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Liquid chromatographic–mass spectrometric determination of the metabolism and disposition of the anti-retroviral nucleoside analogs zidovudine and lamivudine in C57BL/6N and B6C3F1 mice

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Abstract

Transmission of HIV from mother to infant can be effectively prevented by zidovudine (3′-azido-3′-deoxythymidine; AZT) alone or in combination with other anti-retroviral drugs; however, significant evidence for genotoxicity, including transplacental carcinogenicity in mice, has been reported for AZT. A method, based upon solid phase extraction (SPE) in the 96-well format, gradient liquid chromatography (LC), and electrospray mass spectrometry (MS), was developed and validated to measure serum concentrations in maternal C57BL/6N and fetal B6C3F1 mice of the nucleoside analogs AZT, lamivudine ((-)2′,3′-dideoxy-3′-thiacytidine; 3TC), and several metabolites selected based on importance in detoxification and bioactivation reactions. After intravenous (IV) and oral dosing with either 400 mg/kg AZT or 200 mg/kg 3TC, pharmacokinetics were determined for AZT, AZT-5′-glucuronide, 3′-amino-3′-deoxythymidine (AMT), AZT-5′-phosphate, 3TC, and 3TC-5′-phosphate in serum of adult female mice. Pharmacokinetics were also determined in spleen for AZT-5′-phosphate and 3TC-5′-phosphate following IV dosing. In addition, a preliminary assessment was made of placental transfer of AZT and 3TC and the presence of metabolites in the fetal compartment. The method described provides a means to evaluate thoroughly metabolism and disposition of anti-retroviral nucleoside analogs in maternal and fetal mice for comprehensive studies of genotoxicity.

Keywords: Zidovudine; Lamivudine

1. Introduction

Vertical transmission of HIV from mother to infant can be effectively prevented by administration of zidovudine (3′-azido-3′-deoxythymidine; AZT), alone or in combination with related antiviral drugs. However, the use of AZT is a potential concern because it becomes incorporated into DNA [1], increases the mutant frequency and micronucleus formation in bone marrow [2], and increases the tumor incidence in mice after transplacental or neonatal exposures [1,3]. While the use of AZT as a single agent to treat HIV has largely been replaced by more effective combinations of antiviral drugs, the impact of combination therapy on the metabolism, disposition, and transplacental toxicity of AZT is largely unknown. This paper describes the development and validation of an analytical method based on LC with electrospray mass spectrometry (MS) and isotope dilution to provide determinations in mouse serum and spleen of AZT and lamivudine ((-)2′,3′-dideoxy-3′-thiacytidine; 3TC), a related nucleoside analog, along with several toxicologically significant metabolites (see Fig. 1 for structures). This method provided the basis for a preliminary investigation of the metabolism and disposition of parent nucleosides in adult C57BL/6N female mice and their B6C3F1 fetuses.

2. Experimental

2.1. Reagents

AZT and 3TC were obtained from Cipla Ltd. (Mumbai, India); AZT-5′-glucuronide (AZTG), 3′-amino-3′-deoxythymidine (AMT), 3TC ((-)2′,3′-dideoxy-3′-thiacytidine), Cremophor EL, and sweet potato acid phosphatase were

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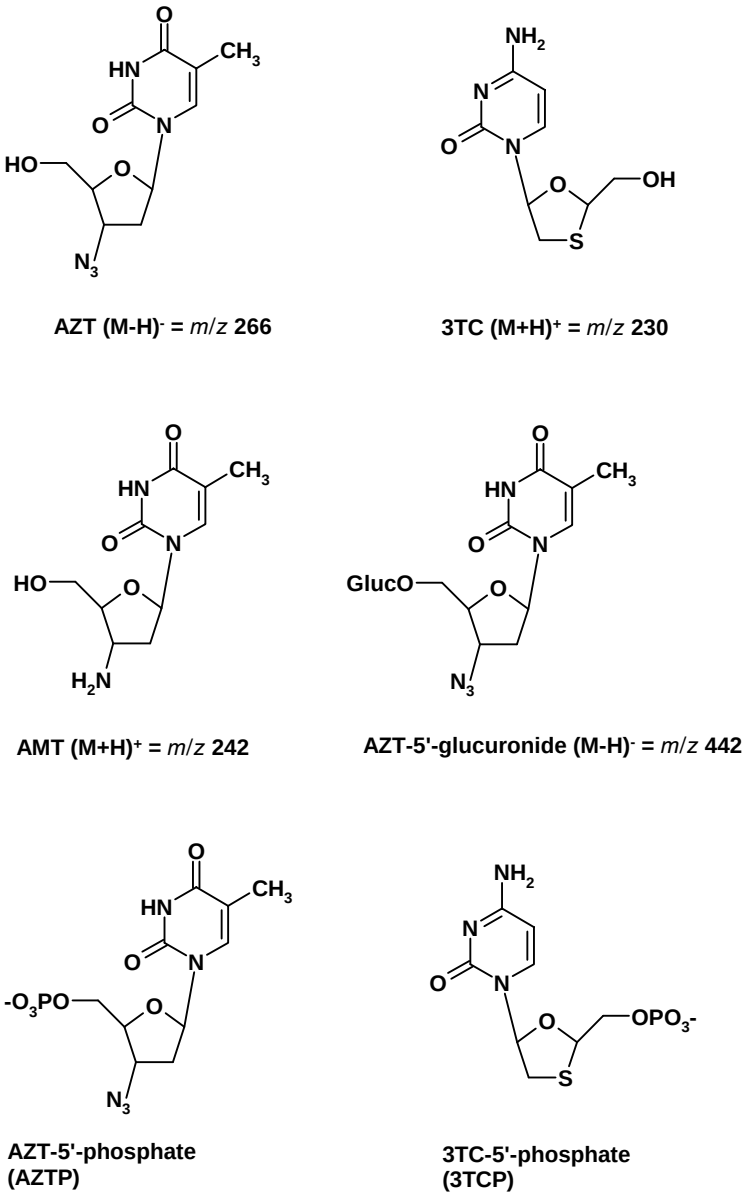

Fig. 1. Structures of nucleoside analogs and metabolites.

purchased from Sigma (St. Louis, MO). AZT-5′-phosphate (AZTP) and 3TC-5′-phosphate (3TCP) were obtained from Moravek Biochemicals (Brea, CA). $^{13}C_1{}^{15}N_1d_3$AZT and $^{13}C_2{}^{15}N_3$3TC internal standards were a gift from Burroughs-Wellcome Co. (Research Triangle Park, NC). All solvents were HPLC grade, and Milli-Q water was used throughout.

2.2. Solid phase extraction (SPE)

2.2.1. Nucleoside analogue extraction

SPE was carried out using the 96-well format under reduced pressure. Isolute ENV+ cartridges (50 mg, 1 cm^3, Jones Chromatography, Lakewood, CO) were used for the extraction. Activation of the cartridges was achieved with 2×1 ml washes of methanol, followed by 2×1 ml washes of 0.1% formic acid. Serum samples (10–100 μl) were spiked with the labeled internal standards ($^{13}C_1{}^{15}N_1d_3$AZT (100 ng) and $^{13}C_2{}^{15}N_3$3TC (either 100 ng for 3TC dosing studies or 10 ng for AZT dosing studies) contained in 10 μl of water) and diluted with 150 μl of 0.1% formic acid prior to extraction. The cartridges, containing sample, were then washed with 1×1 ml water and 1×1 ml 90/10 water/methanol. Elution of the sample occurred with 3×400 μl washes of 10% ammonium acetate (100 mM, pH 4) in methanol. The eluate was reduced to dryness using a centrifugal vacuum concentrator and reconstituted into 200 μl water.

2.2.2. Nucleotide extraction

Nucleotide analysis was achieved in three stages: an initial SPE to extract the phosphates in separate fractions; hydrolysis of the nucleotides to the respective parent nucleoside; and subsequent extraction and analysis of the nucleosides using the method described above. Weak anion exchange SPE used Isolute NH_2 96-well cartridges (50 mg, 1 cm^3, Jones Chromatography, Lakewood, CO) under reduced pressure. The cartridges were activated by 2 × 1 ml washes of methanol, followed by equilibration with 2 × 1 ml washes of 10 mM ammonium acetate buffer, pH 5. Serum samples (100 μl) were diluted with 400 μl of 10 mM ammonium acetate buffer, pH 5, and loaded onto the cartridges. Whole spleen samples (60–90 mg) were weighed, homogenized for 30 s in 2 ml of 10 mM ammonium acetate, pH 5, centrifuged for 10 min at 25,000 rpm, and the resulting supernatant was loaded onto the cartridges. The samples were washed sequentially with 1 ml 10% methanol in 10 mM ammonium acetate, pH 5, and 1 ml 0.1% acetic acid in methanol. The nucleotide fraction was eluted with 5 ml × 1 ml washes of 65 mM ammonium acetate, pH 9. The resultant 5 ml extracts were spiked with the respective nucleoside internal standards (10 ng of $^{13}C_1{}^{15}N_1d_3$AZT and 10 ng of $^{13}C_2{}^{15}N_3$3TC) and acidified to pH 4.8 using 5 M ammonium acetate, pH 3.7. Quantitative hydrolysis of nucleotides was achieved by incubation of the eluted fractions with 10 U of acid phosphatase (50 mM sodium acetate, pH 4.8) for 30 min at 37 °C. After hydrolysis, the respective nucleoside analogues were extracted and analyzed using the method described above.

2.3. Liquid chromatography

Liquid chromatography (LC) was performed using a Waters 2695 liquid handling system (Waters Assoc., Milford, MA). Chromatographic separation was achieved on a Polaris C_{18} analytical column (150 mm × 2.0 mm × 3 μm particles, ANSYS Technologies, Lake Forest, CA), equipped with a C_{18} security guard (4 mm × 2.0 μm, Phenomenex, Torrance, CA), at a flow rate of 200 μl/min. The mobile phase consisted of 0.1% formic acid and acetonitrile. Initial conditions were set to 0% acetonitrile increasing to 31% over 4 min. This gradient was sufficient to separate the AMT peak from the unretained material at the beginning of the run. A fast gradient to 90% acetonitrile at 8.9 min was employed in order to elute 3TC, AZT, and AZTG in the shortest possible time. At 9 min, the gradient was programmed to initial conditions for column equilibration. Injection volumes ranged from 5 to 100 μl and all separations were performed at ambient temperature.

2.4. Mass spectrometry

The entire column effluent was directed into a Platform II single quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface for mass analysis. Because 3TC and AMT were ionized more effectively in positive mode, while AZT and AZT-glucuronide required negative ionization, the chromatographic run was split into three scan events. The first scan event was from 0 to 6 min and monitored selected positive ions (SIM) for the protonated molecule of AMT and its major product ion (*m*/*z* 116). The $[M+H]^+$ ion for AMT (*m*/*z* 242) had optimal ionization at a cone-skimmer potential of 15 V, while the fragment ion required 25 V. The second scan event was from 6.1 to 8.1 min and used positive SIM for the protonated molecules of 3TC, $^{13}C_2{}^{15}N_3$3TC internal standard, and the major fragment ion. The $[M+H]^+$ ion for 3TC (*m*/*z* 230) and its internal standard (*m*/*z* 235) had optimal ionization at a cone-skimmer potential of 15 V, while the fragment ion of 3TC (*m*/*z* 112) required 25 V. In the third scan event, which was from 8.2 to 11 min, the deprotonated molecules $[M-H]^-$ for AZT (*m*/*z* 266), AZTG (*m*/*z* 442), and $^{13}C_1{}^{15}N_1d_3$AZT (*m*/*z* 271) were analyzed with a cone-skimmer potential of 25 V, while the AZT fragment ion (*m*/*z* 233) was analyzed at 40 V. Quality control procedures included concurrent analysis of spiked serum samples (0.1, 1, and 10 μg/ml), blank serum, and a mixture of labeled and unlabeled standards interspersed throughout each sample set.

2.5. Characterization of labeled internal standards

The concentrations of $^{13}C_2{}^{15}N_3$3TC and $^{13}C_1{}^{15}N_1d_3$AZT internal standards were determined by comparing LC-UV (260 nm) responses with the corresponding authentic unlabeled materials that had been prepared by accurate weighing. No unlabeled 3TC or AZT was observed in the respective labeled internal standards. A calibration plot of response ratios for both 3TC and AZT versus concentration ratios was constructed by duplicate injections of six concentrations (1–2000 pg/μl) of AZT and 3TC and a constant amount (50 pg/μl) of $^{13}C_1{}^{15}N_1d_3$AZT and $^{13}C_2{}^{15}N_3$3TC internal standards. Both plots were linear, with 3TC showing a correlation coefficient of 0.999 and a slope of 1.10, and AZT a correlation coefficient of 0.999 and slope of 1.00. Plots of response ratios versus concentration ratios for AZTG and AMT with their respective internal standards ($^{13}C_1{}^{15}N_1d_3$AZT and $^{13}C_2{}^{15}N_3$3TC) were also highly linear in the ranges used in this study (correlation coefficients > 0.99).

2.6. Animal treatments

C57BL/6N female mice were received as weanlings from the NCTR breeding colony. At approximately 9 weeks of age, 24 mice were dosed orally and 24 were dosed by intravenous (IV) injection with a single treatment of 400 mg/kg of AZT. An identical number of mice were treated likewise with 200 mg/kg 3TC. Oral doses were administered to each animal in sterile water containing 0.2% methyl cellulose/0.1% Polysorbate 80 (McTween). IV doses were administered using a solvent vehicle of Cremophor EL/ethanol/water (1:1:8) via tail vein injection. At 5, 10, 15, 30, 45, 60, 90, 120, 180,

240, 360, and 480 min post-treatment, blood was collected by cardiac puncture from two mice per treatment, placed on ice to clot, and serum was prepared by centrifugation. Serum samples were stored at −80 °C until analysis. Spleens were collected immediately following blood collection, frozen on dry ice, and stored at −80 °C until analysis.

For the transplacental pharmacokinetic study, three pregnant C57BL/6N females were gavaged with a mixture of AZT (240 mg/kg) and 3TC (120 mg/kg) on gestational day 17. At 30, 45, or 60 min after treatment, the dams were anesthetized using CO_2, blood was removed by cardiac puncture, and serum was prepared. The placentas were then separated from the B6C3F1 fetuses and blood was collected in a heparinized syringe from each fetus and combined by litter for preparation of plasma. The volume of heparin added to the syringe was subtracted from the total volume to estimate the volume of fetus plasma collected. Serum concentrations of nucleosides and metabolites (duplicate analyses) and nucleotides (single analysis) in the dams were determined using the methods described above. The small volume of fetal plasma collected from each litter made possible only a single analysis of nucleosides, metabolites, and nucleotides.

2.7. Statistical analysis

Serum or plasma levels of the nucleosides, metabolites, and nucleotides were assessed by analysis of variance, with pairwise comparisons being conducted by the Student–Newman–Keuls method. When necessary, the data were natural log transformed before the analysis to maintain a normal distribution. $P < 0.05$ were considered significant.

2.8. Pharmacokinetic determinations

Plots of nucleoside analog and metabolite concentrations in serum or spleen as a function of time were analyzed using the model-independent spreadsheet approach of Ritschel [4]. Natural log-linear plots of IV data for 3TC showed two phases, consistent with distribution and elimination processes, and IV data for AZT showed a single phase, consistent with elimination only (not shown). Oral dosing with AZT or 3TC produced two phases attributed to absorption and elimination, although the absorption phase was too fast to determine accurately the rate. Metabolite time–concentration profiles showed an accumulation phase followed by an elimination phase, although in some cases data scatter did not permit an accurate assessment of elimination rates. The first-order elimination rate constant (k_{elim}) was determined from the slope of the terminal phase of the ln-linear serum concentration–time curve. Internal exposure to AZT and 3TC and their metabolites ($AUC_{0-\infty}$, area under the concentration–time curve from zero to infinity) was determined using the trapezoidal rule [4]. The volume of distribution for the nucleoside analogs (V_d) was calculated from IV data as dose/(AUC × k_{elim}) and total serum clearance was calculated as dose/$AUC_{0-\infty}$. The fraction of parent drug absorbed (f) was calculated by the ratio of oral and IV AUCs.

3. Results

3.1. Method performance

A gradient LC–ES/MS method was developed to separate 3TC, AZT, and their major metabolites in serum. Fig. 2 shows chromatograms for the compounds of interest in blank and incurred mouse serum samples. Precise and accurate quantification was obtained through the use of isotopically labeled internal standards. 3TC was quantified using the response ratio with $^{13}C_2{}^{15}N_3$3TC, while AZT and AZTG were compared with $^{13}C_1{}^{15}N_1d_3$AZT. AMT, although a metabolite of AZT, was compared to the $^{13}C_2{}^{15}N_3$3TC internal standard because it was ionized more effectively in positive ion mode and because it was closer in retention time to 3TC. Analyte recoveries from spiked serum were determined to be 89 ± 1, 92 ± 3, 90 ± 1, and 86 ± 8% for 3TC, AZT, AZTG, and AMT, respectively ($n = 4$). Small but measurable ion suppression was observed with a fortified serum sample (i.e. a blank serum sample processed through the SPE procedure to which an authentic standard was added) when compared with an equivalent amount of the same standard dissolved in water containing a small amount of buffer. AZT and 3TC suppression was estimated to be approximately 15 and 4–5%, respectively; no significant suppression was observed for AZTG or AMT. The limits of quantification (LOQs) for all four analytes, approximated by extrapolating to a signal/noise (S/N) ratio of 10:1, were below 0.1 μg/ml of serum. The performance of the method at the LOQ (i.e. R.S.D. < 9.7%) is shown in Table 1. Similarly, the LODs, approximated by extrapolating to a S/N ratio of 3:1, were approximately 0.03 ng/ml. These values were based on the analysis of 10 μl of serum and injection of 25% of the reconstituted volume. This protocol provided more than adequate responses for all the samples from the mice analyzed in this study. Lower LOQs could readily be achieved by using larger sample and injection volumes for application to typical human dosing protocols (ca. 6 mg/kg AZT and 2 mg/kg 3TC, both of which yield maximal serum concentrations of approximately 1 μg/ml).

Overall recoveries for the nucleotide method for AZTP in mouse serum and spleen were determined to be 87 and 84%, respectively, ($n = 4$) and for 3TCP in serum and spleen, 82 and 77%, respectively ($n = 4$). The LOQs for both AZTP and 3TCP, approximated by extrapolating to a S/N ratio of 10/1, were below 10 ng/ml in serum and 30 ng/g in spleen.

3.2. Method validation

The method was validated for AZT, AZTG, AMT, and 3TC by replicate analysis ($n = 4$) through the SPE and LC/MS procedures on different days using untreated rat

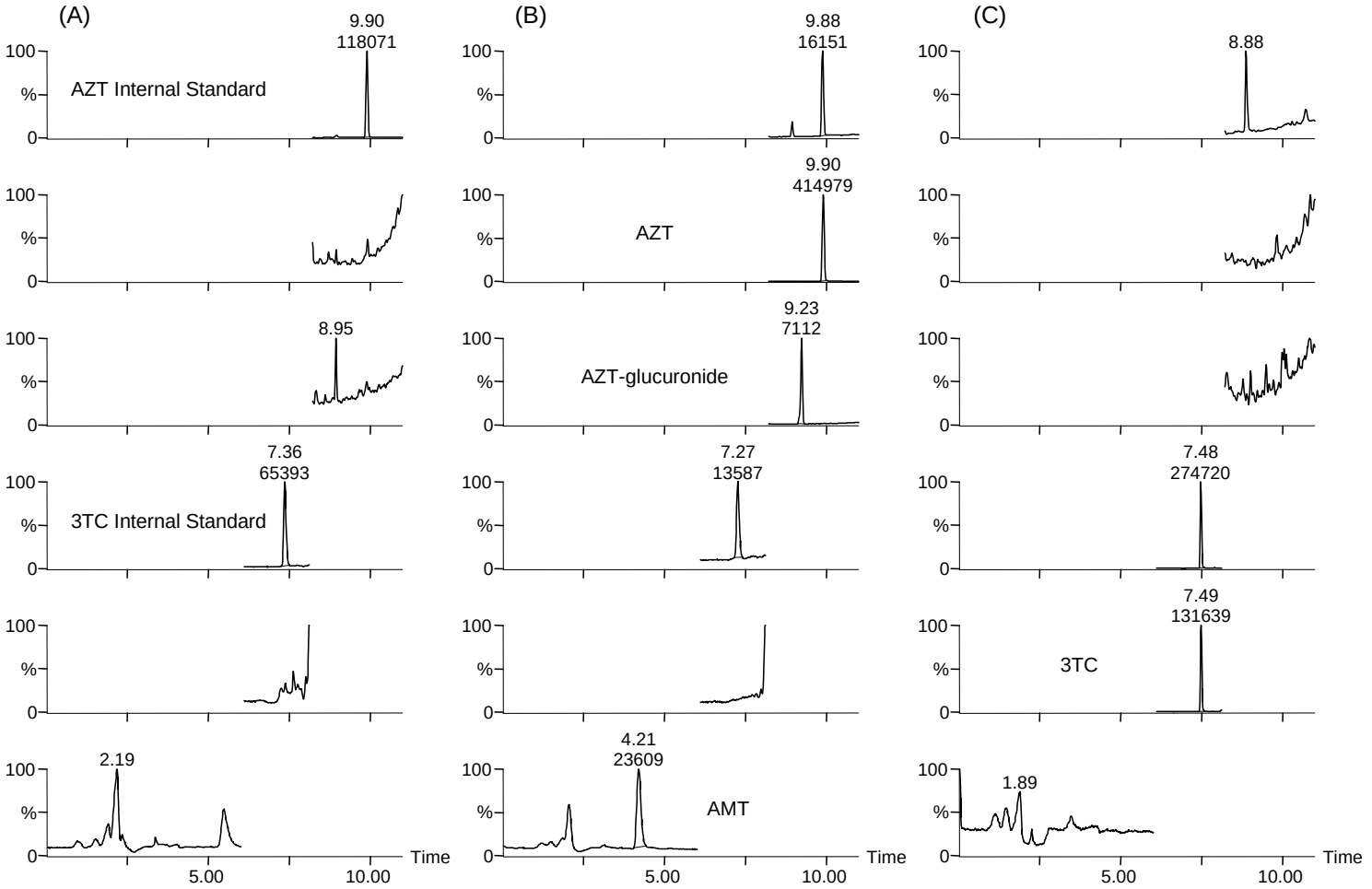

Fig. 2. LC–ES/MS analysis of 3TC, AZT and metabolites in mouse serum. Panel A: untreated mouse. Panel B: AZT-treated mouse (400 mg/kg body weight). Panel C: 3TC-treated mouse (200 mg/kg body weight). The MS traces, from top to bottom, correspond to $^{13}C_1{}^{15}N_1d_3$AZT internal standard (*m*/*z* 271), AZT (*m*/*z* 266), AZTG (*m*/*z* 442), $^{13}C_2{}^{15}N_3$3TC internal standard (*m*/*z* 235), 3TC (*m*/*z* 230), and AMT (*m*/*z* 242), respectively.

Table 1
Method validation summary

Compound	Concentration (μg/ml)	Day 1 precision (R.S.D.%)	Day 1 accuracy (% added)	Day 2 precision (R.S.D.%)	Day 2 accuracy (% added)
AZT	0.1	0.10 ± 0.001 (0.8)	101	0.10 ± 0.0003 (0.3)	101
	1	0.99 ± 0.009 (0.9)	99	1.0 ± 0.01 (1)	100
	10	9.8 ± 0.09 (1)	98	10.1 ± 0.07 (0.68)	101
	200	204 ± 3.2 (1.6)	102	198 ± 0.86 (0.4)	99
AZTG	0.1	0.11 ± 0.006 (5.6)	114	0.10 ± 0.004 (3.8)	104
	1	1.0 ± 0.015 (1.5)	103	1.2 ± 0.11 (9.5)	115
	10	10.4 ± 0.16 (1.5)	104	10.1 ± 0.19 (1.8)	101
AMT	0.1	0.098 ± 0.01 (9.7)	98	0.1 ± 0.002 (1.6)	100
	1	0.91 ± 0.018 (2)	91	1.0 ± 0.019 (1.9)	100
	5	5.0 ± 0.59 (11.7)	100	5.1 ± 0.192 (3.8)	101
3TC	0.1	0.10 ± 0.001 (0.94)	104	0.10 ± 0.003 (3.3)	103
	1	1.0 ± 0.014 (1.4)	100	1.0 ± 0.013 (1.3)	100
	10	10 ± 0.32 (3.2)	100	9.9 ± 0.1 (1)	99
	20	20.3 ± 0.19 (1)	101	19.4 ± 0.23 (1.2)	97

Control rat serum was spiked with various concentrations of AZT, AZTG, AMT and 3TC on two separate days. The values shown are means ± S.D. ($n = 4$) with the relative standard deviation (R.S.D.) shown in parentheses.

serum (10 μl) to which had been added different concentrations of the analytes. As shown in Table 1, acceptable inter- and intra-day precision and accuracy were obtained for all compounds. The data presented in Table 1 were also plotted graphically (not shown) and were found to be highly linear (i.e. correlation coefficients > 0.999) (rat serum was used for the validation because insufficient volumes of untreated mouse serum were available). Untreated mouse serum had no additional interferences and did not affect method performance when assessed using a single spiked concentration of all analytes.

The anion exchange SPE method for the nucleotides did not permit retention of the labeled internal standards. Therefore, it was necessary to spike the extracts from untreated mouse serum and spleen with labeled internal standards after elution of the purified nucleotide fraction from the SPE cartridges just prior to enzymatic dephosphorylation. Repeated analysis of nucleotide hydrolysis reactions showed that complete dephosphorylation to the respective nucleoside occurred (data not shown). Table 2 shows the validation results obtained by spiking serum (100 μl) and spleen (10 mg equivalents) with either AZTP or 3TCP. Acceptable inter- and intra-day precision was obtained. As stated above, the accuracies in this case correspond to the recoveries of sample from the anion exchange SPE since labeled internal standards were not used until the hydrolysis stage.

3.3. Pharmacokinetics

The pharmacokinetics of AZT, AZTG, AMT, and 3TC were determined in serum following IV and oral administration of AZT and 3TC to adult female mice. Additionally, AZTP and 3TCP were measured in serum and spleen following IV administration. The approximate values for maximal serum concentrations (C_{max}) and the time where C_{max} occurred (t_{max}), as determined graphically, are presented in Table 3.

The fraction of AZT absorbed, as determined by the ratio of AUCs from oral (Table 5) and IV (Table 4) administrations was 0.71 (Table 5). AZTG was eliminated at a slightly greater rate than AZT (Table 5), whereas AMT was eliminated approximately three-fold slower than AZT (Table 5). Despite these kinetic differences, the internal exposures (serum AUCs, with the assumption of comparable V_d for all these small highly water-soluble compounds) to AZTG and AMT metabolites were much lower than that for the parent drug (ca. 1%).

Table 2
Nucleotide method validation summary

Compound	Concentration	Day 1 precision (R.S.D.%)	Day 1 accuracy (% added)	Day 2 precision (R.S.D.%)	Day 2 accuracy (% added)
AZTP serum	100 (ng/ml)	92.4 ± 2.3 (2.5)	92	97 ± 2.1 (2.2)	97
AZTP spleen	1 (ng/mg)	0.905 ± 0.03 (2.9)	91	0.91 ± 0.02 (1.7)	91
3TCP serum	100 (ng/ml)	91.6 ± 0.95 (1)	92	92.6 ± 1.7 (1.8)	93
3TCP spleen	1 (ng/mg)	0.87 ± 0.008 (0.9)	87	0.87 ± 0.001 (1.2)	87

Serum and spleen from untreated mice were spiked with either AZTP or 3TCP on two separate days. The values shown are means ± S.D. ($n = 4$) with the relative standard deviation (R.S.D.) shown in parentheses.

Table 3
Estimated values for the maximal serum concentration (C_{max}) and the time where C_{max} occurred (t_{max}) for AZT, 3TC, and metabolites in adult mouse serum and spleen

Drug/metabolite-route of administration	C_{max} (μg/ml)	t_{max} (min)
AZT-IV	470	5
AZTG-IV	6.8	45
AMT-IV	3.0	45
AZTP-IV (serum)	0.11	5
AZTP-IV (spleen)	0.13	15
AZT-oral	260	15
AZTG-oral	5.6	30
AMT-oral	1.4	30
3TC-IV	65	5
3TCP-IV (serum)	0.05	30
3TCP-IV (spleen)	0.36	30
3TC-oral	15	30

AZTP in the serum was eliminated six-fold faster than AZT (Table 4), but AZTP in spleen had a 12-fold lower elimination rate than in serum (Table 4). The AUCs for AZTP in serum and spleen were approximately equal and were more than three orders of magnitude lower than AZT in serum. The observed biotransformations are consistent with conversion of AZT to inactive (AZTG), more toxic (AMT), and activated (AZTP) metabolites and with accumulation in lymphoid tissue (spleen) from the serum of the phosphorylated form.

The elimination of 3TCP also was slower from spleen than from serum (Table 6), but in contrast to AZTP, the AUC for 3TCP was approximately 50-fold greater in the spleen compared to the serum. Nonetheless, the internal exposures to 3TCP in either serum or spleen were far below that for the parent drug (<2%). These data are also consistent with accumulation of phosphorylated 3TC in spleen from the serum. Similar to AZT, the fraction of 3TC absorbed was 0.67 (Table 6); however, the elimination of 3TC was slower and the volume of distribution and the clearance were greater than those for AZT.

A preliminary determination of transplacental distribution and metabolism was made at three time points (30, 45, and 60 min) around the expected peak concentrations following oral administration of a mixture of AZT and 3TC to dams. For comparative purposes, average values were calculated. Table 7 shows that the parent drugs and all metabolites observed in the dam were also observed in the fetus. There was no statistically significant difference in the average levels of AZT or AZTP between the dams and fetuses; however, the average levels of either AZTG (22%) or AMT (51%) were lower in fetuses relative to dams, with the AZTG difference being significant. Levels of 3TC were significantly lower in fetuses (22%) than in dams and although average 3TCP levels were lower in fetuses (39%) than dams, this difference was not significant.

Table 4
Pharmacokinetics of AZT and metabolites in serum following IV administration of 400 mg/kg

PK parameter	AZT	AZTG	AMT	AZTP (serum)	AZTP (spleen)
$t_{1/2}$ elim. (min)	36	NA	NA	6.0	75
$AUC_{0-\infty}$ (mg min/ml)	24	0.54	0.49	0.0064	0.0074
V_d (l/kg)	0.86	–	–	–	–
Cl_{tot} (ml/(min kg))	16	–	–	–	–

NA: insufficient data to determine. The elimination half-life ($t_{1/2}$ elim), area under the concentration–time curve ($AUC_{0-\infty}$), the volume of distribution (V_d), and total serum clearance (Cl_{tot}) values are shown.

Table 5
Pharmacokinetics of AZT and metabolites in serum following oral administration of 400 mg/kg

PK parameter	AZT	AZTG	AMT
$t_{1/2}$ elim. (min)	44	35	141
$AUC_{0-\infty}$ (mg min/ml)	17	0.29	0.23
$AUC_{oral/IV}$ (f)	0.71	–	–
Cl_{tot} (ml/(min kg))	23	–	–

Table 6
Pharmacokinetics of 3TC and metabolites in serum and spleen following oral and IV administration of 200 mg/kg

PK parameter	3TC-IV	3TC-oral	3TCP-IV (serum)	3TCP-IV (spleen)
$t_{1/2}$ distr. (min)	29	NA	NA	NA
$t_{1/2}$ elim. (min)	96	110	27	130
$AUC_{0-\infty}$ (mg min/ml)	4.2	2.8	0.0013	0.078
V_d (l/kg)	3.0	–	–	–
$AUC_{oral/IV}$ (f)	0.67	–	–	–
Cl_{tot} (ml/(min kg))	48	71	–	–

NA: insufficient data to determine.

4. Discussion

This paper describes an LC–MS based method for quantification of antiviral nucleosides and metabolites in mouse serum that was developed and validated for use in a preliminary assessment of mouse metabolism, pharmacokinetics, and placental transfer. The metabolites chosen were AZTG, because this represents a primary detoxification pathway; AMT, because of its increased cytotoxicity relative to AZT [5]; and AZTP, because it is a substrate for the rate-limiting step in synthesis of AZT triphosphate (i.e. thymidylate kinase-catalyzed formation of AZT diphosphate [6]), the ultimate metabolite that inhibits DNA polymerase and becomes incorporated at the end of terminated DNA chains [7]. Although 3TC is minimally metabolized prior to excretion, phosphorylation is an important activation pathway; thus, 3TCP was also measured. The phosphorylated derivatives of these nucleoside analogs were also measured in spleen because of their reported propensity to accumulate in lymphoid tissues [8].

Placental transfer of AZT (fetal/maternal ratio = 0.9) was observed in mice in accord with previous studies in non-human primates [9] and in isolated human placenta [10]. Placental transfer of 3TC was also observed (fetal/maternal ra-

Table 7
Determination of placental transfer of AZT and 3TC and fetal metabolism

	Time (min)	AZT (μg/ml)	AZTG (μg/ml)	AMT (μg/ml)	AZTP (ng/ml)	3TC (μg/ml)	3TCP (ng/ml)
Dam 1	30	114	8.1	2.1	38	19	5.3
Dam 2	45	124	13	3.3	67	26	14
Dam 3	60	117	10	3.9	43	25	10
Litter 1	30	80	0.97	0.87	25	2.6	1.7
Litter 2	45	128	2.9	1.6	68	6.3	4.4
Litter 3	60	124	3.1	2.2	50	6.6	5.4

Pregnant dams were treated on gestational day 17 with a mixture of AZT and 3TC and blood was removed from individual dams and combined for all fetuses in each litter at the indicated time for analysis of AZT, 3TC, and metabolites.

tio = 0.2) but to a lesser degree than AZT. Although the data are limited, it appears that levels of all metabolites increased with time. The lower levels of AZTG (fetal/maternal ratio = 0.2) and AMT (fetal/maternal ratio = 0.5) observed in fetuses are probably due to a lesser degree of fetal metabolism relative to the adult [9], although placental transfer of maternal metabolites cannot be excluded based on small amounts of AZTG that were observed to perfuse through human placenta in vitro [10]. The observation that AZTP levels in the fetus are not significantly different from dams (fetal/maternal ratio = 1) suggests that thymidine kinase activity is present in the fetus, although again placental transfer cannot be excluded. The presence of 3TCP in fetal plasma (fetal/maternal ratio = 0.4), although at levels much lower than AZTP, is also indicative of either fetal phosphorylation or maternal transfer. The observation of phosphorylated metabolites of AZT and 3TC in the fetus, regardless of the source, is significant because this metabolic conversion is a prerequisite for both genotoxicity (incorporation into fetal DNA) and efficacy (inhibition of viral replication) [11] and may be of predictive value.

The data presented here are consistent with previous studies that have measured either parent nucleosides [12] or nucleotides [8] in mice and humans [13] although the doses of AZT and 3TC and the resultant blood levels are significantly higher in this study due to its focus on potential toxicity. The set of analytical methods developed here permits measurement of parent nucleosides and several toxicologically important metabolites in maternal and fetal compartments. This capability will be useful in future studies on metabolism, disposition, and genotoxicity of combinations of anti-retroviral drugs in mice from transplacental and neonatal exposures.

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