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# High-performance liquid chromatographic assay for 3′-amino-3′-deoxythymidine in plasma, with 9-fluorenyl methyl chloroformate as the derivatization agent

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**Abstract**

We have developed a sensitive high-performance liquid chromatographic assay for the determination of the zidovudine metabolite 3′-amino-3′-deoxythimidine (AMT) using fluorescence detection and sensitivity in the picomolar range. Plasma was diluted with 0.05 *M* sodium phosphate buffer pH 7.2 and subsequently prepared for analysis using solid-phase extraction. AMT was derivatized with 9-fluorenyl methylchloroformate and chromatographed using a reversed-phase system. The mobile phase consisted of acetonitrile–0.01 *M* potassium phosphate buffer (pH 7) (32:68, v/v). The fluorescence of the column effluent was monitored at 262 nm (excitation) and 306 nm (emission). Good resolution of AMT from endogenous plasma components was obtained. Within- and between-day variability was less than 10%. The limit of quantitation was 0.9 μg/l. The assay was successfully applied to the determination of AMT in human plasma and in plasma of mice treated with zidovudine.

## 1. Introduction

Studies of human zidovudine metabolism have shown that approximately 80% of the drug is eliminated through glucuronidation. Stagg et al. [1] have identified another metabolite, 3′-amino-3′-deoxythymidine (AMT), which is five times more toxic for bone marrow cells than zidovudine (Fig. 1).

AMT might contribute to zidovudine-induced anemia and neutropenia, and it is thus of interest to measure its plasma concentration during zidovudine therapy. Stagg et al. [1] reported that after intravenous infusion of a standard dose of zidovudine, AMT concentrations were five to ten times lower than those of the parent drug, ranging between 20 and 200 μg/l. However, widespread application of the method used by these authors is limited because of its radiometric detection method. Recently, Burger et al. [2] proposed an HPLC method based on solid-phase extraction with a cation-exchange column, followed by ion-pair chromatography with ultra-

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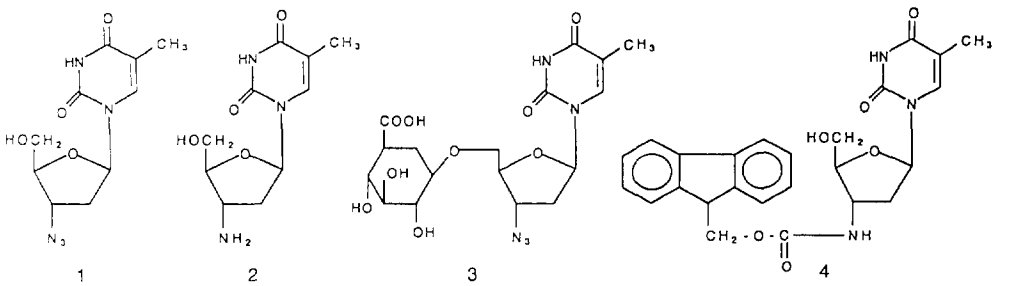


Fig. 1. Structures of zidovudine (1), 3'-amino-3'-deoxythymidine (2), zidovudine 5'-O-glucuronide (3), AMT-FMOC derivative (4).

violet detection. The limit of quantitation was 5 $\mu$g/l.

More recently Zhou and Sommadossi [3] reported an HPLC method for the quantitation of AMT in human plasma after precolumn derivatization with fluorescamine. The limit of quantitation was 3 $\mu$g/l. As the chemical structure of AMT is characterized by the presence of a primary amino group, we developed an HPLC method using 9-fluorenyl methylchloroformate (FMOC-Cl) as the precolumn derivatizing agent. The procedure involves a plasma clean-up/concentration step on a $C_{18}$ column, followed by FMOC-Cl derivatization and reversed-phase chromatography with fluorimetric detection. The method was used to measure AMT in human plasma and in plasma of mice treated subcutaneously with zidovudine.

## 2. Experimental

### 2.1. Reagents, materials and methods.

Zidovudine was from Wellcome (Issy les Moulineaux, France). Dithiothreitol (DTT) was from Sigma (St. Louis, MO, USA). 9-Fluorenylmethyl chloroformate (FMOC-Cl) was from Aldrich (Milwaukee, WI, USA). Acetonitrile, methanol, phosphoric and boric acids, sodium mono- and dihydrogen phosphate, and potassium mono- and dihydrogen phosphate were from Merck (Darmstadt, Germany) and all other reagents were of analytical grade. Bond Elut $C_{18}$ (500 mg) cartridges were from Analytichem International (Harbor City, CA, USA).

A stock solution of 3'-amino-3'-deoxythymidine (AMT) was prepared according to Handlon and Oppenheimer [4]. Zidovudine was mixed under mild conditions with DTT [5 mg of zidovudine (18.7 $\mu$mol) and 14.4 mg of DTT (93.5 $\mu$mol)] were dissolved in 1 ml of 0.1 *M* potassium phosphate buffer pH 7.2 and incubated at 37°C for 18 h under nitrogen. Total reduction of zidovudine was confirmed by HPLC. The reaction product was analyzed by HPLC coupled with mass spectrometry (Nermag-10-10-C, LC/175). The mass spectrum showed a quasi-molecular ion at *m/z* 242 $[M+H]^+$ and two fragments corresponding to [3'-amino-1,2-dideoxyribose-NH4]$^+$ (*m/z* 134) and thymine at *m/z* 127.

The HPLC system consisted of a solvent-delivery pump (LC 9 A) (Schimadzu, Touzart et Matignon, Vitry, France); a Rheodyne 7120 injection valve; an RF 551-Shimadzu fluorescence detector set at 262 nm (excitation) and 306 nm (emission); and an RC-5-A Shimadzu integrator (Touzart et Matignon). The analytical column was a Hypersil ODS-$C_{18}$ (250 × 4.6 mm I.D.; particle size 5 $\mu$m) (HPLC Technology, Touzart et Matignon). The mobile phase was acetonitrile–0.01 *M* potassium phosphate buffer (pH 7) (32:68, v/v) at a flow-rate of 1.1 ml/min. Elution was carried out at room temperature.

### 2.2. Sample preparation

Plasma (0.2 ml) diluted with 0.2 ml of 0.05 *M* sodium phosphate buffer pH 7.2 was extracted with a fresh 3-ml $C_{18}$ cartridge using a Vac Elut vacuum assembly. The cartridge was first acti-

vated with 3 × 1 ml of methanol and rinsed twice with 3 ml of 0.05 *M* sodium phosphate buffer pH 7.2. The sample was then drawn through the column, which was allowed to equilibrate for 5 min prior to washing with 1 ml of buffer. The column was dried by reducing the pressure for 5 min. The sample was eluted with 1 ml of methanol, then evaporated to dryness under a stream of nitrogen and redissolved in 100 μl of 0.2 *M* borate buffer pH 9.5.

### 2.3. Derivatization procedure

To the above sample (100 μl), 200 μl of distilled water and 300 μl of FMOC-Cl solution [2.5 g/l in 50:50 (v/v) acetone–acetonitrile) were added and vortex-mixed immediately for 3 min. The excess of FMOC-Cl was extracted with pentane (2 ml) and the lower layer was diluted 1:5 with mobile phase prior to injection.

The derivatization yield was measured by reversed-phase chromatography (Hypersil ODS column 250 × 4.6 mm I.D.; particle size 5 μm). The mobile phase was 0.05 *M* potassium phosphate buffer (pH 7.1)–acetonitrile (68:32, v/v), with a flow-rate of 1 ml/min. Absorbance was monitored at 265 nm with a SDP6A UV detector (Shimadzu). The retention times were 3.5 and 31.2 min for AMT and the AMT-FMOC derivative, respectively. A derivatization yield of 100% leads to disappearance of AMT and the formation of a single derivative. The AMT concentration range was 0.9 μg/l–9 mg/l.

The stability of the AMT-FMOC derivative obtained after AMT extraction from plasma (45 and 450 μg/l) was studied at −20°C, 4°C, 20°C and 37°C in darkness and at 20°C at ambient temperature for 15 days, during which it was analyzed five times.

### 2.4. Preparation of calibration standards and quality control samples

A stock AMT spiking solution (450 mg/l) was serially diluted with plasma to prepare a nine-point standard curve over the range 0.9 μg/l–9 mg/l. Quality control samples consisted of 9.0 and 225 μg/l of AMT and were analyzed along with the mouse serum samples.

### 2.5. Recovery

Recovery of AMT after solid-phase extraction was calculated by assaying spiked plasma samples (0.9, 90, 900 and 9000 μg/l; $n = 7$ at each concentration). The recovery of AMT from plasma was determined from the response of standards directly derivatized by FMOC-Cl.

### 2.6. Assay validation

The appropriate regression model for the assay was determined from several sets of calibration standards. The concentration and peak-height data were fitted by a weighted least-squares linear regression model with a weighting factor of 1/concentration. The within-day precision was measured by simultaneously assessing samples (90, 450 and 900 μg/l) in replicates of five. The between-day precision was determined on six separate runs by assaying plasma at concentrations of 0.9–9000 μg/l.

Precision was expressed as the coefficient of variation (C.V.) of the means. Accuracy (expressed as % bias) was also calculated for between-day assays. Quality control charts were maintained throughout the animal experiments and used to monitor precision during routine assays.

The lower limit of quantitation was defined as the lowest plasma AMT concentration that could be measured with a relative standard deviation of less than 20%.

Plasma samples from HIV-seropositive patients were analyzed to investigate interference. The following substances were also tested for interference: zidovudine, sulfamethoxazole, trimethoprim, didanosine, zalcitabine, amphotericin B, pyrimethamine, sulfadiazine, isoniazid, ethambutol and rifampicin.

### 2.7. Analysis of mouse and human plasma

Zidovudine solution was injected subcutaneously at a dose of 20 mg/kg to DBA2 mice.

Blood was collected from the retro-orbital sinus (5–6 mice each time) and plasma was stored at −80°C until analysis. Samples were taken 0.5, 1, 2, 4 and 6 h following the subcutaneous injection.

Plasma from ten HIV infected patients who participated in a pharmacokinetic study of zidovudine was used for the determination of AMT after oral administration of zidovudine (250 mg). Plasma zidovudine concentrations were determined with the HPLC method of Good et al. [5].

## 3. Results and discussion

We describe a method for the determination of the zidovudine metabolite AMT in biological samples. Although HPLC assays of AMT have been described [1–3], none combines sample purification by solid-phase extraction on $C_{18}$ columns with derivatization with FMOC-Cl, which results in a low limit of quantitation and makes the method suitable for routine application. FMOC-Cl is suitable for use in assays of primary and secondary amino acids, giving stable and highly fluorescent products [6]. The presence of a primary amine group in AMT made this approach feasible and gave derivatization yields close to 90% in the 0.9–9000 μg/l AMT plasma concentration range (Table 1).

Yields of 88–89% have been reported with preparative derivatization of amino acids with FMOC-Cl [7]. The use of this reagent requires optimization of the reaction co-solvent, the pH of the reaction buffer and the conditions under which the excess of labeling compound is removed. Acetonitrile was used as the reaction co-solvent, as it avoids the problem of precipitation of the derivative. However, as acetonitrile causes derivatization of AMT to proceed slowly, we added acetone as co-solvent [8]. Derivatization was carried out in borate buffer at pH 9.5 for 3 min, a pH at which AMT is nonionized, giving high derivatization yields.

Table 1
Extraction recoveries of AMT from plasma ($n = 7$) and derivatization yield ($n = 5$)

| Concentration (μg/l) | Recovery Mean ± S.D. (%) | Derivatization yield Mean ± S.D. (%) |
|---|---|---|
| 0.9 | 80.3 ± 6.2 | 85.4 ± 4.9 |
| 90 | 90.1 ± 3.8 | 91.1 ± 7.8 |
| 900 | 91.3 ± 5.1 | 90.2 ± 5.4 |
| 9000 | 88.8 ± 3.2 | 87.3 ± 8.2 |

The *n*-pentane extraction was necessary to efficiently remove the excess of reagent and to lower the concentration of the FMOC-Cl hydrolysis product (FMOC-OH) [6]. The FMOC-AMT derivative fluorescence spectrum in the mobile phase was specific for the fluorenyl group and was characterized by excitation and emission wavelengths of 262 and 306 nm, respectively. As Burger et al. [2] showed that AMT was stable in plasma at 60°C for at least 3 h and at −30°C for at least three months, we only investigated the stability of the FMOC-AMT derivative. We demonstrated that the derivative was stable for at least 15 days regardless of temperature and lighting conditions, thereby facilitating automation. Representative chromatograms of blank plasma and plasma from a mouse treated with zidovudine are shown in Fig. 2a,b. The retention time of FMOC-AMT was typically 27–29 min, while the later-eluting peaks corresponded to FMOC hydrolysis by-products (FMOC-OH = 37 min).

Several mobile phases were tested for this analytical separation. Since FMOC-AMT is stable in acetonitrile, this solvent was included in the mobile phase. pH control was required for reproducible separation, and phosphate buffer (pH 7) was thus used as the aqueous component in the mobile phase.

A decrease of 5% in the acetonitrile concentration in the mobile phase increased the capacity factor of FMOC-AMT (50%) but did not affect the selectivity between FMOC-AMT and FMOC-OH.

The pH (in the 5–7 range) did not influence the retention of FMOC-OH, which was easily separated from other compounds at an appropriate pH. Under the proposed conditions, ma-

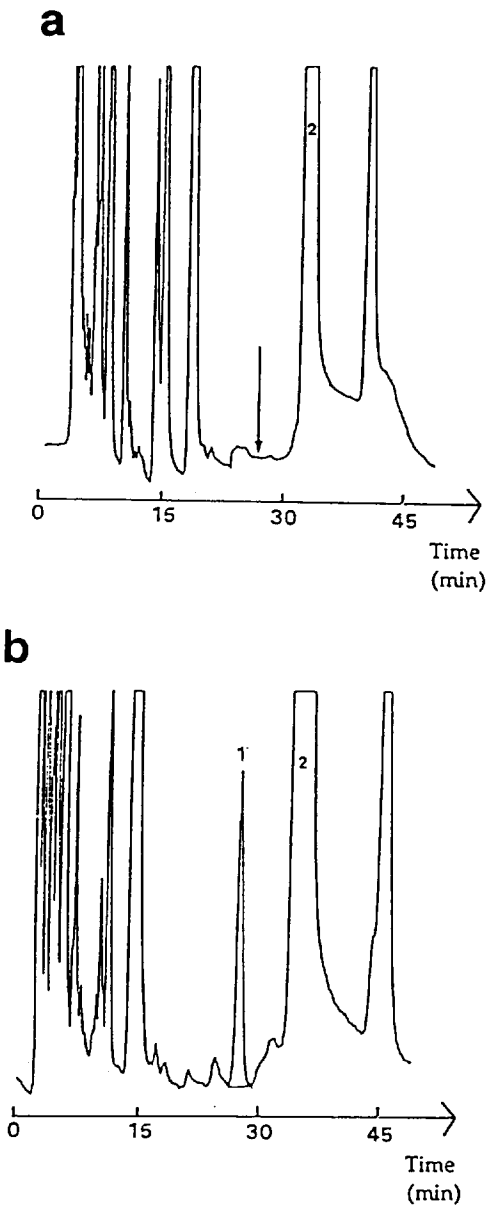


Fig. 2. (a) Chromatogram of a blank plasma. Peak 2 = FMOC-OH. (b) Chromatogram of plasma from a treated mouse. Peaks: 1 = FMOC-AMT, retention time 27.8 min, AMT concentration 2040 μg/l; 2 = FMOC-OH.

trix components do not interfere with the determination of FMOC-AMT.

### 3.1. Validation of the method

The recovery of AMT from plasma is shown in Table 1. More than 80% of the compound was recovered over the 0.9–9000 μg/l range. The C.V. at each concentration was less than 6.2%. Sample pretreatment optimization was based on the conditions proposed for zidovudine [5]. A pH of 7.2 for extraction, at which AMT was nonionized, was considered optimal. At pH < 7.2 the recovery of AMT decreased to 70%.

The within-day and between-day precision and accuracy of the method are shown in Table 2. Precision was within 14.2% and accuracy −11.2% in plasma with low AMT concentrations.

These results were extended by analysis of the quality control data from the assay of plasma from treated mice. In one such study, calculated between-day precision was 7.5 and 8.5% at concentrations of 90 and 225 μg/l, respectively. Given this good precision, an internal standard was considered unnecessary.

Based on the precision and accuracy, the limit of quantitation was set at 0.9 μg/l. This value is ten-fold lower than the sensitivity reported by Burger et al. [2], taking into account the small plasma volume (200 μl) required in our assay. The assay (Fig. 3) is adequate to measure the low AMT concentrations observed after oral zidovudine therapy (6–18 μg/l). The assay was

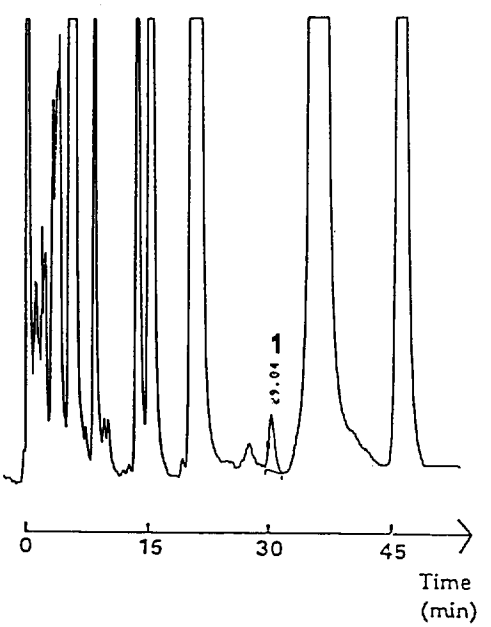


Fig. 3. Chromatogram of a spiked human plasma sample. Peak 1 = FMOC-AMT; AMT concentration = 19 μg/l.

Table 2
Precision and accuracy for the analysis of AMT in plasma

| Concentration ($\mu$g/l) | Precision | | | | Accuracy (%) |
|---|---|---|---|---|---|
| | Within day ($n = 5$) | | Between day ($n = 6$) | | |
| | Mean ($\mu$g/l) | C.V. (%) | Mean ($\mu$g/l) | C.V. (%) | |
| 0.9 | – | – | 0.8 | 14.2 | −11.2 |
| 4.5 | – | – | 5.1 | 2.4 | +13.7 |
| 22.5 | – | – | 24.5 | 4.3 | +8.7 |
| 45 | – | – | 39 | 9.5 | −13.3 |
| 90 | 95 | 9.3 | 85 | 9.8 | −5.5 |
| 450 | 460 | 8.9 | | | |
| 900 | 830 | 8.3 | 930 | 4.6 | +3.3 |
| 4500 | – | – | 4570 | 6.6 | +1.5 |
| 9000 | – | – | 8800 | 1.6 | −2.2 |

linear over the AMT concentration range 0.9–9000 $\mu$g/l. Slope values were $8.39 \cdot 10^{-3} \pm 0.25 \cdot 10^{-3}$ and correlation coefficients ($r^2$) of the regression lines were 0.998. Interferences with zidovudine and AMT could occur in the chain of processing AMT up to detection. We therefore spiked the calibration standards with zidovudine to a concentration of 2 mg/l. No interference due to zidovudine occurred in the chromatogram and no statistical difference was found in the slope of the AMT calibration curves at 5%.

Plasma from AIDS patients treated with didanosine contained no endogenous peaks that interfered with the quantitation of AMT. None of the potentially coadministered drugs and metabolites tested interfered with the assay.

### 3.2. Applications

The method is currently used to measure steady-state AMT concentrations in plasma of mice treated orally or subcutaneously with zidovudine. Fig. 4 shows the zidovudine and AMT plasma concentration–time curves. The maximal concentrations of AMT were in the 1000–2000 $\mu$g/l range. Five to six hour after

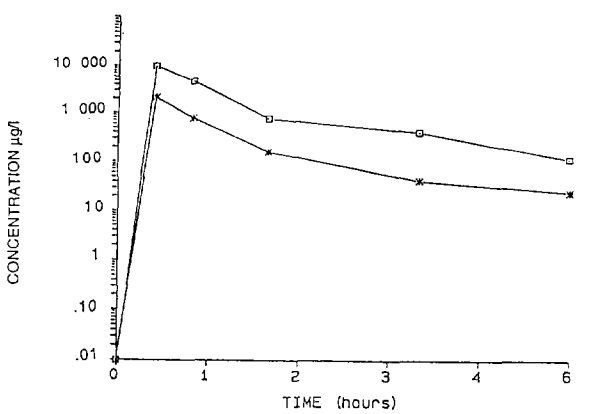


Fig. 4. Time course of zidovudine and AMT plasma concentrations in mice having received a dose of 20 mg/kg of zidovudine subcutaneously. (□) zidovudine, (☆) AMT.

Table 3
Concentrations of zidovudine and of AMT in plasma of patients following a 250 mg oral dose

| Time after the dose (h) | Zidovudine ($\mu$g/ml) | AMT ($\mu$g/l) |
|---|---|---|
| 0.5 | 1.8 | 205 |
| 0.5 | 1.4 | 210 |
| 0.5 | 1.5 | 165 |
| 1.0 | 2.0 | 176 |
| 1.0 | 0.7 | 81 |
| 4.0 | 0.2 | ND |
| 4.0 | 0.05 | ND |
| 6.0 | 0.2 | ND |
| 6.0 | 0.3 | 50 |
| 6.0 | 0.15 | ND |

ND: not detectable.

zidovudine administration, AMT concentrations of 25 μg/l could still be detected in the plasma. The AMT area under the curve (AUC) was about 20% that of zidovudine, in keeping with values reported by Stagg et al. [1] following intravenous zidovudine injection.

The assay was also used to measure AMT plasma levels in patients following oral administration of zidovudine (Table 3). The results were in keeping with values reported by Stagg et al. [1].

## 4. Conclusions

This isocratic HPLC assay with FMOC-Cl as the derivatizing agent can be used to determine AMT, a cytotoxic metabolite of zidovudine, in biological samples. Because the derivatives are stable for at least several days, analysis can be automated. The combined use of solid-phase extraction and reversed-phase chromatography resulted in good resolution. High sensitivity was obtained by labelling and spectrofluorimetric detection. The method is applicable to the analysis of human samples. It can be used to measure AMT concentrations during zidovudine therapy and requires only small sample volumes.

## References

[1] M. Stagg, M. Cretton, L. Kidd, R.B. Diasio and P. Sommadossi, *Clin. Pharmacol. Ther.*, 51 (1992) 668–676.
[2] D.M. Burger, H. Rosing, F.J. Koopman, P.L. Meenphorst, J.W. Mulder, A. Bult and J.H. Beijnen, *J. Chromatogr.*, 622 (1993) 235–242.
[3] X.J. Zhou and J.P. Sommadossi, *J. Chromatogr.*, 656 (1994) 389–396.
[4] A.L. Handlon and J. Oppenheimer, *Pharm. Res.*, 5 (1988) 297–299.
[5] S.S. Good, D.J. Reynolds and P. Demiranda, *J. Chromatogr.*, 431 (1988) 123–133.
[6] S. Einarsson, B. Josefsson and S. Lagerkvist, *J. Chromatogr.*, 282 (1993) 609–618.
[7] M. Makita, S. Yamamoto and M. Kono, *J. Chromatogr.*, 120 (1976) 120–129.
[8] P.A. Haynes, D. Sheumack, J. Kibby and W. Redmond, *J. Chromatogr.*, 540 (1991) 177–185.