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Simultaneous determination of zidovudine and lamivudine in human serum using HPLC with tandem mass spectrometry

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Abstract

A method employing high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS) has been developed and validated for the simultaneous determination of clinically relevant levels of zidovudine (AZT) and lamivudine (3TC) in human serum. The method incorporates a fully automated ultrafiltration sample preparation step that replaces the solid-phase extraction step typically used for HPLC with UV detection. The calibration range of the dual-analyte LC-MS/MS method is 2.5–2500 and 2.5–5000 ng ml^{-1} for AZT and 3TC, respectively, using 0.25 ml of human serum. The lower limit of quantification was 2.5 ng ml^{-1} for each analyte, with a chromatographic run time of approximately 6 min. Overall accuracy, expressed as bias, and inter- and intra-assay precision are $< \pm 7$ and $< 10\%$ for AZT, and $< \pm 5$ and $< 12.1\%$ for 3TC over the full concentration ranges. A cross-validation study demonstrated that the LC-MS/MS method afforded equivalent results to established methods consisting of a radioimmuno-assay for AZT and an HPLC-UV method for 3TC. Moreover, the LC-MS/MS was more sensitive, allowed markedly higher-throughput, and required smaller sample volumes (for 3TC only). The validated method has been used to support post-marketing clinical studies for Combivir™ — a combination tablet containing AZT and 3TC.

Keywords: Zidovudine; Lamivudine; HPLC; LC-MS/MS; HIV; Determination; Human plasma

1. Introduction

Zidovudine (AZT, 3′-azido-3′-deoxythymidine) and lamivudine (3TC, 2′-deoxy-3′-thiacytidine) are synthetic nucleoside analogs with activity against

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the human immunodeficiency virus (HIV). Both medications are phosphorylated intracellularly to active triphosphate metabolites that are potent inhibitors of viral reverse transcriptase and weaker inhibitors of DNA polymerase. AZT and 3TC are front line therapies for the treatment of HIV infection and recently have become available in a combination tablet (Combivir™). Each Combivir™ tablet contains 300 mg of AZT and 150 mg of 3TC.

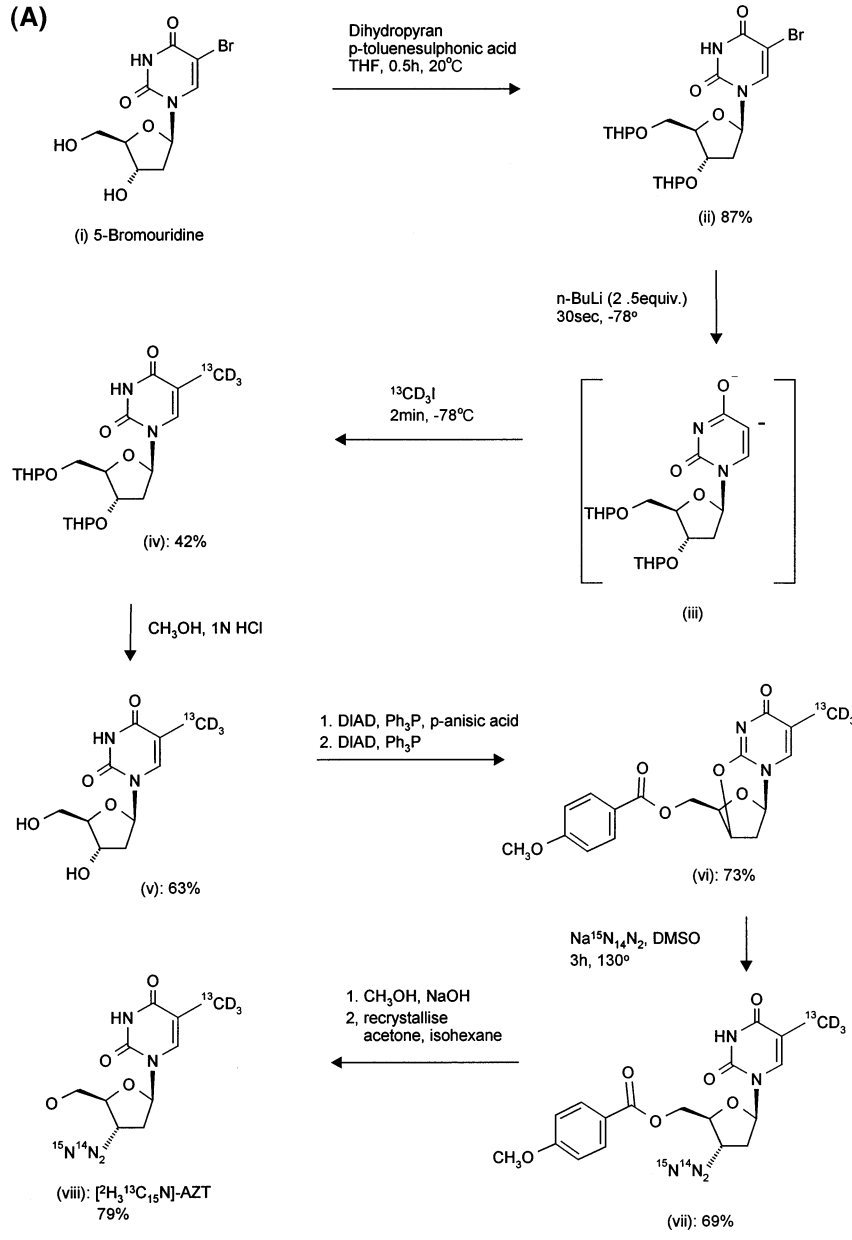

Fig. 1. Synthetic route for the synthesis of the (A) zidovudine (AZT) and (B) lamivudine (3TC) stable isotope labeled internal standards.

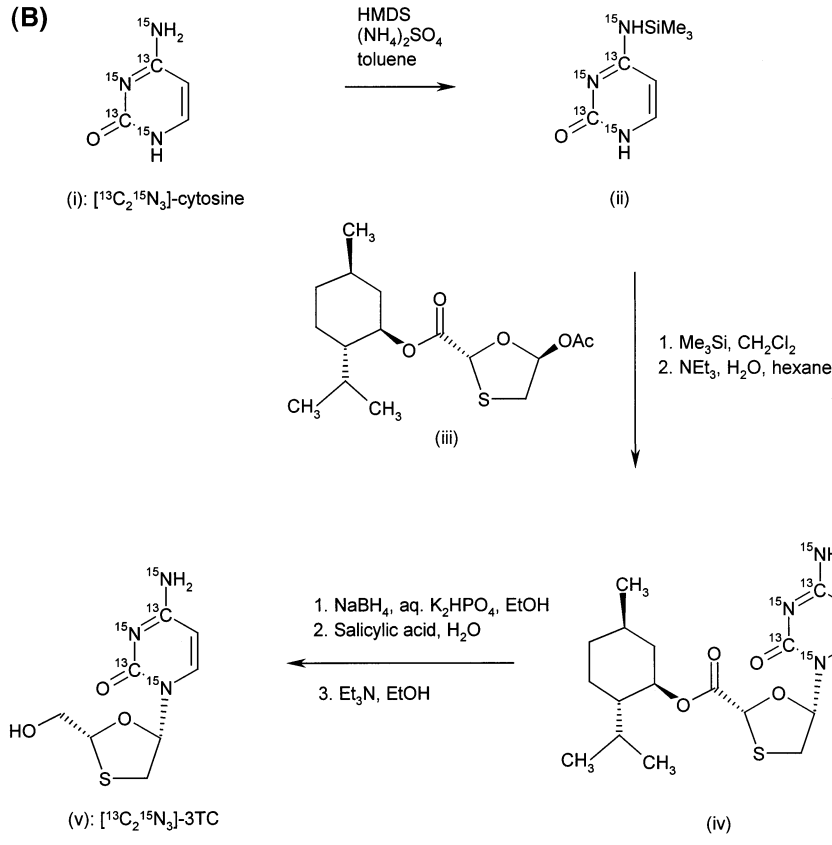

Fig. 1. (*Continued*)

Numerous analytical methods, mostly employing either high performance liquid chromatography (HPLC) with UV detection [1–3] or immunoassay [4–7], have been reported for AZT or 3TC; however, none have been reported using HPLC with tandem mass spectrometry (MS) for the simultaneous determination of these important anti-virals in human serum. Simultaneous determination of both drugs is desirable as this would allow more efficient generation of clinical data and could be performed at more modest cost than separate assays.

This paper describes the development and validation of an LC-MS/MS method for the simultaneous determination of AZT and 3TC in human serum. The validation includes a cross-validation to the established RIA and HPLC-UV methods that were used to support the clinical development program for AZT and 3TC.

2. Experimental

2.1. Reagents and chemicals

All chemicals were of analytical-reagent grade. Ammonium acetate was purchased from Mallinckrodt, and acetonitrile and HPLC grade water were obtained from EM Science. Zidovudine and 3TC were supplied by Glaxo Wellcome Research and Development, UK. Stock analytical standard solutions of both drugs were prepared in water and stored in the dark at approximately +4°C.

2.1.1. Synthesis of a stable isotope labeled AZT internal standard

5-Brom-2′-deoxyouridine (Fig. 1A(i)) was protected by conversion into the bis-tetrahydropyranyl ether (Fig. 1A(ii)). Rapid reaction with

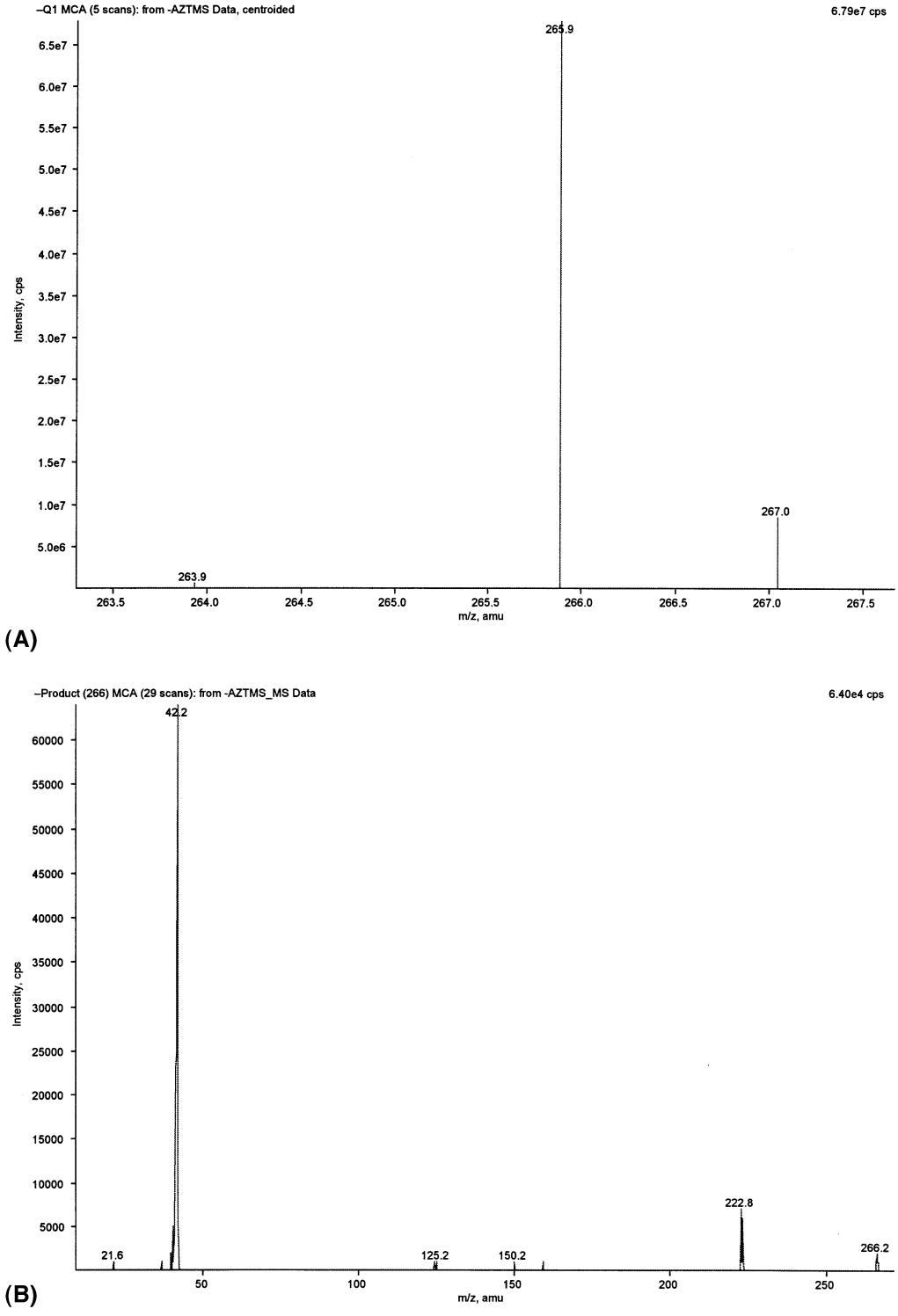

Fig. 2. Negative ion electrospray ionization (ESI)-mass spectra for zidovudine (AZT): (A) mass spectrometry (MS) (Q1) spectrum, (B) product ion scan and C) product ion scan for the AZT internal standard.

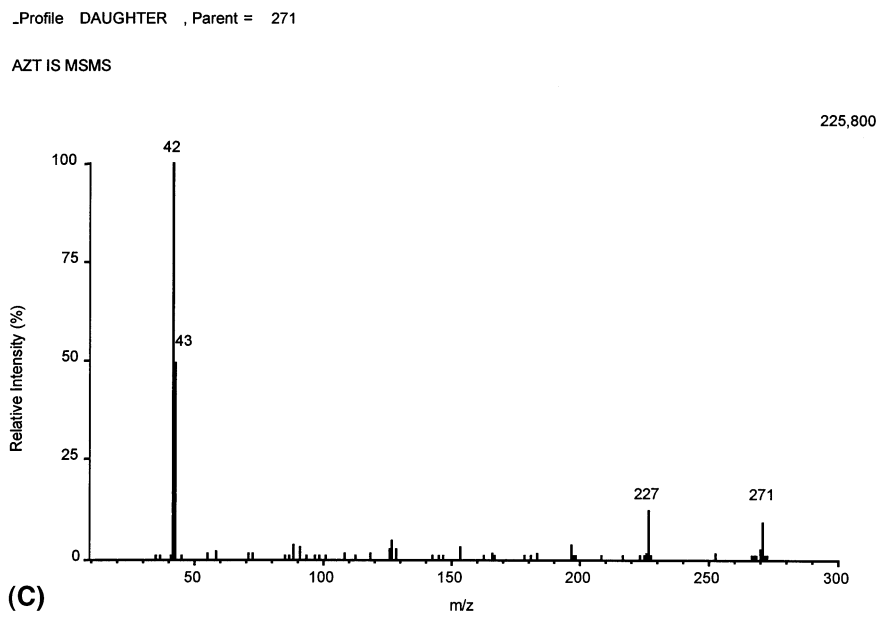

Fig. 2. (*Continued*)

n-butyl lithium at −78°C gave dianion (Fig. 1A(iii)) which was immediately quenched with [$^{13}C^{2}H_3$]iodomethane to give [$^{13}C^{2}H_3$]thymidine bis-tetrahydropyranyl ether (Fig. 1A(iv)). The thymidine bis-ether was purified chromatographically on silica gel before deprotection with methanolic hydrogen chloride to give [$^{13}C^{2}H_3$]thymidine (Fig. 1A(v)) as a crystalline solid. This was converted into labeled AZT using the Czernecki procedure [8]. A double Mitsunobu reaction [8] gave anhydro compound (Fig. 1A(vi)) which crystallised from the reaction mixture in high purity without the need for chromatographic purification. Ring opening with sodium [$^{14}N_2^{15}N$]azide in DMSO at 130°C, afforded AZT *p*-anisate ester (Fig. 1A(vii)) in which one atom of nitrogen-15 was uniformly distributed between the N1 and N3 nitrogen atoms of the azido moiety. The ester was purified chromatographically on silica. Finally, alkaline hydrolysis of the ester afforded the desired [$^{13}C^{2}H_3^{15}N$]AZT (Fig. 1A(vii)) which was purified chromatographically on silica then recrystallised from acetone–isohexane.

2.1.2. Synthesis of stable isotope labeled 3TC internal standard

Commercially available [$^{13}C_2^{15}N_3$]cytosine (Isotec, Miamisburg, OH, Fig. 1B(i)) was silylated with hexamethyldisilazane to give protected cytosine (Fig. 1B(ii)). Coupling with menthyl ester (Fig. 1B(iii)) followed by desilylation afforded ester (Fig. 1B(iv)). Reduction of the ester with sodium borohydride gave [$^{13}C_2^{15}N_3$]3TC which was isolated as salicylate monohydrate (Fig. 1B(iv)). Finally, the salicylate salt was converted into free base [$^{13}C_2^{15}N_3$]3TC (Fig. 1B(v)) with triethylamine in ethanol.

2.2. Instrumentation

High-performance liquid chromatography was performed on a Waters 600 series chromatograph equipped with a column-switching valve that was employed to direct flow either into the mass spectrometer or to waste. Flow was directed to waste during the first 2 min of each run to reduce contamination of the mass spectrometer by poorly

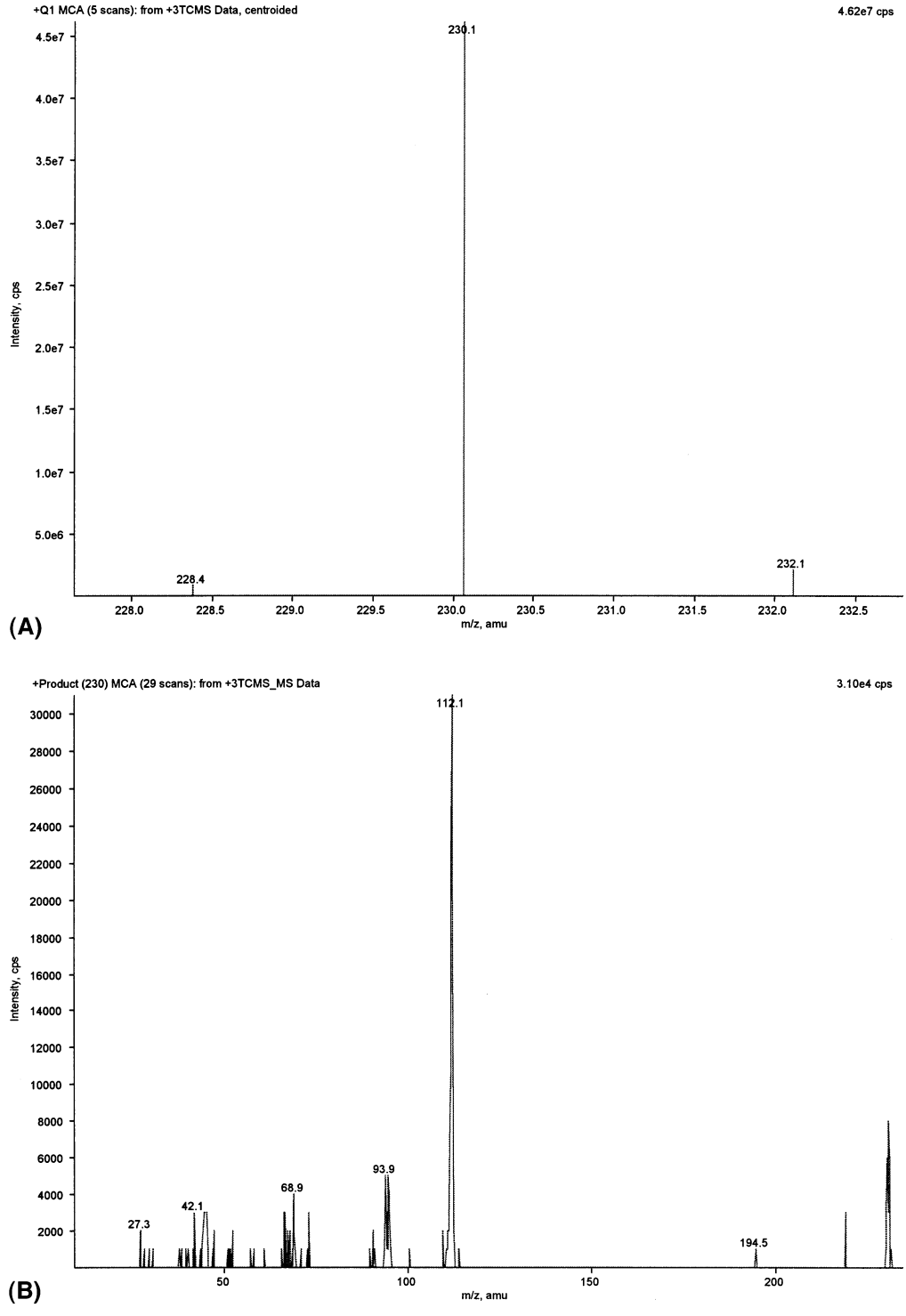

Fig. 3. Positive ion electrospray ionization (ESI)-mass spectra for lamivudine (3TC): (A) mass spectrometry (MS) (Q1) spectrum, (B) product ion scan.

Table 1
Summary statistical data for zidovudine (AZT) validation controls

Validation Run no.	Nominal concentration (ng ml^{-1})	Mean ± S.D. (ng ml^{-1})	Bias (%)	Relative S.D. (%)	*n*
Run 1	2.5	2.5 ± 0.09	0.0	3.6	6
	8	8.3 ± 0.32	3.6	3.9	6
	150	153 ± 3.40	2.1	2.2	6
	1500	1428 ± 13.70	−5.0	1.0	6
	2500	2272 ± 62.50	−10.0	2.7	6
Run 2	2.5	2.5 ± 0.15	1.3	5.9	6
	8	9.2 ± 1.60	15.0	17.6	6
	150	165 ± 2.30	10.0	1.4	6
	1500	1541 ± 19.00	2.7	1.2	6
	2500	2471 ± 60.00	−1.2	2.4	6
Run 3	2.5	2.6 ± 0.21	3.2	8.3	6
	8	8.6 ± 0.41	7.0	4.8	6
	150	162 ± 5.20	7.2	3.2	6
	1500	489 ± 127.00	−0.8	8.5	6
	2500	2452 ± 60.10	−2.0	2.5	6
Run 4	2.5	2.3 ± 0.13	−6.8	5.7	6
	8	8.2 ± 0.62	2.0	7.5	6
	150	152 ± 7.00	1.2	4.6	6
	1500	1358 ± 38.10	−10.4	2.8	6
	2500	2172 ± 51.00	−15.1	2.3	6
Summary statistics (runs 1–4)	Nominal concentration (ng ml^{-1})	Overall bias	Inter-assay precision	Intra-assay precision	
	2.5	0.0	3.0	6.5	
	8	7.0	2.7	9.2	
	150	5.2	3.8	3.0	
	1500	−3.1	5.0	4.8	
	2500	−6.3	6.1	2.5	

retained materials. Flow was maintained to the mass spectrometer during this period using a second HPLC pump that provided 50% (v/v) acetonitrile at 0.35 ml min^{-1}. This solution was diverted to waste when the valve switched to allow column eluant into the instrument. The flow diversion valve was used during all quantitative analyses. All separations were performed with isocratic elution. The mobile phase comprised acetonitrile:water (15:85, v/v). Injection volume was 10–20 μl. Chromatography was performed on a 150 × 2 mm (i.d.), 5 μm Keystone Aquasil C18 column; flow rate was 0.3 ml min^{-1}.

Detection (LC-MS/MS) was performed on a PE-Sciex API III + mass spectrometer equipped with a turbo-ion spray (TISP) source for electrospray ionization. Ionization mode was switched from positive or negative ion mode within the same run. Optimization of instrumental conditions for TISP-MS was performed by constant infusion of test compounds (5 μl min^{-1}, concentration was approximately 10 ng μl^{-1}) through the TISP source using a make-up flow of mobile phase.

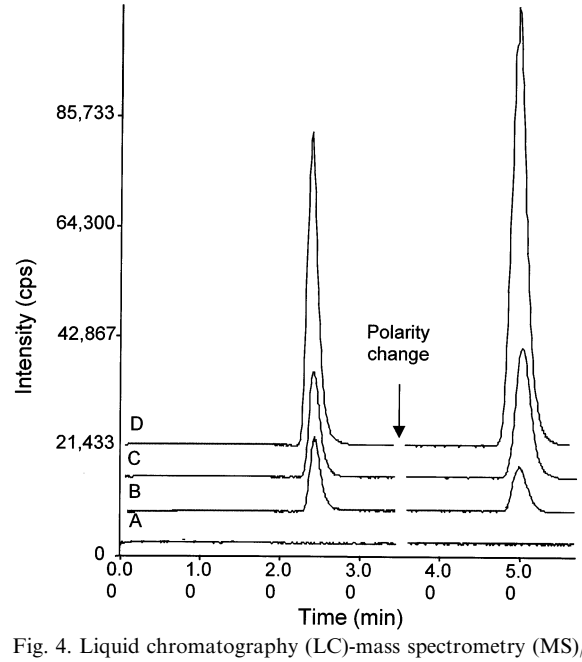

Fig. 4. Liquid chromatography (LC)-mass spectrometry (MS)/MS chromatograms for: (A) drug-free human serum, and human volunteer sera containing (B) 20 and 120 (C) 425 and 322 and (D) 1903 and 1308 ng ml^{-1} of zidovudine (AZT) (5 min) and lamivudine (3TC) (2.5 min), respectively.

Detection by tandem MS was based on precursor ion transitions to the strongest intensity product ions. These corresponded to 266 → 42 for AZT, 271 → 42 for AZT internal standard (IS), 230 → 112 for 3TC, and 235 → 117 for the 3TC IS. Other instrumental conditions were: curtain gas, 1.2 l min^{-1}; auxillary flow, 5500 l min^{-1}; nebulizer temperature, 500°C; nebulizer pressure, 62 psi; interface temperature, 50°C; dwell time, 250 ms; ionization modes, negative ion and positive ion for AZT and 3TC, respectively. Multiple reaction monitoring was employed using argon as collision gas at a density of 300×10^{13} atoms cm^{-2}, with a collision energy of 20 eV for both AZT and 3TC.

Data reduction was performed using Sciex MacQuan V1.4 using linear regression analysis with $1/x^2$ weighting to derive calibration curves

Table 2
Summary statistical data for lamivudine (3TC) validation controls

Validation run no.	Nominal concentration (ng ml^{-1})	Mean ± S.D. (ng ml^{-1})	Bias (%)	Relative S.D. (%)	*n*
Run 1	2.5	2.7 ± 0.18	9.4	6.6	6
	8	8.2 ± 0.30	2.4	3.6	6
	150	152 ± 2.42	0.9	1.6	6
	1500	1513 ± 11.3	0.8	0.7	6
	2500	2559 ± 13.8	2.3	0.5	6
	5000	5064 ± 57.3	1.3	1.1	6
Run 2	2.5	3.0 ± 0.14	17.6	4.5	6
	8	8.7 ± 0.89	7.7	10.2	6
	150	153 ± 2.5	2.0	1.7	6
	1500	1522 ± 36.3	1.5	2.4	6
	2500	2583 ± 118	3.2	4.6	6
	5000	5170 ± 117	3.3	2.3	6
Run 3	2.5	2.3 ± 0.24	−7.9	10.4	6
	8	8.0 ± 0.36	−0.4	4.5	6
	150	155 ± 2.5	3.3	1.6	6
	1500	1535 ± 19.9	2.3	1.3	6
	2500	2582 ± 27.9	3.2	1.1	6
	5000	5059 ± 45.1	1.2	0.9	6
Run 4	2.5	2.4 ± 0.13	−5.9	5.7	6
	8	8.1 ± 0.51	1.4	6.3	6
	150	151 ± 2.6	1.1	1.8	6
	1500	1517 ± 17.2	1.1	1.1	6
	2500	2527 ± 22.2	1.1	0.9	6
	5000	5007 ± 48.5	0.1	1.0	6
Summary statistics (runs 1–4)	Nominal concentration (ng ml^{-1})	Overall bias	Inter-assay precision	Intra-assay precision	
	2.5	4.9	12.1	7.2	
	8	3.0	2.3	6.5	
	150	1.9	0.9	1.6	
	1500	1.4	0.2	1.5	
	2500	2.5	0.2	2.4	
	5000	1.5	1.2	1.4	

Table 3
Method comparison results for zidovudine (AZT) in human serum

Period	Time (h)	Reference RIA result (ng ml^{-1})	Test LC-MS/MS result (ng ml^{-1})	C_t/C_r
Period 1	0	<10	<2.5	
	0.25	2327.4	2367.9	1.02
	0.5	1770.9	1689.2	0.95
	0.75	1501.2	1550.6	1.03
	1	728.2	817.7	1.12
	1.5	511.1	578.1	1.13
	2	321.5	347.0	1.08
	3	134.9	149.0	1.11
	4	86.1	83.0	0.96
	6	31.4	30.0	0.96
	8	19.7	16.3	0.83
	10	10.1	9.9	0.98
	12	<10	5.5	
	16	<10	2.7	
	20	<10	<2.5	
	24	<10	<2.5	
Period 2	0	<10	<2.5	
	0.25	70.5	76.8	1.09
	0.5	1091.5	1098.3	1.01
	0.75	1505.2	1558.7	1.04
	1	1412.8	1371.1	0.97
	1.5	777.7	898.2	1.15
	2	604.6	634.9	1.05
	3	244.9	275.3	1.12
	4	154.6	160.7	1.04
	6	44.6	44.0	0.99
	8	20.7	18.4	0.89
	10	10.1	9.7	0.97
	12	<10	5.6	
	16	<10	2.6	
	20	<10	<2.5	
	24	<10	<2.5	
Period 3	0	<10	<2.5	
	0.25	11.5	12.1	1.05
	0.5	80.0	78.3	0.98
	0.75	95.2	97.9	1.03
	1	165.0	171.6	1.04
	1.5	530.0	569.2	1.07
	2	525.8	553.8	1.05
	3	407.6	467.6	1.15
	4	249.5	262.3	1.05
	6	85.7	85.2	0.99
	8	36.6	32.5	0.89
	10	18.0	17.3	0.96
	12	10.2	9.8	0.96
	16	<10	3.8	
	20	<10	<2.5	
	24	<10	<2.5	

using analyte/internal standard peak area ratios. Concentrations of validation controls (VCs) and clinical samples used were interpolated from their respective daily calibration curves.

2.3. Sample preparation for quantitative analysis

Automation of the liquid handling steps during sample preparation was performed on a Packard

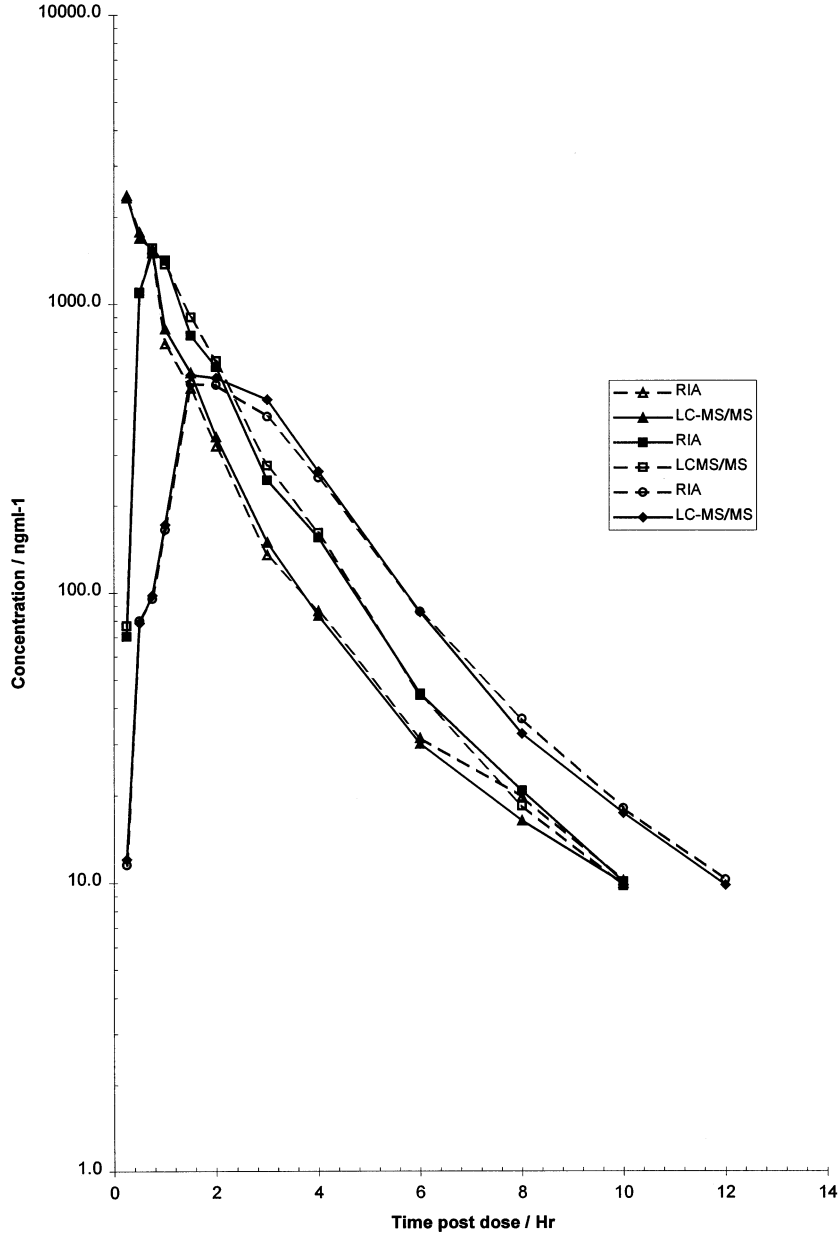

Fig. 5. Pharmacokinetic profiles for zidovudine (AZT), measured by radioimmunoassay (RIA) and liquid chromatography (LC)-mass spectrometry (MS)/MS.

Table 4
Summary data for the cross-validation of zidovudine (AZT) methods

Mean C_t/C_r ratio	1.02
S.E.	0.0135
S.D.	0.0774
Confidence level (95.0%)	0.0275
Confidence intervals:	
LCL (mean–confidence level)	0.993
UCL (mean+confidence level)	1.047
95% confidence interval of the slope of the linear regression:	
Lower 95%	−0.00002
Upper 95%	0.00007

Multiprobe 104DT robotic sample processor running EasyPrep V1.0, and involved serum or plasma ultrafiltration using disposable Centricon 30 ultra-filtration units (molecular weight cutoff, 30 000 Da).

Clinical samples, calibration standards, validation (VC) or quality control (QC) samples (0.25 ml), 25 mM ammonium acetate diluent (0.25 ml) and internal standard (0.1 ml of a 0.5 μg ml^{-1} solution) were dispensed into ultrafiltration units for centrifugation at $3000 \times g$ for 30 min in a fixed angle centrifuge. The solutions of ultrafiltrate (200 μl) were transferred to WISP injector vials for subsequent analysis by LC-MS/MS. VCs were prepared over the range 2.5–2500 (5 levels) and 2.5–5000 ng ml^{-1} (6 levels) in drug-free serum for AZT and 3TC, respectively. Quality control samples, at 8, 150 and 1500 ng ml^{-1} of AZT and 3TC, replaced VCs during clinical study support. Calibration standards were prepared containing either: 2.5, 5.0. 10, 50, 100, 500, 1000 or 2500 ng ml^{-1} of AZT and 3TC. An additional standard was prepared containing 5000 ng ml^{-1} of 3TC only. Calibration standards, and validation and quality control samples contained both analytes, except for the 5000 ng ml^{-1} standard that contained 3TC only.

2.4. Radioimmunoassay (RIA) of AZT

Competitive RIA of AZT was performed, according to manufacturer's instructions, by means of the Incstar (Stillwater, Minnesota, USA) ZDV-Trac assay kit. The method used a [^{125}I]ligand and afforded an LLOQ of 10 ng ml^{-1} of AZT in plasma.

2.5. HPLC-UV method for 3TC [2]

Determination of 3TC by HPLC-UV was performed using a 250 mm × 4.6 mm (i.d.) Hypersil BDS column and a mobile phase comprising methanol:acetonitrile:acetic acid: 0.1 M ammonium acetate (8:1:0.1:90.9; v/v/v/v) at a flow rate of 1 ml min.$^{-1}$. Serum (1 ml) was mixed with an equal volume of 1% (v/v) acetic acid and the solution is applied to a Certify™ (Varian) mixed-bed solid-phase extraction cartridge that had been conditioned previously with 2 ml of methanol and 2 ml of 1% (v/v) acetic acid. After washing steps (2 ml deionized water, 2 ml methanol–10% acetic acid (9:1 v/v) and a further 2 ml deionized water), the sample was eluted with 4 × 0.5 ml volumes of methanol–triethylamine (9:1 v/v). The eluent was evaporated to dryness at 70°C under nitrogen and the residue reconstituted with 200 μl of mobile phase by vortex-mixing; a 100 μl aliquot was injected onto the HPLC column. The lower limit of quantification is 3 ng ml^{-1}, with an HPLC run time of 30 min; absorption wavelength was 270 nm.

3. Results and discussion

3.1. MS of AZT and 3TC

Electrospray ionization (ESI) mass spectra (Fig. 2A and 3A) obtained by scanning Q1 indicated either deprotonated or protonated molecular ions at 266 $(M-H)^-$ for AZT and 230 $(M+H)^+$ for 3TC. Different polarities were employed because the highest product ion intensities were obtained using positive ionization for 3TC and negative ionization for AZT. Each compound could be detected by positive and negative polarities on precursor scans; however, the intensity of product ions was weak using polarities opposite to those indicated above. Selection of a common polarity

Table 5
Method comparison results for lamivudine (3TC) in human serum

Period	Time (h)	Reference HPLC result (ng ml^{-1})	Test LC-MS/MS result (ng ml^{-1})	C_t/C_r
Period 1	0	<5	<2.5	
	0.25	441.6	422.5	0.96
	0.5	1044.5	941.9	0.90
	0.75	1104.6	1015.1	0.92
	1	957.9	894.2	0.93
	1.5	624.4	754.7	1.21
	2	681.2	646.5	0.95
	3	611.5	615.3	1.01
	4	609.3	559.4	0.92
	6	487.5	498.7	1.02
	8	228.9	248.8	1.09
	10	145.4	140.0	0.96
	12	88.0	81.4	0.93
	16	37.6	39.4	1.05
	20	29.1	28.1	0.96
	24	22.1	21.2	0.96
Period 2	0	<5	<2.5	
	0.25	329.4	325.7	0.99
	0.5	937.7	912.9	0.97
	0.75	1369.9	1316.3	0.96
	1	1641.7	1570.3	0.96
	1.5	1358.7	1216.4	0.90
	2	1111.7	989.0	0.89
	3	993.7	910.1	0.92
	4	835.8	777.3	0.93
	6	548.6	517.3	0.94
	8	338.6	321.4	0.95
	10	177.3	170.9	0.96
	12	103.7	100.5	0.97
	16	46.6	43.3	0.93
	20	32.0	30.4	0.95
	24	26.6	22.9	0.86
Period 3	0	<5	<2.5	
	0.25	<5	3.7	
	0.5	18.2	19.3	1.06
	0.75	98.6	105.8	1.07
	1	150.3	163.3	1.09
	1.5	678.5	677.6	1.00
	2	1118.5	1147.4	1.03
	3	1174.6	1194.6	1.02
	4	857.5	857.5	1.00
	6	509.6	510.9	1.00
	8	324.8	304.2	0.94
	10	202.4	198.3	0.98
	12	105.6	104.4	0.99
	16	45.1	46.7	1.03
	20	29.8	30.8	1.03
	24	22.6	22.4	0.99

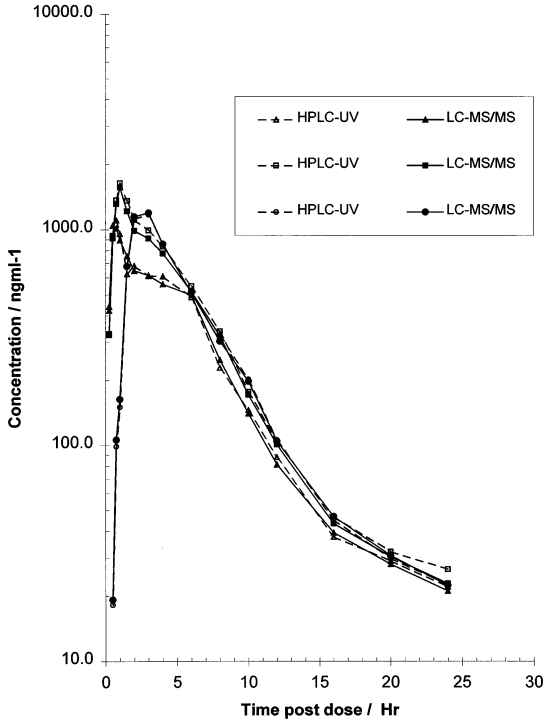

Fig. 6. Pharmacokinetic profiles for lamivudine (3TC), measured by high performance liquid chromatography (HPLC)-UV and LC-mass spectrometry (MS)/MS.

Table 6
Summary data for the cross validation of lamivudine (3TC) methods

Mean C_t/C_r ratio	0.979
S.E.	0.01
S.D.	0.064
Confidence level (95.0%)	0.019
Confidence intervals:	
LCL (mean − confidence level)	0.959
UCL (mean + confidence level)	0.998
95% confidence interval of the slope of the linear regression:	
Lower 95%	−0.00007
Upper 95%	0.00002

would have afforded inadequate sensitivity for one of the compounds in the clinical method. The difference in optimum polarity probably arises from the presence of the primary amino group on the 3TC pyrimidine base moiety. The product ion spectra indicated that the compounds undergo different collision induced dissociations (CID). The ESI-MS/MS spectrum (Fig. 2B) for AZT shows a major product ion at m/z 42 which Font et al. [9] have attributed to the 3′-azido group. The spectrum (Fig. 3B) for 3TC indicates an ion fragment at m/z 112 corresponding to the pyrimidine base moiety.

The ESI-MS/MS spectrum (not shown) for the stable isotope labeled 3TC internal standard was similar to 3TC except that each ion of interest occurred, as expected, at m/z +5. Interestingly the product ion spectrum for the stable isotope labeled AZT internal standard (AZT-IS, Fig. 3C) revealed ions at m/z 42 and 43. The ion at m/z 42 was unexpected because the stable isotope labeled azido group should have produced a single ion at 43. This suggests that two isobaric product ions may be produced during CID of AZT. The presence of the ion at m/z 43 for AZT-IS confirms the presence of the azido group. The ion at m/z 42 suggests that the CNO^- group on the base moiety may be a further product ion. This fragment is unlabelled and would thus appear at m/z 42 for both AZT and its stable isotope labeled internal standard. This product ion was selected for the quantitative method because of its greater intensity.

3.2. Sample preparation and HPLC

Zidovudine and 3TC are polar compounds displaying low protein binding and are consequently amenable to isolation from serum by ultrafiltration. Ultrafiltration has been employed previously by Nebinger and Koel [3] for AZT analysis, and we used a similar method for the simultaneous determination of AZT and 3TC. Our method differed in that ammonium acetate was added to the serum samples to improve the chromatographic behavior of 3TC on the Aquasil column. In the absence of ammonium acetate 3TC was poorly retained on the column, eluting near the solvent front. Extraction efficiencies for both compounds spiked into drug-free human serum indicated a mean ± S.D. recovery of 104.7 ± 5.9 and 99.7 ± 6.7% over the concentration range 25–2000 ng ml^{-1} for AZT and 3TC, respectively.

HPLC was performed on a 2 mm internal diameter column to improve method sensitivity and to decrease mobile phase flow rate (0.3 ml min^{-1}) into the range suitable for direct introduction into the TISP source without the need for splitting flow to waste.

Fig. 4 presents typical chromatograms for drug-free serum and serum containing AZT and 3TC at the lower, middle and upper limits of quantification. Drug-free serum was typically clear of interfering peaks.

3.3. Bioanalytical method validation

Experiments were performed to establish the accuracy, precision, specificity, recovery and limits of quantification of the LC-MS/MS method for AZT and 3TC. These investigations were complemented by studies to confirm the stability of the analytes in serum, plasma, sample extracts and during freeze–thaw cycles. Finally, the LC-MS/MS method was cross-validated to established methods employing either RIA or HPLC with UV detection for the determination of AZT and 3TC, respectively.

Data from four consecutive analytical runs were used to determine accuracy (as bias) and precision. Each validation run comprised two complete sets of calibration standards, with one set placed at the beginning and the other set at the end of the run. VC samples, in replicates of six were distributed between the two calibration curves.

3.3.1. Acceptance criteria for calibration standards

To be acceptable, each run must contain at least 75% of calibration standards back calculating to within 20% of their nominal concentrations. The calibration curve must contain at least one standard at both the lower (LLOQ) and upper (ULOQ) levels of quantification (see Section 3.3.3).

3.3.2. Acceptance criteria for VCs

The overall accuracy of the method was determined at each concentration by assessing the agreement between the measured (mean) and the nominal concentrations of the analytes in VC samples. Acceptable bias was $< \pm 15\%$ at all VC concentrations.

The precision of the method was determined by assessing the agreement between replicate measurements of VC samples. The data for individual analytes were analyzed by one way ANOVA to give estimates of both the intra- and inter-assay precision of the method at each concentration. The precision was estimated for each concentration by calculating the percentage relative standard deviation (RSD). Acceptable precision was $< 15\%$ at all VC concentrations.

3.3.3. Accuracy and precision

Tables 1 and 2 present the back calculated concentrations for the AZT and 3TC VCs over the four validation runs. The controls at 2.5 and either 2500 or 5000 ng ml^{-1} for AZT or 3TC, respectively, were included in the method validation to define the lower (LLOQ) and the upper (ULOQ) limits of quantification for the analytical method. The overall bias (shown as % nominal) of the controls for both compounds at all concentrations is $\leq 7\%$. The overall intra-assay precision (as RSD) is $< 10\%$, and the overall inter-assay precision is $< 6.1\%$ for AZT and $< 12.1\%$ for 3TC. The bias and the precision of the calibration standards (using back calculated data, not shown) is < 8 and $< 7\%$ for AZT and < 3 and $< 4\%$ for 3TC. These results meet the validation requirements of bias and precision of $< 15\%$ for VC samples. Consequently, the LLOQ and ULOQ are 2.5 and 2500 ng ml^{-1} for AZT and 2.5 and 5000 ng ml^{-1} for 3TC.

3.3.4. Specificity results

The specificity of the method for AZT and 3TC was assessed by analysis of blank human plasma and serum samples collected from six drug-free volunteers.

Chromatograms of sera and plasma from all drug-free volunteers were examined for endogenous materials that may co-elute with 3TC or AZT. No interfering components were found. A typical chromatogram for a drug-free serum is presented in Fig. 4A.

3.3.5. Stability studies

The stability of AZT and 3TC was assessed in analytical standard solutions, processed sample extracts, and biological matrix (also after freeze–thaw cycles and during heat inactivation of plasma) by comparison to freshly made standards or freshly spiked plasma or serum samples. A two sample one-sided t-test (95% confidence levels) was performed on the data to determine whether the analyte concentration had changed by more than 15%. All results, other than those rejected for analytical reasons or identified as outliers by a statistical test, were used in the calculation. If the results of the t-test showed the results the two sets of data were equivalent then the analyte was deemed stable in the particular matrix for the time interval and conditions of the test.

3.3.5.1. Stability of analytical standard solutions. The stability of solutions of AZT and 3TC in water solutions stored at 10°C was examined for 7 months. Standard solutions of AZT and 3TC were prepared throughout the method development phase of this study and stored at 10°C when not in use. These solutions were compared to a freshly prepared standard of AZT and 3TC. Standard solutions of AZT and 3TC prepared in water and stored at 10°C were stable for at least 7 months.

3.3.5.2. Stability of processed sample extracts. The stability of AZT and 3TC during a period between extraction and analysis was investigated. The 150 and 1500 ng ml^{-1} serum controls were extracted in replicates of six and stored for approximately 72 h at room temperature. These samples were then assayed and results compared to freshly treated controls. Results for stored extracts were equivalent to those for freshly prepared samples; consequently, AZT and 3TC are considered stable in sample extracts, stored at room temperature, for at least 72 h after extraction.

3.3.5.3. Stability in biological matrix. Stability of AZT and 3TC in serum or plasma was assessed after storage under several conditions: approximately −40°C with periodic assay of aliquots over 6 months, room temperature in the light for 24 h, room temperature in the dark for 24 h, and refrigerated at 10°C for 6 days. In each instance there was no apparent loss of either compound over the designated periods.

3.3.5.4. Stability during freeze–thaw cycles. The stability of AZT and 3TC in serum was assessed through three freeze–thaw cycles. Serum samples were frozen at approximately −40°C and thawed at room temperature. This cycle was repeated to obtain three freeze–thaw cycles. Serum samples were extracted and analyzed prior to freezing and after the three freeze–thaw cycles. There was no apparent loss of either compound after three freeze–thaw cycles.

3.3.5.5. Stability during heat inactivation. Sera samples were inactivated by heating for 5 h at 58°C dry heat in an oven. There was no apparent loss of either compound after heat treatment.

3.4. Cross validation with an RIA for AZT and an HPLC-UV method for 3TC

Cross validation studies are performed to determine whether two bioanalytical methods generate equivalent results. The cross-validation protocol involves analyzing between 24 and 72 authentic study samples, together with six pre-dose samples, by both methods. Study samples are selected such that analyte concentrations are within the calibration range of the test and reference methods. A pre-requisite of cross-validation is that both methods have been successfully validated.

There are two acceptance criteria for the cross validation procedure. For the first, a confidence interval is calculated for the true mean value of the concentration ratio C_t/C_r; where C_t is the test method concentration and C_r is the reference method result. The overall confidence level of the concentration ratio test is set at 95%. If the entire calculated confidence interval lies within the preset acceptance range of 0.85–1.15 then the concentration ratio test is satisfied. For the second criterion, a graph is constructed of the C_t/C_r ratios versus C_r. If the two methods are equivalent the data will be scattered randomly about a hori-

zontal line at a C_t/C_r value of 1. A deviation from the horizontal would indicate a systematic error. The 95% confidence intervals for the slope are calculated and the limits should include zero for the test to be satisfied.

Forty eight serum samples were selected for the current cross validation study. These samples represented full pharmacokinetic profiles taken from volunteers who had received 300 mg AZT and 150 mg 3TC in an oral bioequivalency study. Each sample was assayed by the LC-MS/MS method and the results compared to those obtained by RIA for AZT and by HPLC-UV for 3TC.

3.4.1. AZT results

The results of the method comparison study for AZT are shown in Table 3 and in Fig. 5. Table 3 presents the concentration results for the reference method (RIA) and for the test method (LC/MS) and the ratio, C_t/C_r. The closer the C_t/C_r ratios are to unity, the more likely the two analytical methods are equivalent.

The C_t/C_r ratios obtained in Table 3 were used to generate the summary data presented in Table 4. Acceptable precision limits (RSD, 15%) within each method, indicate that the 95% confidence intervals of the C_t/C_r ratio must be bracketed by the values 0.85 and 1.15. The measured confidence limits for AZT are 0.993 and 1.047. This interval lies within the range 0.85–1.15, which demonstrates that results generated by each method are equivalent.

For the second acceptance criteria, the value of zero must be contained within the 95% confidence intervals for the slope of the C_t/C_r versus C_r line. This was achieved as zero falls between the lower (-0.00002) and the upper (0.00007) confidence levels (Table 4).

The data used for cross-validation are presented in Fig. 5 as pharamacokinetic profiles (serum concentration vs. time) obtained by each method. The near superimposable profiles from both analytical methods confirm they are equivalent.

3.4.2. 3TC results

The results of the method comparison study for 3TC are shown in Table 5 and in Fig. 6. The summary data for C_t/C_r ratios presented in Table 6 confirm acceptable confidence intervals (0.959 and 0.998) around the mean. The confidence levels of the slope were also acceptable as zero fell between the lower (-0.00007) and the upper (0.00002) limits.

Fig. 6 presents pharamacokinetic profiles for 3TC using the HPLC method and the LC-MS/MS method. Similar to AZT, the profiles are virtually superimposable confirming the methods are equivalent.

4. Conclusions

An LC-MS/MS method using an ultrafiltration extraction step has been developed and validated for the simultaneous determination of AZT and 3TC in human serum. The validation included cross-validations to established methods employing RIA and HPLC-UV, respectively. The LC-MS/MS method afforded a lower limit of quantification (2.5 ng ml^{-1}) for each analyte, with a chromatographic run time of approximately 6 min. This method has been used routinely to support post-marketing pharmacokinetic studies during the further development of Combivir™.

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