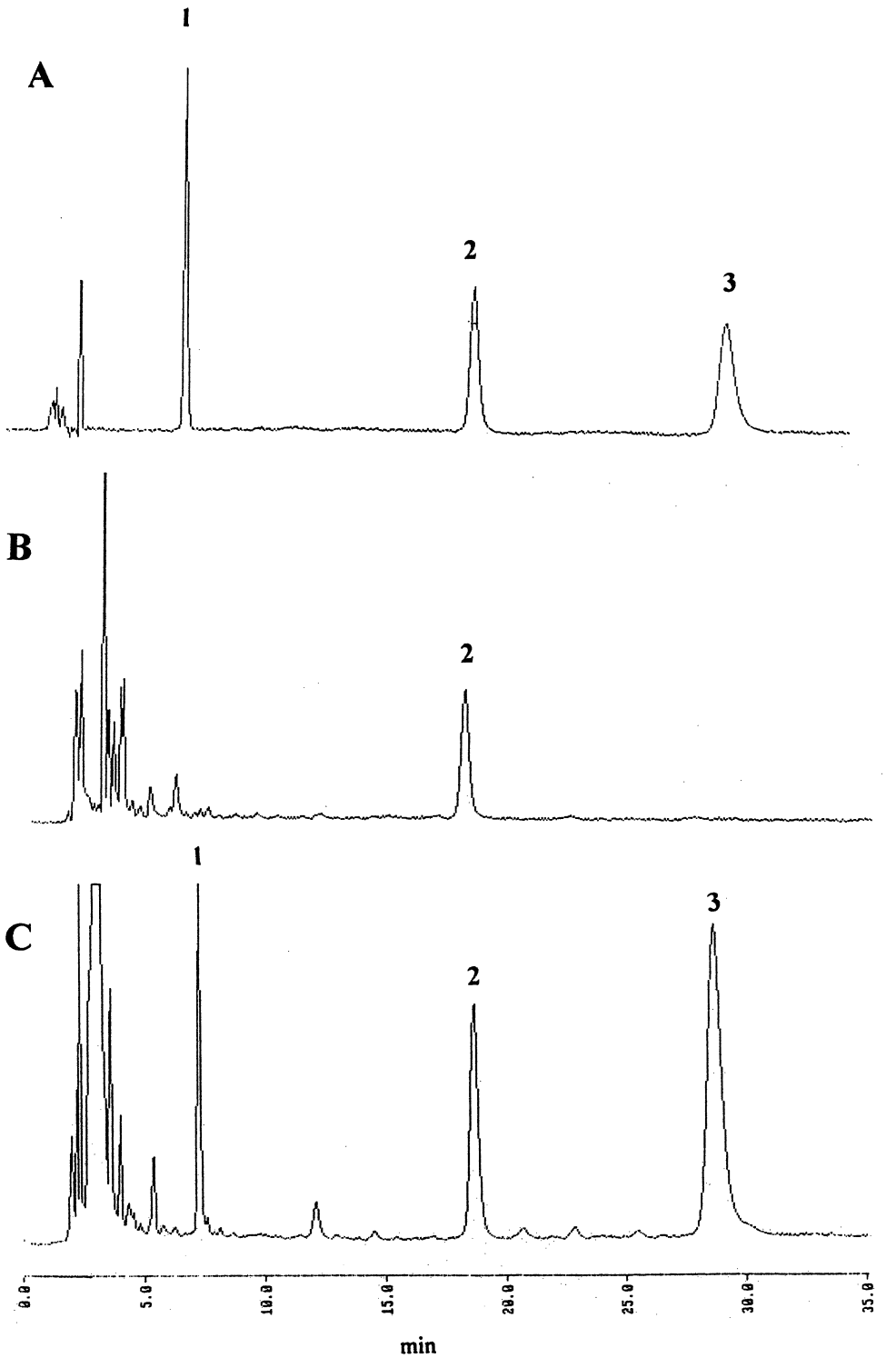

Fig. 1. Chromatograms showing: (A) responses of a standard extract; peak 1 AZT; peak 2 internal standard and peak 3 NVP; (B) blank human plasma spiked with internal standard; (C) sample extract. Column: Zorbax SB-C18 column, 4.6 × 250 mm; mobile phase: potassium dihydrogen phosphate (10 mM; pH 6.5)–acetonitrile (83:17, v/v); isocratic flow: 1 ml/min.

is a lipophilic molecule, easily extractable in organic solvents like methyl *tert*-buthyl ether [12], consequently liquid–liquid extraction was rejected. In preliminary studies the removal of interfering plasma components was attempted by SPE washing the column with eluents at different pH values. Washing with acetic acid 5% or NH_4OH 5% in methanol–water (65:35, v/v) resulted in low recoveries and/or a lot of interferences in chromatograms. The largest recoveries and the cleanest extraction procedure were achieved washing the column with 1 ml of potassium phosphate buffer (pH 6.8; 10 mM) and 1 ml of a mixture of potassium phosphate buffer (pH 6.8; 10 mM)–methanol (95:5, v/v).

Chromatographic conditions were based on the method described by Moyer et al. [10]. The use of a longer column allowed to get a better separation from possible interferences.

The quantitative determination of AZT and NVP in human plasma was performed on ten patients. Results are given in Table 1. A NVP plasma concentration between 2.81 and 7.53 μg/ml and an AZT plasma concentration between 0.05 and 0.85 μg/ml were found. Plasma concentration of NVP and AZT were in agreement with the concentration range reported in literature [10]. The chromatogram of a patient sample is shown in Fig. 1C.

The method was validated with regard to specificity, linearity, limits of detection and quantitation, recovery, precision, accuracy and ruggedness.

3.1. Specificity

Blank samples showed no interfering endogenous substances eluting at the retention time of AZT and NVP (Fig. 1B).

Other NRTIs (didanosine, lamivudine and stavudine) are extracted by this procedure. Nevertheless, under the chromatographic conditions described, these compounds elute at retention times different from those of AZT and NVP and therefore do not interfere with the analysis.

Potentially co-administered antiretroviral drugs tested either had different retention times (didanosine 3.08 min, lamivudine 3.13 min and stavudine 3.26 min) or were not detected under the described analytical condition (indinavir, saquinavir, ritonavir).

In the paper by Moyer et al. [10] numerous drugs have been tested as possible interferences. Among these, phenacetin and penicillin V for NVP and hydrochlorothiazide and sulfapyridine for AZT would seem to cause an apparent increase in plasmatic concentrations of the two drugs. It could be possible to draw wrong conclusions about their plasmatic concentration if confounding factors are not taken into consideration. Consequently, also these possible interfering drugs were chromatographed by the method here described. They were or eluted at different retention times or extracted with neglectable recoveries.

Peak purity evaluation was used to confirm the lack of interferences by hidden compounds in patient samples. Spectra were acquired across the AZT and NVP peaks, normalised and mathematically compared. The purity index calculated by the software clearly showed the purity of the peaks. Peak purity was checked also by using the Ratio Signal Plot technique. Two wavelengths for each compound were chosen (the maximum at 270 nm and the minimum at 235 nm for AZT; the two maxima at 235 and 290 nm for NVP). The absorbance ratio between the two wavelengths resulted constant over the elution profile for both the compounds, giving further confirmation of the peaks purity.

3.2. Linearity

The linearity of the method was determined by injection of AZT and NVP standard extracts at six concentration levels in the range 0.05–5 and 0.150–10 μg/ml, respectively. Each concentration was tested in triplicate. Least-square regression calibration curves were constructed by plotting peak-area ratios of AZT and NVP to internal standard as a function of the drug concentration in the standard working solution. The following calibration equations were obtained: $y = 1.4719x - 0.0108$, $r^2 = 0.999$ for AZT and $y = 0.7174x - 0.0293$, $r^2 = 0.999$ for NVP. The standard error of slopes was 0.011 for AZT and

Table 2
Absolute recovery of AZT and NVP after extraction from human plasma

Concentration AZT (μg/ml)	Recovery[a] (% ± SD)	Concentration NVP (μg/ml)	Recovery[a] (% ± SD)
0.05	96.4 ± 1.3	0.150	89.3 ± 0.8
0.1	91.4 ± 1.4	0.5	97.3 ± 1.5
0.5	100.1 ± 0.3	1	92.3 ± 2.3
1	96.7 ± 1.3	2	93.0 ± 0.6
2	98.3 ± 0.4	5	94.5 ± 2.3
5	95.8 ± 0.8	10	91.2 ± 0.4

[a] Results are the mean of three experiments.

0.008 for NVP; the standard error of intercepts was 0.005 for both AZT and NVP. Linear regressions were used to calculate the drug concentration in the samples.

3.3. Limits of detection and quantitation

The detection limit (DL) and quantitation limit (QL) were calculated as follows:

$$\text{DL or QL} = K(\sigma/S)$$

where $K = 3.3$ for DL and 10 for QL, σ is the SD of response and S is the slope of calibration curve [22].

For AZT the DL was 0.025 μg/ml and QL was 0.05 μg/ml; for NVP the DL was 0.05 μg/ml and QL was 0.150 μg/ml. QL levels were included in the calibration curves as the lowest concentration level.

3.4. Recovery

The absolute recovery was calculated by comparing the area under the peaks obtained from standard working solutions with the peak-areas from standard extracts (Table 2). The average recovery of the internal standard was 88.5 ± 5.3%.

3.5. Precision and accuracy

Intra- and inter-day precision and accuracy were studied at three different concentrations. Precision was calculated as:

$$\%\text{CV} = (\text{SD}/\text{mean})100$$

Accuracy was calculated using the expression:

$$\%\text{BIAS} = [(\text{measured value} - \text{true value})/\text{true value}]100$$

The results are shown in Table 3. For all compounds both %CV and %BIAS were $< 15\%$.

3.6. Ruggedness

Slight variations in retention times were observed using mobile phases prepared on different days. The column-to-column reproducibility was evaluated injecting the samples on two columns from different manufacturers and containing the same brand of packing material. The elution order and the resolution of compounds were not affected and only slight variations in retention times were observed.

3.7. Stability

Stability of AZT and NVP in solution and in biological matrix was determined at different temperatures. No significant changes in the concentration of AZT and NVP at 4, 25 and 60 °C were observed during a week time. The test performed in triplicate for one low and one high concentration of each compound ensures that no degradation occurred during the step, before loading the plasma into an extraction column. AZT and NVP showed to be stable also in the eluate from the extraction column. No degradation were noticed over a 24 h interval.

Table 3
Precision and accuracy

Nominal concentration (μg/ml)	AZT			Nominal concentration (μg/ml)	NVP		
	Calculated concentration (μg/ml ± SD)	Precision (CV%)	Accuracy (Bias%)		Calculated concentration (μg/ml ± SD)	Precision (CV%)	Accuracy (Bias%)
Intra-day[a]							
0.1	0.087 ± 0.002	2.42	9.1	0.5	0.503 ± ± 0.0052	1.03	0.5
0.5	0.517 ± 0.035	6.85	3.38	1	1.08 ± 0.102	9.47	8.1
1	0.999 ± 0.052	5.15	0.01	2	1.84 ± 0.265	12.8	7.7
Inter-day[b]							
0.1	0.095 ± 0.0034	3.6	5.1	0.5	0.487 ± 0.028	5.8	2.6
0.5	0.535 ± 0.034	6.3	6.9	1	0.923 ± 0.185	2.0	7.7
1	0.960 ± 0.065	6.8	3.0	2	1.86 ± 0.178	8.8	7.0

[a] Results are the mean of five experiments.
[b] Results are the mean of two experiments a day over a period of 5 days.

4. Conclusions

Dosing of antiretroviral agents is quite complex, being therapies generally based on the simultaneous administration of three or more drugs in replicate doses. Consequently, a relatively simple LC–UV assay to be routinely used with standard laboratory equipment is advisable as an alternative to more time-consuming and expensive techniques. The method here described showed to be specific, precise and accurate for the simultaneous determination of AZT and NVP in human plasma with clinically relevant sensitivity. Consequently, it can be used both for monitoring plasma NVP and AZT concentrations and for pharmacokinetic studies in HIV-1 infected individuals. Thanks to the absence of interference with other antiretroviral drugs, the method is usable on patients treated with combined antiretroviral therapy. Additional studies involving a larger number of antiretroviral agents and patients are under way.

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References

[1] J. Darbyshire, Trop. Med. Int. Health 5 (2000) A26–A31.
[2] W.G. Powderly, J. Neurovirol. 6 (2000) S8–S13.
[3] R.M.W. Hoetelmans, Curr. Opin. Infect. Dis. 12 (1999) 593–595.
[4] X. Tan, F.D. Boudinot, J. Chromatogr. B 740 (2000) 281–287.
[5] S.M. Hammer, K.E. Squires, M.D. Hughes, J.M. Grimes, L.M. Demeter, J.S. Currier, J.J. Eron, J.E. Feinberg, H.H. Balfour, L.R. Deyton, J.A. Chodakewitz, M.A. Fischl, N. Engl. J. Med. 337 (1997) 725–733.
[6] R.A. Koup, F. Brewster, P. Grob, J.L. Sullivan, AIDS 35 (1993) 305–308.
[7] Q. Chan Li, T. Tougas, K. Cohen, R. Lee, P. Meagan, M. Corson, T. Muchnick, J. Chromatogr. Sci. 38 (2000) 246–254.
[8] J.W. Pav, L.S. Rowland, D.J. Korpalski, J. Pharm. Biomed. Anal. 20 (1999) 91–98.
[9] R.P.G. Heeswijk, R.M.W. Hoetelmans, P.L. Meenhorst, J.W. Mulder, J.H. Beijnsen, J. Chromatogr. B 713 (1998) 395–399.
[10] T.P. Moyer, Z. Temesgen, R. Enger, L. Estes, J. Charlson, L. Oliver, A. Wright, Clin. Chem. 45 (1999) 1465–1476.
[11] X. Tan, F.D. Boudinot, C.K. Chu, D. Egron, C. Perigaud, G. Gosselin, J.L. Imbach, Antivir. Chem. Chemother. 11 (2000) 203–211.
[12] G. Aymard, M. Legrand, N. Trichereau, B. Diquet, J. Chromatogr. B 744 (2000) 227–240.
[13] I. Schrive, J.C. Plasse, J. Chromatogr. B 657 (1994) 233–237.
[14] F. Kamali, M.D. Rawlins, J. Chromatogr. 530 (1990) 474–479.
[15] C. Lacroix, T.P. Hoang, F. Wojciechowski, H. Duwoos, J. Nouveau, B. Diquet, J. Chromatogr. 525 (1990) 240–245.
[16] S.S. Good, D.J. Reynolds, P. De Miranda, J. Chromatogr. 431 (1988) 123–133.
[17] S.A. Wring, R.M. O'Neill, J.L. Williams, W.N. Jenner, M.J. Daniel, M.R.D. Gray, J.J. Newman, G.N. Wells, D.R. Sutherland, J. Pharm. Biomed. Anal. 12 (1994) 1573–1583.
[18] R.P. Quinn, B. Orban, S. Tadepalli, J. Immunoassay 10 (1989) 177–189.
[19] S.M. Tadepalli, L. Puckett, S. Jeal, L. Kanics, R.P. Quinn, Clin. Chem. 36 (1990) 897–900.
[20] S.M. Tadepalli, R.P. Quinn, J. Acquir. Immune Defic. Syndr. 3 (1990) 19–27.
[21] K.B. Kenney, S.A. Wring, R.M. Carr, G.N. Wells, J.A. Dunn, J. Pharm. Biomed. Anal. 22 (2000) 967–983.
[22] European Pharmacopoeia Commission: Technical Guide for the Elaboration of Monographs, third ed., February 1999.