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Analysis of 3′-azido-3′-deoxythymidine levels in tissues and milk by isocratic high-performance liquid chromatography

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SUMMARY

A sensitive high-performance liquid chromatographic (HPLC) assay was established to analyze levels of the antiretroviral agent 3′-azido-3′-deoxythymidine (AZT, zidovudine) in serum, milk and tissue extracts. After methanol precipitation, serum samples could be injected directly into the HPLC apparatus, whereas tissue extracts required further clarification. Recovery of AZT was virtually complete. Isocratic elution with a mobile phase consisting of 6% acetonitrile and 0.1 *M* ammonium acetate, pH adjusted to 4.5 with glacial acetic acid, resulted in good resolution of AZT and its metabolites; retention times for AZT and the internal standard, *p*-nitrophenol, were 20 and 37 min, respectively. Using this method, we have demonstrated that AZT crosses both the blood–brain and placental barriers and is excreted into milk at high levels.

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INTRODUCTION

Maternal transmission of the human immunodeficiency virus type 1 (HIV-1) [1–3], the causative agent of the acquired immunodeficiency syndrome (AIDS), is a growing problem worldwide. In January 1988, 1/61 newborn infants in the New York City area carried antibodies to HIV-1 [4]. About 30–50% of these infants are truly infected [5]. The World Health Organization predicts that by 1992, about 250 000 infants will develop AIDS due to perinatal infection in Africa [6]. Presently, no prophylactic therapy is available to prevent infection of newborn infants.

The nucleoside analogue 3′-azido-3′-deoxythymidine (AZT, zidovudine) [7–10] is thus far the only federally approved drug for treatment of AIDS and the AIDS-related complex (ARC). We have tested the toxicity and efficacy of transplacental AZT therapy in several murine systems [11–13]. Our data indicate that AZT administered to pregnant mice beginning at midgestation and continuing throughout lactation results in significant survival benefits for offspring infected with the neurotropic retrovirus Cas-Br-E [11]. We observed no significant embryo toxicity. Likewise, oral AZT therapy given to lactating mice resulted in prolonged survival of pups infected in the neonatal period with the same virus. Similar results were obtained in transgenic Mov mice that express the T-cell tropic Moloney Murine Leukemia Virus (Mo-MuLV) during late fetal stages or in the neonatal period [12]. In a third series of experiments in chronically viremic female BALB/c mice, known to pass Mo-MuLV to their pups via the milk, oral AZT therapy during gestation and lactation prevented viremia in 75% of their offspring [13].

Methods for determining AZT levels in serum or spinal fluid have been published earlier [14–18]. Here we present our data on a fast, convenient method using isocratic high-performance liquid chromatography (HPLC) to measure AZT levels in body fluid as well as in tissues. We have employed this method successfully to measure AZT levels in fetuses of pregnant mice given AZT during gestation and in the milk of lactating mice treated with AZT. We demonstrate that this method can be applied to measure AZT levels in tissue extracts reproducibly.

EXPERIMENTAL

Materials

AZT was a gift of Dr. Sandra Nusinoff-Lehrman (Burroughs Wellcome, Research Triangle Park, NC, U.S.A.). The AZT derivatives (AZT monophosphate, AZT diphosphate, AZT triphosphate and 3′-azido-3′-deoxy-5′-β-D-glucopyranuronosylthymidine, the 5′-O-glucuronide of AZT) were a gift of Dr. Steven Good (Burroughs Wellcome). The internal standard, *p*-nitrophenol, was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.). All

other chemicals and solvents were of analytical-reagent grade and were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Animals

BALB/c, 129/J and SWR/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, U.S.A.). All animals were held in quarantine at least one week prior to initiating any experimental procedures. The origin of the transgenic Mov 9 and Mov 18 mice was described earlier [12]. The animals were mated and bred in the mouse colony at the Whitehead Institute.

Apparatus

Chromatographic separation and peak detection of AZT and of the internal standard, *p*-nitrophenol, was carried out on a Waters Assoc. HPLC system, which consisted of a Model 440 fixed-wavelength detector at 280 nm set at a sensitivity of 0.01 absorbance units full scale (a.u.f.s.), a Model 6000A pump, a U6K injector and a radial compression module 100 equipped with a 10 cm × 5 mm I.D. analytical column with a 5 μm particle size (Radial-Pak NovA C_{18} cartridge) and a C_{18} Guard-Pak precolumn insert, which is a disposable plastic insert prepacked with C_{18} with a 10 μm particle size. This insert needs to be used with a modified inlet connector or with a Guard-Pak precolumn module, both available from Waters Assoc.

The mobile phase consisted of 6% acetonitrile and 0.1 *M* ammonium acetate, pH adjusted to 4.5 with glacial acetic acid. The solution was degassed using a Millipore solvent clarification kit. The flow-rate was 1.0 ml/min.

Animal procedures

For experiments involving the transgenic Mov 9 and Mov 18 mice, Mov strain males were mated with uninfected females of the identical genetic background, i.e. 129J mice, to avoid passage of virus from a viremic mother to the offspring in the perinatal period. Viremic BALB/c female mice were generated by intraperitoneal (i.p.) inoculation with approximately 0.05 ml of Mo-MuLV with a titer of 10^6 plaque-forming units (pfu) per ml within the first 24 h after birth. Mo-MuLV was prepared and titers were determined by XC plaque assay as described previously [19]. The mice were tested for viremia by radioimmunoassay for the viral p30 protein at one month of age [20], and viremic female mice were mated with uninfected BALB/c males, beginning at five to six weeks of age.

Oral administration of AZT was carried out by dissolving the drug in the drinking water according to our earlier protocols [11–13]. For oral therapy of pregnant mice, AZT was dissolved in the drinking water at the concentrations indicated in the text. The animals were given AZT for several days prior to the collection of blood samples and sacrifice. The embryos were dissected immediately and stored at −70 °C prior to analysis. Intraperitoneal injections of

timed-pregnant or lactating females were given at 200 mg/kg AZT, and serum, milk, embryos or maternal organ samples were collected at the time points indicated and stored at −70 °C until analysis.

To examine the AZT concentration in the blood and milk of lactating females, the females were preconditioned between postnatal days 15 and 22 for milking by removal from their suckling young overnight. Mice were injected i.p. with AZT (200 mg/kg), and blood and milk were collected at 30 min, 1 h and 2 h thereafter. At 10–20 min prior to milking, the mice were injected i.p. with 0.4 units of oxytocin (Sigma, St. Louis, MO, U.S.A.) to provide stimulation for milk production. Milk was collected with a device built according to the specifications of McBurney et al. [21]. Milk was collected, quick frozen on dry ice, and stored at −70 °C until analysis. Mice were bled from the retro-orbital plexus and serum was collected and frozen at −70 °C until analysis.

Extraction with trichloroacetic acid

The internal standard *p*-nitrophenol was added to the weighed tissue samples to give an injection concentration of $5 \cdot 10^{-4}$ *M* (150 μl of $1 \cdot 10^{-3}$ *M*). The tissue samples were then homogenized with 10 ml of water per gram. A saturated solution of trichloroacetic acid (TCA) was added to yield a concentration of 10%. The sample was thoroughly mixed and incubated at room temperature for 30 min. Subsequently, the homogenate was centrifuged for 15 min using an IEC centrifuge at 4 °C, and the supernatant was removed and saved. The pellet was resuspended in 1.0 ml of 10% TCA and incubated for 5 min. After a repeat centrifugation, the supernatants were pooled. The 1.0 ml of 10% TCA wash was repeated two more times. The pH of the combined supernatants was adjusted to 7.0 with 5 *M* sodium hydroxide. The solution was then loaded onto a C_{18} Sep-Pak (Waters Assoc.) which was prepared by washing first with 2.0 ml of methanol, followed by one washing with 5.0 ml of water and two washes with 5.0 ml of buffer consisting of 4% acetonitrile and 0.05 *M* ammonium acetate, pH adjusted to 4.5 with glacial acetic acid. After the sample was loaded onto the C_{18} Sep-Pak, it was washed with 5.0 ml water, followed by three 5.0-ml aliquots of the above buffer. The sample was then eluted from the C_{18} Sep-Pak with 2.0 ml methanol and dried on the Speed-Vac evaporator with gentle heating. The residue was dissolved in 150 μl of the HPLC mobile phase. The injection volumes ranged between 5 and 25 μl.

The standard curves for each tissue were obtained by adding known amounts of AZT to the serum and tissue samples and using the extraction method described above. Care was taken to obtain standard curves within the linear range, in general within one log range of the AZT concentration of unknown samples. The retention time for AZT was 20 min and for the internal standard (*p*-nitrophenol) 37 min.

Methanol extraction method

In a second approach for extracting AZT from tissues, two volumes of methanol were added to the serum of tissue homogenates, assuming 1.0 g of tissue equals 1.0 ml. The samples were mixed well and incubated at 4°C for 1 h, followed by centrifugation in a Fisher Model 235B micro-centrifuge at 4°C. At this point, serum supernatants could be injected directly into the HPLC apparatus. In contrast, tissue supernatants were transferred to a new tube, centrifuged again to remove residual debris and injected. The internal standard, *p*-nitrophenol, was added just prior to injection because of the complete recovery of AZT from the samples as well as the need to analyze samples containing unknown concentrations over a wide range [22]. The injection volumes ranged from 10 to 50 μl. Standard curves were prepared by adding known amounts of AZT to serum and tissue samples and subjecting them to the extraction procedure described above.

Determination of AZT levels in milk

AZT was extracted from mouse milk by precipitating the weighed sample of milk (the mouse milk was thick and sometimes curd-like) with four volumes of methanol, assuming 1 g of milk equals 1 ml. The samples were worked up as described for the methanol method. The internal standard, *p*-nitrophenol, was added just before injection. Human milk was spiked with known amounts of AZT and precipitated with two volumes of methanol. Recovery of AZT from both mouse and human milk was determined by adding known amounts of AZT to the milk samples and working them up by methanol precipitation. The resulting peak areas were compared to those obtained when a standard amount of AZT was added to a volume of water equal to the amount of milk and either four or two volumes of methanol added for mouse or human milk, respectively.

Data analysis

The recovery of AZT from serum and tissue samples was analyzed by adding known amounts of AZT to either serum or weighed samples of tissue and using the extraction procedures described above. The resulting peak areas were compared to those obtained from a standard amount of AZT added directly to the mobile phase. To generate standard curves, the results were subjected to linear regression analysis using a VAX-11/750 computer according to the equation $y=ax+c$, where y stood for the peak-height ratio of AZT to *p*-nitrophenol, a for the slope, x for the known concentration of AZT and c for the $y=$intercept.

RESULTS

Recovery of AZT from tissue extracts

The recovery of AZT from serum, brain, embryonic tissues and milk is summarized in Table I. While both the TCA and the methanol precipitation meth-

TABLE I

RECOVERY STUDIES

Samples obtained from brain, embryos, serum or milk were spiked with known amounts of AZT and subjected to the extraction procedures according to the protocols outlined in Experimental. The percentage of AZT recovered as compared to the amount added is given, together with the coefficient of variation. The data represent the mean of three independent experiments, with the exception of results marked with an asterisk which represent the mean of three repeated chromatograms of the same sample.

Method	Source	Concentration (μg/g)	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)
TCA	Mouse brain	2	41.8 ± 0.2	0.5
		5	53.7 ± 0.1	0.2
	Mouse embryo	2	33.1 ± 3.1*	9.4
		5	40.7 ± 2.1*	5.2
Methanol	Mouse brain	2	111.0 ± 6.1	5.5
		5	91.9 ± 6.3	6.9
	Mouse embryo	2	95.2 ± 0.4	0.5
		5	117.4 ± 2.5	2.1
	Mouse serum	2 μg/ml	103.0 ± 6.6	6.4
		50 μg/ml	95.9 ± 5.6	5.9
	Mouse milk	2 μg/ml	101.4 ± 1.8*	1.8
		100 μg/ml	105.1 ± 0.2*	0.2
	Human milk	2 μg/ml	96.0 ± 0.2	0.2
		50 μg/ml	97.0 ± 0.2	0.2

ods yielded reproducible recovery rates, methanol precipitation was clearly superior, as indicated by the near complete recovery from all samples tested. Thus, all subsequent analyses were carried out using the methanol precipitation method, unless noted otherwise. In addition, the methanol precipitation method proved to be faster and more cost-effective. For both methods, the lower limit of detection was 0.15 μg of AZT per gram of tissue.

Chromatograms

The retention times of AZT and the internal standard, *p*-nitrophenol, were 20 and 37 min, respectively (Fig. 1A–F). The use of *p*-nitrophenol as an internal standard may be somewhat surprising given the structural differences between AZT and *p*-nitrophenol as well as the long retention time. However, our aim was to find a system allowing analysis of tissue extracts under isocratic conditions, and *p*-nitrophenol was the only compound that yielded excellent, reproducible separation from both AZT and interfering peaks after a search involving several other compounds. Additionally, the separation time of 37 min allowed the column to be freed of tissue-derived impurities. After the methanol

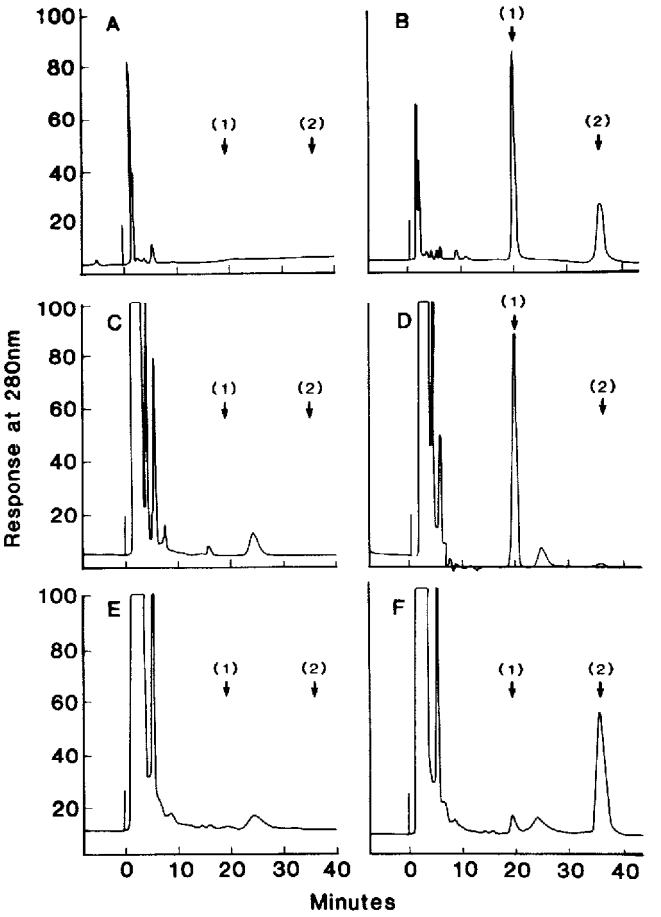

Fig. 1. Representative chromatograms of mouse serum, brain and embryo extracts obtained by the methanol precipitation method. HPLC analysis of (A) blank mouse serum, (B) serum from a pregnant mouse 30 min after receiving 200 mg/kg AZT i.p. and *p*-nitrophenol added as internal standard at 500 μM, (C) blank brain sample, (D) brain sample from a pregnant mouse 30 min after receiving 200 mg/kg AZT i.p. and *p*-nitrophenol added as internal standard at 100 μM, (E) blank embryo sample and (F) experimental embryo sample containing 1.5 μg/g AZT and *p*-nitrophenol added as internal standard at 50 μM. Peaks: 1 = AZT; 2 = *p*-nitrophenol.

precipitation method was applied to serum and tissue samples, no materials co-eluted with either AZT or *p*-nitrophenol in mouse serum (Fig. 1A), mouse brain (Fig. 1C), mouse embryos (Fig. 1E) and human serum (Fig. 2A). The corresponding chromatograms of AZT (peak 1) and the internal standard *p*-nitrophenol (peak 2) extracted from the various tissues are shown in Figs. 1B, D, F and 2B, respectively. For comparison, chromatograms showing AZT and the internal standard in the running buffer only are shown in Fig. 2. Chromatography profiles of samples extracted by the TCA precipitation method were

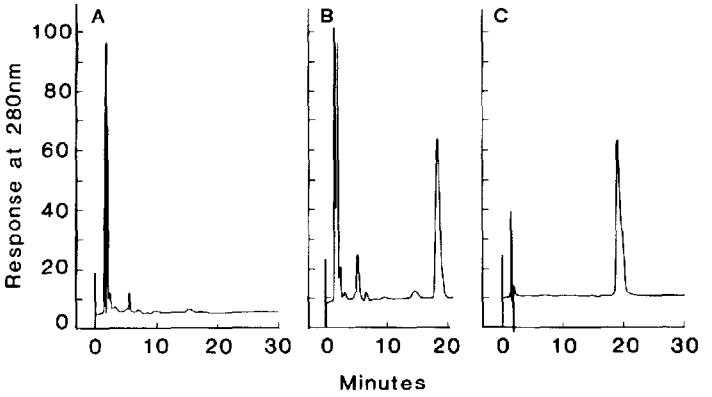

Fig. 2. Chromatograms of spiked human serum. HPLC analysis of (A) human serum alone, (B) a spiked human serum sample containing 50 μg/ml AZT, and (C) HPLC mobile phase spiked with AZT at 50 μg/ml. The samples were prepared according to the methanol precipitation method.

similar to the ones extracted by the methanol method; no other materials co-eluted with the AZT or *p*-nitrophenol peaks, respectively (data not shown).

Our chromatography protocol was able to separate AZT and the internal standard, *p*-nitrophenol, from the known metabolites AZT mono-, di-, and triphosphate as well as 5′-O-glucuronide of AZT. Thus, the retention time for AZT monophosphate was 4.5 min, for AZT diphosphate 1.8 min, for AZT triphosphate 3.0 min, for the 5′-O-glucuronide of AZT 5.25 min, for AZT itself 19.25 min and for *p*-nitrophenol 34.75 min, when the compounds were run in mobile phase (data not shown).

Standard curves and reproducibility

Separate standard curves were generated for each set of extracts derived from tissues or body fluids. In general, unknown samples were analyzed first for peak-height ratios, and standard curves were obtained within one log range. The standard curves were subjected to linear regression analysis, which yielded *r* values ranging from 0.986 to 0.999 for the TCA method, and 0.984 to 1.000 for the methanol precipitation method. The lower limit of detection was judged to be 0.15 μg/g for tissue extracts as well as serum or milk derived by the TCA or methanol precipitation methods.

AZT levels in maternal brain and embryonic tissues

Next, we analyzed the passage of AZT through the placental or blood–brain barriers. Random samples of serum and tissues were collected from timed-pregnant mice receiving AZT therapy which was administered orally via the drinking water. The AZT levels of the fetuses and maternal sera are shown in Table II. It should be noted that the experimental animals consumed the AZT solution ad libitum, and, therefore, the time of the last AZT intake prior to

TABLE II

AZT LEVELS IN EMBRYOS AND SERUM OF PREGNANT MICE TREATED WITH ORAL AZT

Timed-pregnant SWR/J mice were given AZT at 0.2 or 1.0 mg/ml via the drinking water. Serum samples were collected from the pregnant females under anaesthesia, and the embryos were removed after sacrifice. The samples were extracted as indicated in the Experimental section Individual standard curves were run for sera as well as for embryo extracts.

Tissue	AZT concentration (mg/ml)	AZT tissue level (μg/g)	Method of analysis
Embryo	0.2	0.23	TCA
	1.0	4.32	TCA
	1.0	3.77	TCA
Mother's serum	0.2	<0.15	Methanol
	1.0	0.19	Methanol

TABLE III

AZT LEVELS IN EMBRYOS, SERUM AND TISSUES OF PREGNANT MOV MICE GIVEN 200 mg/kg AZT

Thirty min after AZT injection, serum samples were collected from the pregnant females under anaesthesia, and the animals were sacrified. The AZT was extracted according to the methanol precipitation method. Serum levels were converted to μg/g.

Strain	Maternal tissue	Embryos (n)	AZT level (μg/g)	Percentage of maternal serum
Mov 9	Serum		159.5	-
	Brain		18.9	11.8
	Spleen		125.4	78.6
	Liver		128.6	80.6
	-	3	89.8 ± 20	56.3 ± 12.7
Mov 18	Serum		161.5	-
	Brain		19.3	12.0
	Spleen		126.7	78.5
	Liver		149.0	92.3
	-	10	85.1 ± 30.2	52.7 ± 18.7

sample collection was unknown. This explains the high degree of fluctuation of AZT levels, which is not unexpected considering the short half life of the drug.

The analysis was repeated using a high dose of AZT given as a one-time intraperitoneal injection to the timed-pregnant females at time 0; 30 min later, serum samples were collected from the pregnant females, all animals were sacrificed, and their tissues were quick-frozen. The AZT levels in various mater-

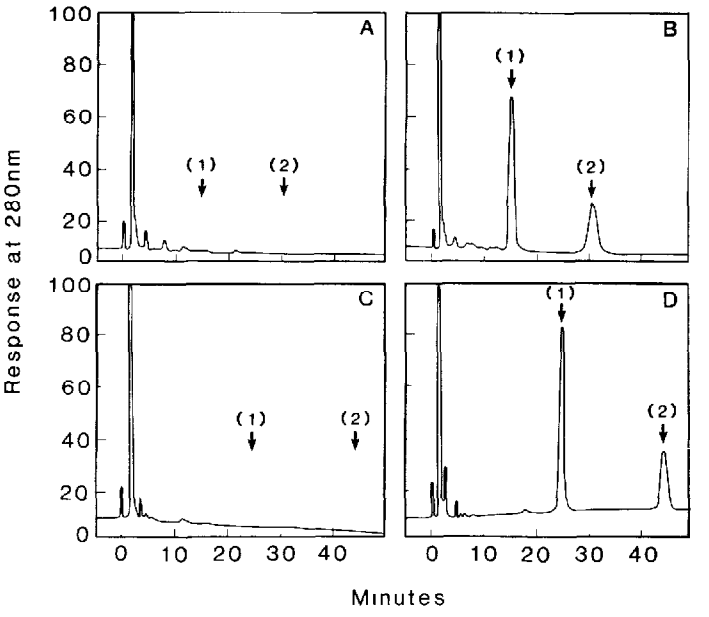

Fig. 3. Chromatograms of mouse and human milk extracts. HPLC analysis of (A) blank mouse milk, (B) an experimental mouse milk sample from a mouse 30 min after receiving a 200 mg/kg intraperitoneal injection of AZT and *p*-nitrophenol added as internal standard at 400 μM, (C) blank human milk and (D) a spiked human milk sample containing 50 μg/ml AZT and *p*-nitrophenol at 100 μM. Peaks: 1 = AZT; 2 = *p*-nitrophenol. All samples were prepared by the methanol precipitation method.

TABLE IV

AZT LEVELS IN SERUM AND MILK OF BALB/c MICE GIVEN 200 mg/kg AZT

Serum and milk samples were collected simultaneously from one lactating mouse as described in the Experimental Section per time point indicated following the i.p. administration of AZT The AZT was extracted according to the methanol precipitation method. Separate standard curves were generated for milk and serum samples.

Time after AZT (min)	Serum (μg/ml)	Milk (μg/ml)	Percentage of serum
30	58.5	324.5	555
60	26.1	78.4	300
120	5.6	8.5	152

nal organs as well as in fetal tissues are given in Table III. High levels of AZT were detected in liver and spleen, whereas the tissue level of AZT in maternal brain was only 12% of the corresponding serum level at 30 min post-injection. AZT penetrated the placental barrier effectively, as demonstrated by the fetal tissue levels which had reached over 50% of the maternal serum levels. It should

also be noted that lower doses of AZT (0.1–0.4 mg/ml AZT in drinking water), administered to timed-pregnant mice carrying fetuses exposed to the neurotropic retrovirus Cas-Br-E, showed highly significant therapeutic benefits and resulted neither in increased fetal loss nor in congenital malformations [11]. In these studies, a total of 23 pregnant female mice were subjected to AZT therapy starting at midgestation, and a total of 139 offspring were born without signs of embryo toxicity. In a parallel series of experiments, pregnant transgenic Mov mice whose fetuses begin to express the T-cell tropic Mo-MuLV during gestation, were treated with AZT throughout the later part of pregnancy and throughout the period of lactation. Again, highly significant survival gains were observed in the AZT-treated offspring without evidence of teratogenicity or toxicity during early post-natal development [12].

AZT levels in mouse and human milk

Using the methanol precipitation method, AZT could be recovered at virtually 100% from mouse as well as human milk. Again, the chromatograms showed no materials co-eluting with either AZT or with the internal standard, *p*-nitrophenol (Fig. 3). In Table IV, the kinetics of AZT clearance from the serum and milk are shown. Lactating females were injected at time 0 with 200 mg/kg AZT, and at 30, 60 or 120 min post-injection, simultaneous serum and milk samples were obtained. Of note is that milk levels declined much more slowly than serum levels; up to five times higher concentrations are noted in milk as compared to the serum (Table IV). In our previous experiments, oral AZT therapy of lactating mothers showed significant therapeutic benefits in mice infected as neonates with the neurotropic retrovirus Cas-Br-E [11] and in transgenic mice that begin to express endogenous proviral sequences of Mo-MuLV in the early post-natal days [12]. The high AZT levels of maternal milk explain the clinical benefit we had seen in our earlier studies [11–13].

DISCUSSION

After methanol extraction of body fluids or tissue homogenates, we have demonstrated that AZT levels can be measured by isocratic HPLC analysis in a mobile phase buffered to pH 4.5, which results in an excellent separation of AZT from its metabolites as well as from the internal standard, *p*-nitrophenol. Because tissue extracts were used as test material in contrast to serum or cerebrospinal fluid, our limit of detectability was approximately five times higher as compared to the two previously published methods for HPLC analysis of serum or CSF [14,15]. Additionally, our analysis was carried out with a fixed-wavelength detector set at 280 nm, rather than the variable-wavelength detector set at the absorption maximum of AZT, which lies at about 267 nm. Our fast, convenient method of analysis can detect AZT tissue levels that can be achieved during therapeutic trials in vivo, as our data indicate [11–13].

We have applied this method to study the passage of AZT across the placental or blood–brain barriers, and we have measured simultaneous AZT levels in serum and milk. Thus, this method proved to be a valuable tool for determining pharmacokinetic data for our pre-clinical studies on the therapeutic efficacy and toxicity of perinatal AZT therapy [11–13].

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