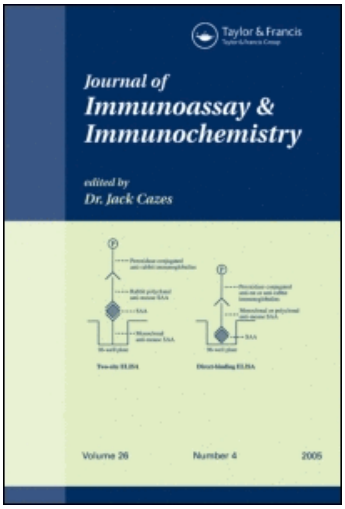

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Quantitation of 3'-Amino-3'-deoxythymidine (AMT), a Toxic Catabolite of 3'-Azido-3'-deoxythymidine (AZT) by competitive ELISA

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QUANTITATION OF 3'-AMINO-3'-DEOXYTHYMIDINE (AMT), A TOXIC CATABOLITE OF 3'-AZIDO-3'-DEOXYTHYMIDINE (AZT), BY COMPETITIVE ELISA

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ABSTRACT

In the present study, a competitive ELISA technique was developed to specifically quantitate 3'-amino-3'-deoxythymidine (AMT), a toxic catabolite of 3'-azido-3'-deoxythymidine (AZT) detected in serum from AZT-treated

patients. In order to eliminate cross-reacting AZT, serum sample was extracted with ethylacetate and then AMT was acetylated (Ac-AMT). A 5'-hemisuccinate-AMT-horseradish peroxidase conjugate was used as a tracer in the presence of anti-AMT rabbit antibodies which were raised against a 5' hemisuccinate-AMT-bovine serum albumin immunogen. Bound/free separation was achieved with an anti-rabbit IgG mouse monoclonal antibody insolubilized onto a microtiter plate. The limit of quantification of Ac-AMT was as low as 0.4 ng/ml in serum samples. This ELISA technique was applied for monitoring AMT plasma levels in patients receiving AZT therapy. The intra and inter-individual variations of the AZT/AMT plasma concentration ratios underlined the need for such a specific test in studying the formation of this toxic catabolite.

(Key words: ELISA, AMT, AZT, 3'-amino-3'-deoxythymidine, 3'-azido-3'-deoxythymidine, High Pressure Liquid Chromatography).

INTRODUCTION

Among the antiviral nucleoside analogues currently used in the treatment of AIDS, 3'-azido-3'-deoxythymidine (AZT) still remains the first line therapy. Although patients receiving AZT therapy exhibit a decreased number of opportunistic infections, (1) and a partial improvement in neurologic manifestations, (2) clinical benefits of AZT have been limited by toxic side effects, including neutropenia (3) and anemia (4) possibly leading to blood transfusions. AZT is rapidly and extensively converted in the liver to a 5'-O-glucuronide derivative (GAZT) which is mostly excreted into urine, and to a lesser extent to 3'-amino-3'-deoxythymidine (AMT, M.W. 241) through a microsomal reduction involving cytochrome P 450 (5;6). Substantial plasma levels of AMT ranging from 90 to 230 ng/ml have

been detected in humans (7), after intravenous administration of tritiated AZT. In that study, a glucuronide derivative of AMT (GAMT) was also detected in bile. The kinetic profile of AMT has also been established in rhesus monkeys after subcutaneous administration of radio-labeled AZT (8). AMT was demonstrated in vitro to be at least five to seven-fold more toxic for colony-forming units granulocyte-macrophage and burst-forming unit erythroid than AZT (9), suggesting that this metabolite may play a role in the AZT-induced myelosuppression occurring in patients receiving AZT therapy. Therefore, there is a need to characterize the in vivo formation of AMT in patients with assessment of its pharmacokinetic parameters which should in turn help in determining its role in the development of the AZT-induced myleosuppression. Recently, a high pressure liquid chromatography (HPLC) technique was reported which involves, after extraction of AMT, a derivatization with the fluorescent compound, fluorescamine, in order to allow a limit of quantification below 3 ng/ml (10). In the present study, using rabbit anti-AMT antibodies, a horseradish peroxidase-AMT conjugate as a tracer and a mouse monoclonal anti-rabbit IgG, insolubilized onto microtiter plate as a separator system, a specific and sensitive competitive ELISA method was developed for assaying AMT in serum. Cross-reacting AZT was eliminated by extraction of serum samples with ethyl acetate and the remaining AMT was acetylated to improve both the specificity and sensitivity of the assay. This method was applied to the measurement of AMT in serum obtained from HIV-seropositive patients treated with AZT. The lack of correlation between AZT and AMT serum levels confirmed

recent investigations (11) and underlines the usefulness of an AMT-ELISA test to monitoring the in vivo formation of this toxic catabolite.

MATERIALS AND METHODS

Flat-bottom Imulon 4 microtiter plates and plastic sealers were purchased from Dynatech, Dekendorf (Germany) and monoclonal mouse anti-rabbit IgG was supplied by Dr. J. Grassi (Service de Pharmacologie et d'Immunologie, CEN Saclay Gif/Yvette, France). Bovine serum albumin (BSA) fraction V and horseradish peroxidase (HRP) grade I 250 U/mg were obtained from Boehringer Mannheim, France, Grenoble (France). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), o-phenylene dihydrochloride (OPD), hydrogen peroxide, and acetic anhydride were purchased from Sigma, l'Isle d'Abeau Chesnes (France). All other reagents were of analytical grade. AZT was purchased from Sigma (St. Louis, MO, U.S.A.)

Synthesis of AMT 5'-OH Hemisuccinate Derivative

In order to prevent succinylation of the 3'-amino group, AZT was firstly converted to 5'-OH-hemisuccinate-AZT (AZT-HS) which was then reduced to the 3'-amino derivative (AMT-HS). Briefly, 267 mg of AZT, dissolved in 10 ml pyridine were reacted with 130 mg of succinic anhydride for 17 h. The formation of AZT-HS was monitored by thin-layer chromatography. After evaporation of pyridine, the dried residue was dissolved in 2 ml of acidified methanol and the AZT-HS was further

purified by step-wise column chromatography on silica using dichloromethane/methanol as eluent (95 : 5 to 90 : 10 v/v). Yield was about 85% and the product was characterized and identified by mass spectrometry, N.M.R. and I.R. AZT-HS, dissolved in methanol was then reduced to AMT-HS using $SnCl_2$. After neutralization and drying, the product was dissolved in distilled water, extracted with ether/dichoromethane (1 : 1, v/v) and the aqueous phase containing the AMT-HS derivative was subsequently vacuum-dried. Lastly, the product was repurified by flash chromatography on silica (CH_2Cl_2/MeOH, 9 : 1 to 6 : 4 v/v), characterized and identified as described above. The yield was approximately 80 %.

Synthesis of AMT 5'-OH Hemisuccinate Bovine Serum Albumin Immunogen

AMT-HS (30 mg dissolved in 2 ml of dimethyl acetamide) was mixed with 68 mg of BSA, previously dissolved in 15 ml of distilled water. The mixture was supplemented with 17 ml of 0.15 M NaCl and 36 mg of EDC were added and reacted overnight at room temperature. The pH was adjusted to 5.5. Non-reacted material was removed by extensive dialysis against saline and the immunogen solution was stored at -20° C.

Preparation of AMT 5'-OH Hemisuccinate Horse Radish Peroxidase Conjugate (AMT-HS-HRP)

AMT-HS (8 mg) was coupled with HRP (4 mg) by the mixed anhydride method using a procedure previously

described in detail (12). After coupling, the AMT-HS-HRP conjugate (final volume 2 ml) was extensively dialyzed against PBS, supplemented with 1% BSA and 0.01% thimerosal, mixed with 1 volume glycerol and aliquots were stored at -20° C. The conjugate was stable throughout the whole study and was referred to as the stock solution, under these conditions.

Synthesis of AMT Standard

AMT used to calibrate the ELISA was synthesized by direct reduction of AZT as previously described (8). Its concentration was verified by spectroscopy, assuming a ε molar coefficient (266 nm) of 10,000, and by HPLC.

Anti-AMT Rabbit Antibodies

Two female hybrid rabbits were immunized at 4-week intervals with 0.5 mg of AMT-HS-BSA immunogen, dissolved in 0.5 ml of saline, and emulsified with an equal volume of complete Freund's adjuvant. Multisite, subcutaneous injections were performed in the back and the animals were bled regularly from the marginal ear vein or by heart puncture, 4-21 days after each injection. The different anti-AMT antiserum batches were stored at -20°C before testing.

Reverse Phase HPLC Analysis of Serum Samples

AZT and GAZT were separated by HPLC with a reverse phase C 18 column (RP 18 Licrospher Merck). Serum (0.5

ml) was first extracted using RP 18 disposable columns (Adsorbex, Merck). After washing with PBS, bound material was eluted with methanol and the solvent was subsequently evaporated. The dried residue was then dissolved in 0.15 ml of 90 : 10 v/v 2.5 mM ammonium phosphate buffer pH 2.4/acetonitrile (mobile phase) and 50 µl of the solution was injected on the column. Absorbance was monitored at 267 nm and elution was carried out at a flow rate of 1 ml/min. Retention times of GAZT and AZT were 9-9.5 min and 14.5-15 min, respectively.

Extraction and Acetylation of Serum Samples

In preliminary experiments, normal human serum samples were spiked with known amounts of AZT and/or AMT, extracted with ethyl acetate and the organic phase content was analyzed by HPLC or ELISA. Ethyl acetate extracted all AZT, but only 10-20% of AMT. Therefore, potential cross-reacting AZT was routinely removed from serum samples prior to acetylation, as follows : serum (0.5 ml) was deproteinized by addition of 0.5 ml of acetonitrile. The mixture was stirred for 30 s, centrifuged and the supernatant extracted for 5 min with 5 ml of ethylacetate. After centrifugation, the organic phase was removed and the aqueous phase evaporated under nitrogen. The dried residue was finally dissolved in 0.25 ml of 50 mM phosphate buffer pH 7.2. Fifty µl of 4 N KOH and 12.5 µl of acetic anhydride were then added sequentially and the mixture stirred for 15 s. Lastly, 12.5 µl of 4 N KOH was added. An aliquot of the AMT standard (10 µg/ml in phosphate buffer) was acetylated

using the same procedure. Recoveries of the AMT from serum matrices were calculated in each series by analyzing two different normal serum samples, previously spiked with 10 ng/ml and 100 ng/ml of AMT standard, and treated under the conditions established above.

Measurement of AMT in serum patient samples

17 blood samples originating from 12 patients (6 males and 6 females, aged 18-43 years) who received 200 mg to 400 mg AZT orally twice a day, were assayed by ELISA.

ELISA Procedure

Anti-rabbit IgG Mab was diluted to 20 µg/ml with 0.1 M phosphate buffer pH 7.2. (PB) and 0.15 ml of this solution was dispensed into the wells of an Immulon 4 microtiter plate. After incubation for 18 h at 25°C, unbound material was removed by aspiration and the plate washed three times with the PB. The plate was then saturated for 10 min at 25°C with 0.2 M Tris-HCl buffer pH 8.2 containing 1% BSA and 10 µg/ml phenol red (assay diluent). The plate coated with anti-rabbit IgG Mab was quickly washed with PB and duplicate 50 µl aliquots of Ac-AMT standards (prepared in assay diluent, ranging from 0 to 100 ng/ml) or acetylated unknowns (diluted at least 1:4 with the assay diluent) were added to the wells. This was followed immediately by 50 µl of AMT-HS-HRP conjugate (diluted 1/15000 from the stock solution) and 50 µl of rabbit anti-AMT antibody (diluted 1/5000

from the selected batch with assay diluent). The plate was covered with a plastic sealer, incubated for 2 h at 25°C and extensively washed with running tap water. Bound enzymatic activity was measured by standard procedure using 0.15 ml of OPD/H_2O_2 solution as the substrate (12) and the reaction was stopped with 0.1 ml of 2 N H_2SO_4. Absorbance was monitored at 492 nm with an automatic plate reader (MR 5000, Dynatech).

RESULTS

Rabbit anti-AMT Antibodies

Antibody titers were measured by ELISA as previously described (12). Briefly, the various anti-AMT antiserum batches were serially diluted with the assay diluent, incubated with AMT-HS-HRP (with or without an excess of 100 μg/ml of AMT) and bound/free separation was achieved using insolubilized anti-rabbit IgG Mab. One rabbit failed to respond to AMT whereas the other one exhibited a particularly high and sustained anti-AMT response (antibodies still detectable at 1/100 000 dilution) 82 days after the initial injection. Therefore, this anti-AMT sample was selected for the studies reported herein.

AMT Standard Curve and Assay Specificity

Fig. 1a shows a typical standard curve for Ac-AMT. The limit of sensitivity calculated as the Ac-AMT concentrations which statistically inhibited (with 95%

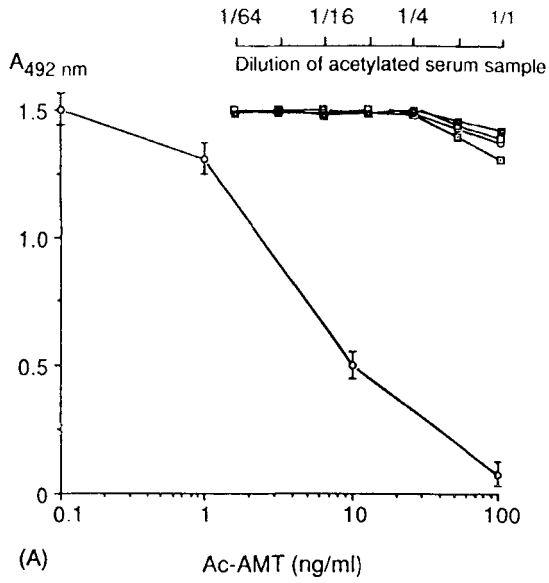

Figure 1 : a)Semi-log representation of displacement curves obtained with Ac-AMT and dilutions from 4 normal human serum previously deproteinized, extracted and acetylated. Each bar represents two S.D. b)Logit-log representation of standard curves derived with Ac-AMT standards (open circles) and dilutions of 3 serum from AZT-treated patients and processed as described in Materials and Methods. Slopes obtained from equations of the regression curves were -2.27, -2.18, -2.16 and -2.21 for Ac-AMT standards and samples, respectively.

confidence) or decreased by 10%, the maximal absorbance, were 0.2 ng/ml and 0.65 ng/ml, respectively. Half-inhibition was observed at concentrations ranging between 3 and 4 ng/ml. Of note, normal human serum previously extracted and acetylated and diluted 1/2 - 1/4 with the assay diluent exhibited absorbance values close to that of zero standard (Fig. 1a). Therefore, acetylated samples to be analyzed were routinely diluted at least 1/4 with the assay diluent prior to testing.

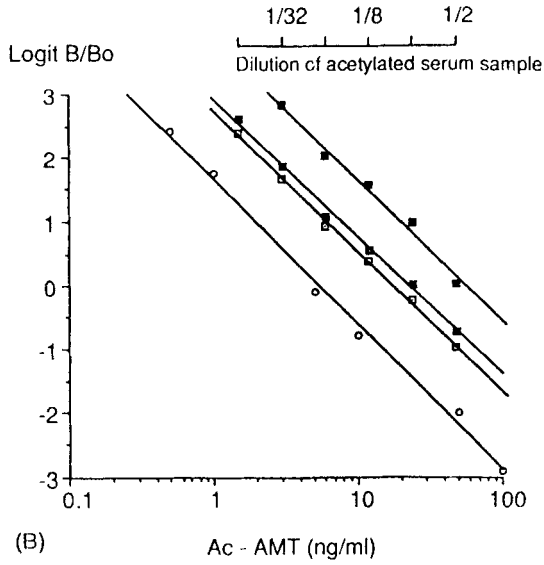

Figure 1 Continued

Acetylation being performed on 2-fold concentrated samples, the AMT detection limit was 0.4 ng/ml in serum samples. The curves derived from dilutions of extracted and acetylated serum samples of AZT-treated patients were parallel (Fig 1b), strongly suggesting that no cross-reacting or interfering material was present in serum samples previously processed as described above. The specificity of the method was also investigated by examining the eventual cross-reactivity of various synthetic or naturally occurring nucleoside analogues, possibly present in serum samples of AZT-treated patients. Table 1 shows that under these assay conditions and using non-acetylated compounds, AMT-HS represented the best competitor. AZT and thymidine to a lesser extent substantially cross-reacted. The low

TABLE 1

Cross-reactivity studies of AMT analogs.

	50% INH (μM)	% Cross Reaction	
AMT	1.24	100	1.29
AZT	4.66	26.6	0.34
Thymidine	8.26	15.0	0.19
GAZT	96	1.3	0.016
AMT-HS	0.029	4275	55
D4T*	312.5	0.39	0.005
DDC**	>4132	<0.03	$<3.8 \times 10^{-4}$
Ac-AMT	0.016	7750	100 (x 77.5)
Ac-AZT	0.55	225.4	2.9 (x 8.5)
Ac-Thymidine	0.30	413	5.33(x 28.0)
Ac-AMT-HS	0.0052	23846	307 (x 5.6)

*2',3'-didehydro-2',3'-dideoxythymidine
** 2',3'-dideoxycytidine

Cross-reactivity was calculated as:
Concentration of AMT or Ac-AMT (μM) at 50 % inhibition x 100
Concentration of analog (μM) at 50 % inhibition
Numbers in parenthesis indicate the increase in reactivity observed following acetylation of products and calculated as:
Cross-reactivity of acetylated compound
Cross-reactivity of non-acetylated compound

cross-reactivity of GAZT (1.3%) as compared to that of AZT (26.6%) confirmed the contribution of the 5'-OH hemisuccinate spacer to the antibody affinity. Conversely, when acetylated, reactivity of these compounds was dramatically improved but still quite variable (Table 1). The 77.5 and 28-fold enhancement observed with AMT and thymidine, respectively, which

both possess two potential acetylation sites at the 5' and 3' positions of the sugar moiety indicated that at least, part of the anti-AMT antibody, was directed towards 5' OH, 3' NH_2 di-acetyl AMT. This result was confirmed by the moderate increase observed with AMT-HS (5.6-fold) and AZT (8.5-fold) in which, acetylation can only occur at the 3' NH_2 or at the 5'OH position, respectively. Although following acetylation, AZT only cross-reacted at 2.9 %, removal of AZT prior to AMT quantitation was mandatory. Indeed, AZT serum concentration being as much as 68-fold higher than that of AMT in serum samples of AZT-treated patients (Table 2) would certainly lead to overestimated AMT levels.

Assay Precision and Recovery Test

Three different serum samples were spiked with pharmacologically relevant AMT concentrations of 10 and 100 ng/ml, followed by extraction and acetylation steps described above. These samples were assayed five times in the same series or on five different occasions. Within-run and day-to-day coefficients of variation ranged from 5.1 to 7.2 % and 8.5 to 9.7 % respectively. Recovery approximated 62 to 68 % with AMT concentrations ranging from 10 to 100 ng/ml. Therefore, AMT values measured by the ELISA method were corrected using a multiplying factor ranging between 1.47 and 1.61.

Analysis of AMT by ELISA in Serum Samples of AZT-treated Patients

This newly developed methodology was used in monitoring AMT levels present in the serum of a 5-year

TABLE 2

Measurement of AMT and AZT levels in various serum patient samples.

Sample n°	AZT (ng/ml)	AMT (ng/ml)	AZT/AMT ratio
1 t -15	<50	3.75	<13.3
1 t 45	206	6.75	30.5
2 t -15	<50	3.75	<13.3
2 t 45	327	4.80	68.1
3 t -15	<50	6.80	<7.4
3 t 45	543	15.00	36.2
4 t -15	<50	4.50	<11.1
4 t 45	385	10.50	36.6
5 t -15	<50	5.30	<9.4
5 t 45	962	15.00	64.1
6 t 45	1289	20.20	63.8
7 t -15	<50	NDa	----
8 t 45	775	12.00	64.5
9 t 120	215	11.20	19.1
10 t 45	900	18.00	50.0
11 t 180	<50	3.20	<15.6
12 t 150	130	5.60	23.2

NDa: Not detectable, below the detection limit of the technique (< 0.4 ng/ml).
Blood samples were drawn 15 min. (residual AMT) before or 45 min. (corresponding to AZT Cmax) to 180 min after AZT administration. AMT and AZT concentrations were determined by ELISA and reverse phase HPLC, respectively.

old HIV seropositive patient receiving 180 mg of AZT three times a day orally (Fig. 2). Residual AMT was observed at baseline and after AZT administration, AMT reached a Cmax of about 24 ng/ml within 0.5 h, plateaued for 2 hrs, and was subsequently slowly eliminated. These results are in agreement with the pharmacokinetics

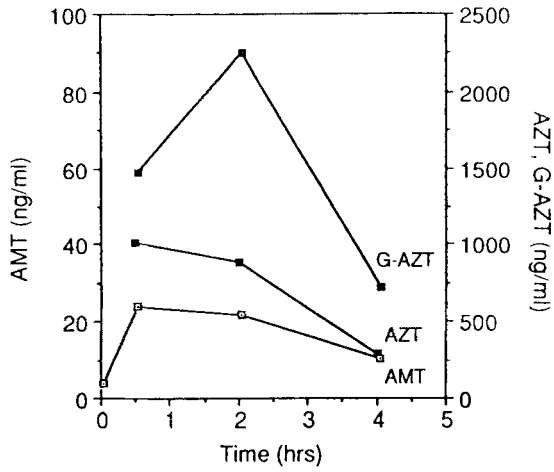

Figure 2 : Serum levels of AZT, GAZT and AMT in a 5-year old patient with advanced HIV infection after oral administration of 180 mg AZT. At indicated times, AMT was assayed by ELISA and AZT and GAZT were quantitated by reverse-phase HPLC as described in Materials and Methods. At t=0, AZT and GAZT levels were below the sensitivity threshold of the HPLC technique (<50 ng/ml).

parameters of AMT reported in monkeys after a subcutaneous injection of tritiated AZT (8), and in humans (11). In addition, AZT/AMT concentration ratios decreased with time, thus confirming that elimination of AMT was slower than that of AZT (9,11). This ELISA was also applied to the measurement of AMT in various serum samples of patients receiving AZT orally, twice a day. In most cases, residual AMT was detectable at baseline and AMT levels were comprised between 3.2 and 20.2 ng/ml at times ranging from 45 min to 180 min after drug administration (Table 2). Interindividual variability in the AZT/AMT concentration ratio ranging from 30.5 to 68.1 at the AZT Cmax was also observed, suggesting that

AMT serum concentrations could not be extrapolated from the AZT dose. These results are in agreement with recent data obtained with a modified HPLC technique (11) which also demonstrates a large interpatient variation and a nonlinearity in AMT formation in 6 AZT naive patients after oral administration of 100 and 500 mg of drug (AUC(AZT) = 618.2 ± 148.9 ng/ml x h, AUC(AMT) = 145.4 ± 15.2 ng/ml x h and AUC(AZT) = 2 954.3 ± 521.8 ng/ml x h, AUC(AMT) = 92.4 ± 44.6 ng/ml x h at 100 and 500 mg dose levels, respectively).

DISCUSSION

In this study, a specific and sensitive ELISA test was developed for the measurement of AMT, a toxic catabolite of AZT, in serum of AZT-treated patients. In preliminary investigations, using unextracted samples and non-acetylated compounds, the assays displayed a poor sensitivity with a half-inhibition of 300 ng/ml and an important cross-reactivity with unchanged AZT. The various attempts, including removal of AZT by various processes, deproteinization of serum samples and previous analysis of AMT by various methods, were too cumbersome and unsuccessful to overcome these drawbacks. The high cross-reactivity of AZT with AMT was unexpected since the reduction of previously succinylated AZT provided an AMT-HS derivative with a free 3′ amino group. Following acetylation of the competitors, a markedly different pattern of cross-reactivity was observed. In particular, AMT and thymidine, in which the 3′ and 5′ positions can be simultaneously acetylated, exhibited dramatically enhanced reactivity, whereas AMT-

HS and AZT (which both possess only one possible acetylation site) demonstrated a moderately improved reactivity. These results clearly demonstrated that the anti-AMT rabbit antiserum was, at least partly, directed against 5′-HS, 3′ acetyl-AMT and consequently, during immunogen synthesis, even at an acidic pH of 5.5, the 3′ amino group of AMT-HS reacted in the presence of the carbodiimide with carboxylic groups of the BSA carrier. The acetylation procedure led to an improved sensitivity of AMT by as much as 77.5-fold while reducing the cross-reactivity of AZT to less than 2.9%. However, in light of the high AZT/AMT concentration ratios observed in serum of AZT-treated patients, the parent drug would certainly interfere with AMT analysis. Therefore, AZT was previously eliminated from samples to be assayed by an ethyl acetate extraction. Of note, once extracted and acetylated, AZT could be assayed separately by the AMT-ELISA, with a sensitivity close to that of most available specific radioimmunoassays. Cross-reactivity of GAMT was not assessed in this study. This metabolite which has been only detected in bile of patients receiving AZT, (7) is not likely to interfere with this ELISA technique. Indeed, the steric hindrance introduced by the glucuronic residue would probably markedly decrease its reactivity in a similar manner to that observed with GAZT. This newly developed immunoassay test was applied in monitoring AMT serum levels in AZT-treated patients with advanced HIV infection. In most patients receiving AZT twice a day by oral administration, residual AMT, as well as significant intra and inter-individual variation of the AZT/AMT concentration ratio were detected. This latter emphasizes the usefulness of such an immunoassay in

studying AMT formation in humans. This ELISA test may be useful in evaluating the AMT formation in placenta of seropositive pregnant women after the recent demonstration of the decreased rate of HIV feto-maternal transmission by AZT.

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