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Determination of 3′-amino-3′-deoxythymidine, a cytotoxic metabolite of 3′-azido-3′-deoxythymidine, in human plasma by ion-pair high-performance liquid chromatography

D.M. Burger*, H. Rosing and F.J. Koopman

Department of Pharmacy, Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam (Netherlands)

P.L. Meenhorst and J.W. Mulder

Department of Internal Medicine, Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam (Netherlands)

A. Bult

Department of Pharmaceutical Analysis, Utrecht University, P.O. Box 80082, 3508 TB Utrecht (Netherlands)

J.H. Beijnen

Department of Pharmacy, Slotervaart Hospital/Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam (Netherlands)

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ABSTRACT

A sensitive high-performance liquid chromatographic assay has been developed to determine the levels of 3′-amino-3′-deoxythymidine (AMT), a cytotoxic metabolite of 3′-azido-3′-deoxy-thymidine (AZT, zidovudine), in human plasma. The sample pretreatment involved solid-phase extraction using cation-exchange extraction columns. Chromatography was carried out on a C_8 column, using a mobile phase of methanol–0.01 *M* ammonium acetate (pH 5)–0.25 *M* sodium dioctylsulfosuccinate (60:40:4, v/v/v) and ultraviolet detection at 265 nm. The method has been validated, and stability tests under various conditions have been performed. The lower limit of quantitation is 5 ng/ml (using 500-μl human plasma samples). The bioanalytical assay has been used for the determination of AMT in patients with AIDS who used AZT.

INTRODUCTION

3′-Amino-3′-deoxythymidine (AMT, Fig. 1) was first synthesized by Miller and Fox in 1964 [1]. In the early 80's the group of Prusoff demonstrated that this nucleoside analogue had potent antineoplastic activity [2–4], but, as far as we know, AMT has never been evaluated in clinical trials.

In 1991, interest for AMT rekindled as it was discovered that AMT is a catabolic metabolite

* Corresponding author.

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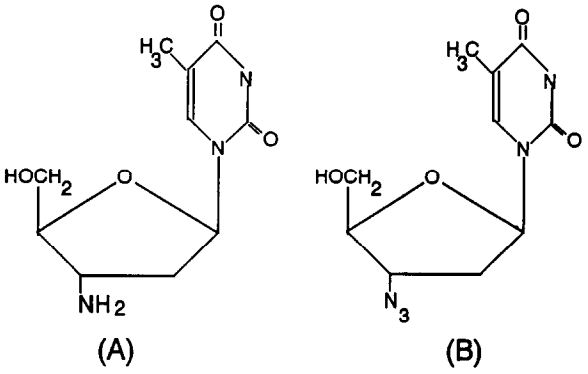

Fig. 1. Structures of 3'-amino-3'deoxythymidine (AMT; A) and 3'-azido-3'-deoxythymidine (AZT; B).

of the antiretroviral agent 3'-azido-3'-deoxythymidine (AZT, zidovudine, Retrovir; Fig. 1). Formation of AMT by reduction of the azido-function of AZT was demonstrated in rat hepatocytes and liver microsomes [5,6], in rhesus monkeys [7], and, finally, in humans [8].

Determination of AMT in human plasma may be important as AMT appears to be five- to sevenfold more cytotoxic to human bone marrow progenitor cells than AZT [5]. Administration of AZT to patients infected with the human immunodeficiency virus (HIV) often leads to bone marrow suppression [9], and a role for AMT within this respect has been suggested [5].

Cretton and co-workers [5–7] and Stagg *et al.* [8] determined AMT in plasma by reversed-phase high-performance liquid chromatography (HPLC) and radiochemical detection. This procedure, however, is only useful after intravenous administration of radiolabeled AZT, which makes the method less appropriate for clinical pharmacokinetic studies.

We have developed an HPLC method for the determination of AMT in human plasma using solid-phase extraction with cation-exchange columns as a highly selective pretreatment procedure, followed by ion-pair chromatography and ultraviolet detection. The usefulness of the method was subsequently tested by the determination of AMT in plasma samples from patients with the acquired immunodeficiency syndrome (AIDS) who used AZT.

EXPERIMENTAL

Equipment

The HPLC system consisted of a Model 510 pump (Waters, Milford, MA, USA), a Spectra 100 programmable wavelength detector (Spectra-Physics, Santa Clara, CA, USA), a Model 8880 automatic sample injection device (Spectra-Physics) and a Model 4270 integrator (Spectra-Physics). The analytical columns used were a μBondapak Phenyl (300 × 3.9 mm I.D.; particle size 10 μm) (Waters) and an Apex Octyl column (150 × 4.6 mm I.D.; particle size 5 μm) (Jones Chromatography, Hengoed, Mid Glamorgan, UK), both protected by a home-packed guard column (Corasil; 24 × 9 mm I.D.; particle size 37–50 μm; Waters). Analytical runs were processed by the Autolab Software Winner 386 system (Spectra-Physics). UV spectra of AMT solutions in methanol were recorded with a SP8-400 UV/Vis spectrophotometer (Pye Unicam, Cambridge, UK).

Chemicals

3'-Amino-3'-deoxythymidine was purchased from Sigma (St. Louis, MO, USA). Methanol (Chromar) was purchased from Promochem (Wesel, Germany), potassium dihydrogenphosphate p.a., disodium hydrogenphosphate dihydrate p.a., and ammonium acetate p.a. from Merck (Darmstadt, Germany). Sodium dioctylsulfosuccinate and 25% ammonium hydroxide were purchased from Baker (Deventer, Netherlands). Deionized water was used throughout. Blank plasma was obtained from the Central Laboratory of Blood Transfusion Service (Amsterdam, Netherlands).

Drug solutions

Stock solutions of AMT (1 μg/μl) were prepared by dissolving the appropriate amount of the drug, accurately weighed, in 50% methanol. For the construction of calibration curves fresh solutions were used. Drug solutions for interference analysis were obtained from the hospital pharmacy, either as solutions for injections or after dissolving solid material (*e.g.* tablets, cap-

sules) in 50% methanol (concentration: 20–30 μg/ml in 50% methanol).

Sample preparation

For the preparation of the standard samples stock solutions of AMT were diluted with methanol. To achieve AMT calibration concentrations of 5–200 ng/ml appropriate quantities of the various diluted stock solutions were added to 2-ml Eppendorf tubes (Merck). The solutions were evaporated to dryness under a nitrogen stream at 60°C. Next, to each tube 500 μl of plasma were added and vortex-mixed for 60 s. Then, 500 μl of phosphate buffer pH 5 (composed of equal volumes of 0.1 *M* potassium dihydrogenphosphate (adjusted to pH 5) and 0.1 *M* disodium hydrogenphosphate dihydrate) were added and mixed on a vortex-mixer for 60 s. The tubes were then centrifuged for 90 s at 10 500 *g* and the diluted plasma samples were subjected to solid-phase extraction. Prior to this, silica gel (3 ml capacity; Bondelut, Analytichem International, Rotterdam, Netherlands), CN (500 mg, Merck) or SCX extraction columns (aromatic sulfonic acid, 500 mg; Bakerbond SPE, J.T. Baker, Philipsburg, NJ, USA) were placed on a vacuum elution manifold (Baker 10-SPE System), and rinsed with 5 × 1 ml of 2.5% ammonium hydroxide in methanol, followed by 1 min continuous vacuum suction. The flow-rate was maintained at 0.3 ml/min. Next, the columns were conditioned with 1 ml of methanol, and rinsed twice with 1 ml of 0.1 *M* phosphate buffer pH 5. Care was taken that the columns did not run dry. Next, 1-ml aliquots of the diluted plasma samples were transferred onto the columns and drawn into them by applying reduced pressure. Subsequently, the columns were washed with 2 ml of phosphate buffer pH 5, 2 ml of distilled water and 2 ml of methanol, and allowed to dry for 1 min under continuous vacuum suction. Elution of the absorbed analytes was performed with 1 ml of 2.5% ammonium hydroxide in methanol into Eppendorf tubes and evaporated to dryness under a gentle stream of nitrogen at 60°C. The residues were redissolved in 150 μl of mobile phase, mixed on a vortex-mixer for 30 s and centrifuged for 5 min at 10 500 *g*. The supernatants were transferred to autosampler vials with inserts.

Chromatography

Chromatographic analysis on Phenyl columns was performed with a mobile phase of 0.005 *M* phosphate buffer pH 5–methanol (90:10, v/v). When using the Octyl columns, the chromatographic analysis was performed with a mobile phase of methanol–0.01 *M* ammonium acetate pH 5–0.25 *M* sodium dioctylsulfosuccinate (DOSS) in water (60:40:4, v/v/v). Prior to use, the mobile phase was filtered under reduced pressure through a 0.2-μm cellulose-acetate filter (Sartorius, Spruyt-Hillen, Utrecht, Netherlands). The absorbance was monitored at 265 nm. Flow-rate was maintained at 1.0 ml/min. Aliquots of 100 μl were injected into the chromatograph.

Specificity and selectivity

The interference from endogenous components was investigated by the analysis of 6 different blank plasma samples in duplicate. The following substances were investigated for interference with the analytical method: acyclovir, AZT, AZT-glucuronide, didanosine, fluconazole, folic acid, ganciclovir, ketoconazole, methadon, oxazepam, ranitidine, sulphamethoxazole, trimethoprim, and zalcitabine.

Limit of detection and limit of quantitation

The lower limit of detection (LOD) was defined by the concentration at a signal-to-noise ratio of 3. At this concentration a significant difference between the spiked sample and the blank has to be observed (6 different blank plasma samples; paired student's *t*-test, $p < 0.05$).

The lower limit of quantitation (LOQ) was investigated in plasma samples from 6 different donors. Each sample was spiked to contain 1, 2, or 3 times the LOD concentration of AMT. For the concentration to be accepted as the lower LOQ, the percentage deviation of the nominal concentration (R.C.R.) and the relative standard deviation (R.S.D.) are to be less than 20%. The upper LOQ was arbitrarily defined as 1000 ng/ml, but this was subsequently diminished to 200

ng/ml as patient samples appeared to be lower than expected.

Accuracy, precision and recovery

Accuracy and precision of the method were determined by assaying 5 replicates of plasma samples with 3 different AMT concentrations (45, 75, and 175 ng/ml). Accuracy, between-day and within-day precision were determined on 3 subsequent occasions. An estimate of the between-day precision was obtained by ANOVA for each test concentration using the run day as the classification variable. The day mean square (DayMS), the error mean square (ErrMS), and the grand mean (GM) of the observed concentrations across run days were calculated using the NCSS package (Version 5.0, J.L. Hintze, East Kaysville, UT, USA, 1991). The between-day precision was calculated as

$$\frac{[(\text{DayMS} - \text{ErrMS})/n]^{0.5}}{\text{GM}} \cdot 100\%$$

where n is the number of replicates within each day. The estimate of the within-day precision was calculated as:

$$\frac{(\text{ErrMS})^{0.5}}{\text{GM}} \cdot 100\%$$

Recovery of AMT from the solid-phase extraction pre-treatment was determined and calculated by comparing the slope of a standard curve prepared in plasma with the slope of a curve for non-processed samples prepared in 50% methanol. The recovery was determined in three analytical runs.

Stability

Drug-free plasma samples were spiked with an aliquot of an AMT stock solution in methanol to give initial concentrations of 45, 75, and 175 ng/ml. These samples were stored for 1, 2, and 3 h at 60°C, and for 1 and 3 months at −30°C. After the storage period the samples were analyzed immediately.

The stability of AMT following one freeze-thaw cycle was investigated at AMT concentrations of 45, 75, and 175 ng/ml. After 1 h at −30°C, the samples were thawed and were analysed immediately.

Analysis of patient samples

Plasma from a patient with AIDS who received 2.5 mg/kg of AZT (Retrovir) intravenously (infusion time: 1 h) was analysed for AMT. Plasma from 7 HIV-infected patients who participated in a population-based pharmacokinetic study of AZT was also used for the determination of AMT after oral ingestion of AZT. Only samples at the time at which maximal concentrations of AZT were achieved (as determined before) were analysed for AMT.

RESULTS

Chromatography and detection

Chromatography was initially performed using a Phenyl analytical column in combination with a mobile phase of phosphate buffer pH 5–methanol. Several methods of sample pretreatment were investigated; however, in all cases the blank samples contained interfering substances around the retention time of AMT (7 min). In contrast, analysis on an Octyl column with DOSS as an ion-pairing agent added to the mobile phase resulted in the absence of interfering substances in blank plasma samples (retention time of AMT: 9 min).

For the determination of AMT, UV detection at 265 nm, the wavelength of maximal absorbance (molar absorptivity 7859 in methanol), was used.

Sample pretreatment

Solid-phase extraction was chosen as sample pretreatment since this procedure earlier had resulted in an effective clean-up of plasma samples used for the analysis of related compounds such as didanosine [10] and stavudine [11]. Among the tested extraction columns (silica, CN, and SCX), only the cation-exchange (SCX) columns yielded acceptable recoveries of AMT. The optimal pH during the solid phase extraction procedure was 5. At pH > 6 the recovery of AMT almost decreased to 0.

Experiments with 0.1 *M* ammonium acetate buffer and 0.1 *M* phosphate buffer, both at pH 5, revealed that the latter gave substantially less interference.

Based on these results, the validation of the

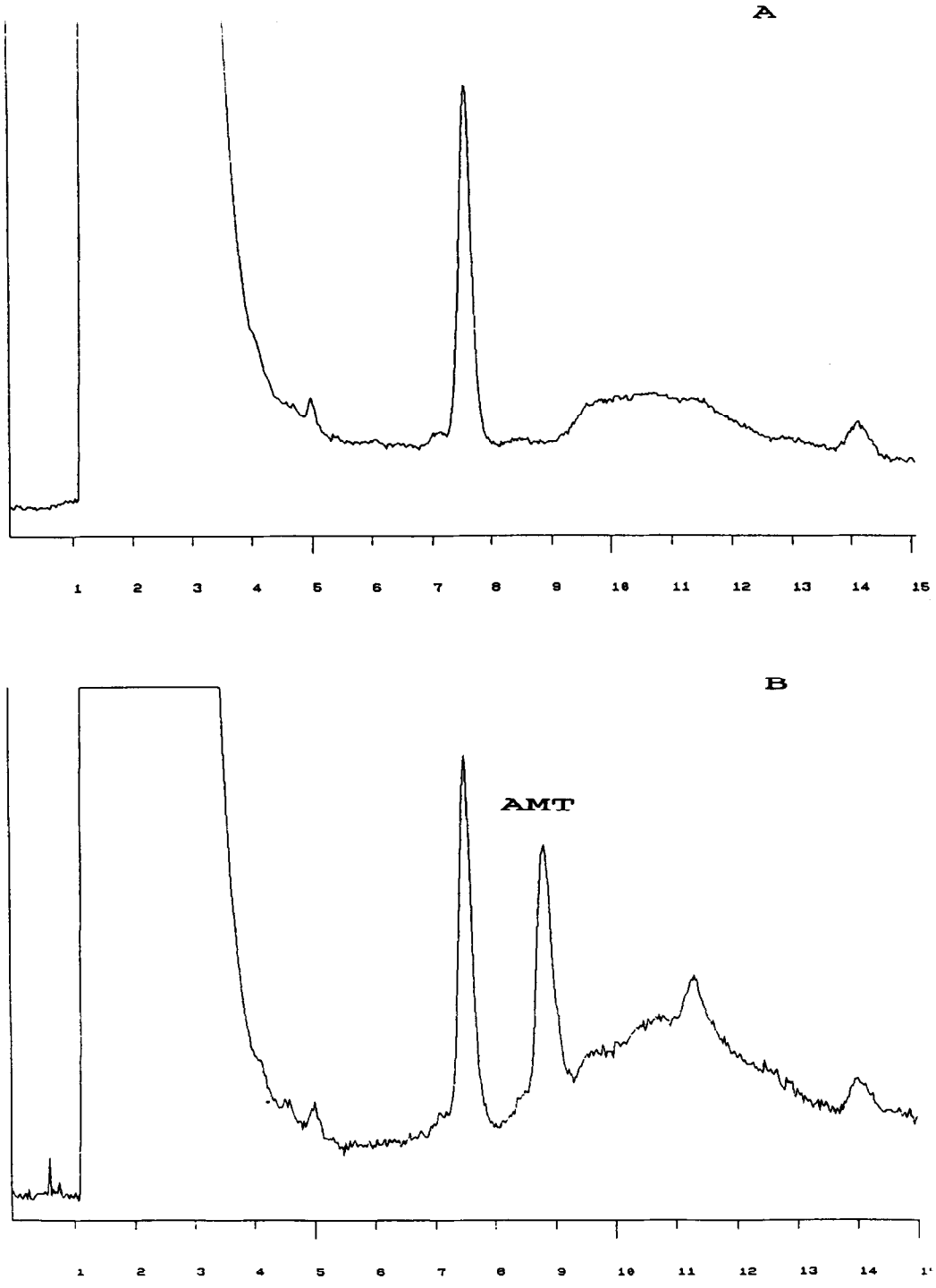

Fig. 2. Chromatograms of blank plasma sample (A) and a spiked plasma sample of 25 ng/ml AMT (B) (absolute amount injected onto the column: 8 ng). Chromatographic conditions: see text.

analytical method was performed using an Octyl column, a mobile phase containing DOSS, a sample pretreatment procedure consisting of solid-phase extraction with SCX extraction columns at pH 5, and a solvent consisting of 2.5% ammonium hydroxide in methanol.

Specificity and selectivity

Blank plasma samples from 6 different donors showed no substances interfering with the analysis of AMT (Fig. 2A). All potentially co-administered drugs or metabolites tested had retention times that were different from that of AMT. Trimethoprim had a retention time of 36 min, which necessitated a run time of 40 min for the analysis of patient samples.

Limit of detection and limit of quantitation

The LOD was determined to be 3.75 ng/ml. At this concentration the signal-to-noise ratio was 3. In addition, the response was significantly different from that of the blank plasma samples ($p < 0.05$). At 5 ng/ml both the R.S.D. and the R.C.R. were less than 20%. Thus, 5 ng/ml was estimated to be the lower LOQ. At the upper LOQ (200 ng/ml) both the R.S.D. and R.C.R. were also less than 20%. A typical chromatogram from analysis of a plasma sample with 25 ng/ml AMT (absolute amount 8 ng) is shown in Fig. 2B.

Validation: accuracy, precision, recovery and stability

The results from the validation of the method are listed in Table I. The use of the peak height in combination with a weighting factor of [1/(conc)^2] resulted in minimal deviation from the nominal concentrations. The method was shown to be accurate (average accuracy at 3 concentrations: 97–114%) and precise (between-day and within-day variation $\leq 15\%$). The recovery was estimated to be $79 \pm 5\%$. Slope values averaged 0.4939 ± 0.0746 [mm/(ng/ml)]. Correlation coefficients (r^2) were ≥ 0.995.

The results from stability studies (Table II) showed that AMT was stable at 60°C for at least 3 h, and at −30°C for at least three months. AMT appeared also to be stable after a freeze–thaw cycle.

Analysis of patient samples

Analysis of the plasma samples from a patient who received 2.5 mg/kg of AZT intravenously showed that maximal concentrations of AMT were achieved at the end of the infusion (16 ng/ml) and that they were below the detection limit at 3 h after cessation of infusion (Fig. 3). Analysis of samples from patients who had ingested AZT orally revealed that AMT levels ranged between 6–18 ng/ml (Table III).

DISCUSSION

We have developed a sensitive and validated analytical method for the determination of AMT in human plasma. The use of solid-phase extraction in combination with ion-pair chromatography resulted in a relatively low limit of detection.

The determination of AMT in human plasma

TABLE I

ACCURACY, PRECISION, AND RECOVERY FOR THE ANALYSIS OF AMT IN HUMAN PLASMA

Concentration (ng/ml)	Accuracy (%)	C.V. (%)	n^a	Precision (%)	
				Between-day	Within-day
45	109	11	15	11	11
75	114	12	15	4	11
175	97	14	15	15	6
Recovery (mean ± S.D.) (%) 79 ± 5%					

[a] n = number of replicates, divided over three runs.

TABLE II

STABILITY OF AMT IN HUMAN PLASMA

Temperature	Concentration (ng/ml)	n^a	Percentage of initial concentration (mean ± S.D.) at the indicated time		
			1 h	2 h	3 h
60°C	175	2	123 ± 4	114 ± 9	107 ± 8
			1 month	3 months	
−30°C	45	5	105 ± 21	83 ± 17	
	75	5	100 ± 7	91 ± 10	
	175	5	112 ± 11	85 ± 7	
			1 cycle		
Freeze–thaw	45	5	125 ± 6		
cycle	75	5	105 ± 10		
	175	5	100 ± 6		

[a] n = number of replicates.

has only been described by Stagg *et al.* [8]. They used reversed-phase high-performance liquid chromatography with radiochemical detection as they administered radiolabeled AZT intravenously to cancer patients. We have described a method for the determination of AMT in plasma from HIV-infected patients who use unlabeled AZT. Knowledge on the pharmacokinetics of AMT may extend the insight into a potential role of this metabolite in AZT-induced myelosuppression.

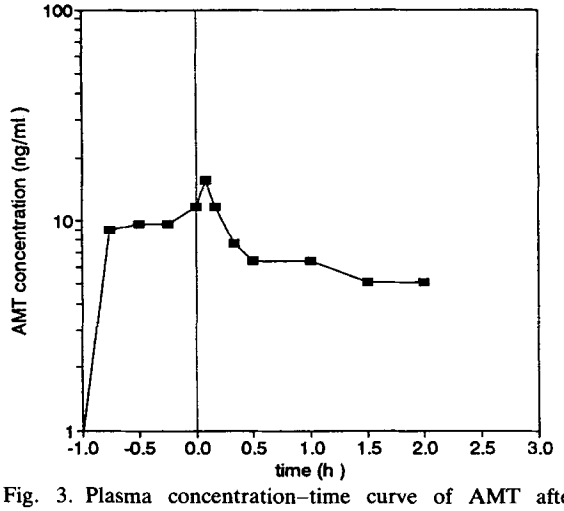

Fig. 3. Plasma concentration–time curve of AMT after intravenous administration of 2.5 mg/kg of AZT in a patient with AIDS (body weight 80 kg).

TABLE III

PLASMA CONCENTRATIONS OF AMT IN PATIENTS WITH AIDS

Patient code	AZT dose (mg)	Time[a] (min)	AMT concentration (ng/ml)
A	250	30	9
B	300	34	10
C	300	47	18
D	250	120	6
E	300	20	16
F	250	30	14
G	300	45	6

[a] Time after ingestion of AZT (Retrovir) capsules.

Compared with the results of Stagg *et al.* the concentrations of AMT we found in plasma from patients with AIDS were much lower. While Stagg *et al.* found AMT concentrations ranging from 40 to 160 ng/ml after intravenous injection of 2.5 mg/kg radiolabeled AZT [8], the maximum plasma concentration of AMT was 16 ng/ml in one of our patients who received the same intravenous dose of unlabeled AZT. In addition, plasma samples from patients who orally ingested 250–300 mg of AZT demonstrated comparable maximum concentrations (Table III).

Although we could not find an explanation for these differences, the observed concentrations may still be relevant. Significant cytotoxicity was

found *in vitro* at AMT concentrations ≥ 25 ng/ml (0.1 μM) [5]. Such concentrations were nearly reached in our patients. Furthermore, AMT has been shown to be a linear non-competitive inhibitor of HIV reverse transcriptase with an activity in the nanomolar range, thereby possibly interfering with AZT's anti-HIV activity [12].

CONCLUSION

We have described a method for the determination of AMT, a cytotoxic metabolite of AZT. The combined use of solid-phase extraction with SCX cation-exchange columns and ion-pair chromatography with DOSS as the ion-pairing agent resulted in a sensitive and validated procedure. Using this method we have demonstrated that AMT concentrations can be detected in patients with AIDS who orally ingested 250–300 mg of AZT.

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