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Journal of Chromatography B, 656 (1994) 389–396

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Quantification of 3′-amino-3′-deoxythymidine, a toxic catabolite of 3′-azido-3′-deoxythymidine (zidovudine) in human plasma by high-performance liquid chromatography using precolumn derivatization with fluorescamine and fluorescence detection

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(First received November 12th, 1993; revised manuscript received February 14th, 1994)

Abstract

A specific and sensitive high-performance liquid chromatographic (HPLC) assay with precolumn derivatization and fluorescence detection was developed for the determination of 3′-amino-3′-deoxythymidine (AMT), a recently characterized toxic catabolite of 3′-azido-3′-deoxythymidine (zidovudine, AZT). After extraction with *tert.*-butyl methyl ether–1-butanol (6:4, v/v) and back-extraction into basified aqueous phase, the solution was neutralized with phosphate buffer and the compound derivatized with fluorescamine. Fluorescamine-labeled AMT was chromatographed on a reversed-phase C_{18} column using a mixture of phosphate buffer–methanol–acetonitrile (47:48:5, v/v/v) as mobile phase and fluorescence detection (excitation wavelength 265 nm, emission wavelength 475 nm). The limit of quantification was 3 ng/ml using a 200-μl plasma sample. The recovery of the extraction procedure averaged at 94.4% in the range of 3–200 ng/ml. The HPLC analysis time required per sample was 16 min. Replicate analyses of quality control samples (5–80 ng/ml) gave satisfactory intra- and inter-assay precision (coefficient of variation varied from 1.9 to 6.7%) and accuracy.

1. Introduction

The nucleoside analog, 3′-azido-3′-deoxythymidine (zidovudine, AZT) was the first drug approved for treatment of patients with acquired immunodeficiency syndrome (AIDS). Clinical benefits including decreased number of opportunistic infections, and partial improvement in neurologic manifestations have been reported [1,2]. The clinical use of AZT is, however, limited by its myelosuppressive effects, manifested by anemia and neutropenia [3,4]. Our recent pharmacokinetic and metabolic studies performed in monkeys and in humans as well as *in vitro* investigations using human hepatic microsomes demonstrated that, besides a 5′-O-glucuronidation process representing a major AZT metabolic pathway [5–7] and resulting in the formation of 5′-O-glucuronide-AZT (GAZT), the parent drug was also substantially

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0378-4347/94/$07.00
SSDI 0378-4347(94)00118-O

reduced to 3′-amino-3′-deoxythymidine (AMT) [8–10]. Using human bone marrow clonogenic assays, this catabolite, when tested *in vitro* was five to seven-fold more toxic for colony-forming unit granulocyte-macrophage and burst-forming unit erythroid than AZT [8]. These results suggest that formation of this metabolite in patients treated with AZT may lead to toxic effects on bone marrow cells, accounting partially for AZT induced toxicities. Therefore, monitoring plasma levels of AMT in patients may be important in order to improve AZT therapeutic regimen.

We previously reported a high-performance liquid chromatographic (HPLC) method for simultaneous assay of radiolabeled AZT, GAZT and AMT in human plasma by using liquid scintillation detection [10]. The use of radioactive drug, however, prevents its application to a larger number of patients. In addition, the expected plasma concentration of AMT being low (<200 ng/ml) as compared to AZT and GAZT, a sensitive assay for AMT was necessary. This paper describes a sensitive HPLC method for the quantification of AMT in human plasma. The procedure consists of extraction of plasma samples with a mixture of *tert.*-butyl methyl ether–1-butanol (6:4, v/v) and back-extraction into basified aqueous phase followed, after neutralization with phosphate buffer, by precolumn derivatization with fluorescamine, HPLC separation and fluorescence detection.

2. Experimental

2.1. Materials and reagents

AMT and fluorescamine were purchased from Sigma (St. Louis, MO, USA). AMT was 99% pure as ascertained by the HPLC technique described below. Potassium dihydrogenphosphate (HPLC grade), potassium hydroxide and HPLC grade organic solvents (*tert.*-butyl methyl ether, 1-butanol, methanol, acetonitrile and acetone) as well as disposable 5-ml borosilicate glass tubes (12 × 75 mm) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Disposable 15-ml polypropylene tubes (17 × 130 mm) were purchased from Baxter Diagnostics (McGaw Park, IL, USA). Water was bidistilled from a glass apparatus. Helium was supplied by Airco (Murry Hill, NJ, USA).

2.2. Identification of AMT fluorophor and optimization of reaction conditions

The identity of fluorescamine labeled AMT was checked by FAB-MS analysis. The FAB mass spectrum of the compound suspended in glycerol was obtained using a Varian MAT 311A (San Jose, CA, USA). Xenon was used as the bombardment gas.

The optimal excitation and emission wavelengths of the AMT fluorophor were determined by using the scan utility of the HP1046A programmable fluorescence detector (Hewlett-Packard, Palo Alto, CA, USA). The pH of the reaction medium is important to achieve a high fluorescence. The optimal pH value was determined by monitoring the fluorescence intensity of the AMT fluorophor resulting from reaction media (0.25 *M* phosphate buffer) with pH ranging from 3 to 11. Subsequently, the amount of fluorescamine required for a complete fluorogenic reaction was assessed by measuring the fluorescence resulting from reactions with addition of increasing amounts of the compound (10–500 μg dissolved in anhydrous acetone). Finally, in order to allow overnight autosampling the stability of fluorescamine labeled AMT was checked every hour by examining its peak area using the HPLC method described below.

2.3. Preparation of solutions and standards

The back-extraction solution was prepared by dissolving 2.81 g of potassium hydroxide in 500 ml bidistilled water (0.1 *M*) containing 5% NaCl (w/v), and the neutralizing buffer by dissolving 17.01 g of potassium dihydrogenphosphate in 500 ml bidistilled water (0.25 *M*, pH 4.3). Fluorescamine solution (0.2 mg/ml) was freshly made by dissolving the compound in anhydrous acetone.

Stock solutions of AMT were prepared in bidistilled water and their concentrations were

determined spectrophotometrically based on the molar absorption in 0.1 *M* hydrochloric acid at 265 nm ($\epsilon = 9400$ l mol^{-1} cm^{-1}) and in 0.1 *M* sodium hydroxide at 267 nm ($\epsilon = 7400$ l mol^{-1} cm^{-1}). Stock solutions of AMT at 0.1 and 1.0 μg/ml were prepared and stored in 500-μl aliquots for the preparation of a standard curve and quality control samples. The standard curve (3–200 ng/ml) and quality control samples (5, 40 and 80 ng/ml) were prepared by combining AMT stock solutions (different batches) with normal human plasma. Usually, 25 ml of each concentration of the quality control samples were prepared and divided into 250-μl aliquots. Stock solutions and quality control samples were stored at −20°C until use.

2.4. Sample preparation and derivatization

A 200-μl plasma sample (blank plasma, spiked plasma and patient plasma samples) and 50 μl bidistilled water were vortex-mixed in a 15-ml polypropylene tube to which were then added 5 ml of *tert.*-butyl methyl ether–1-butanol (6:4, v/v). The tube was shaken (15 min) and centrifuged (2000 *g*, 10 min). This procedure led to two phases: the upper homogenous liquid phase consisting of the aqueous/organic mixture and the lower solid phase representing precipitated proteins. A single liquid phase was formed because the 5 ml of the organic phase were miscible with the 250 μl aqueous solution (200 μl plasma plus 50 μl water). The liquid phase (5.1 ml) was transferred into another 15-ml polypropylene tube containing 200 μl back-extraction solution (0.1 *M* potassium hydroxide). After back-extraction by shaking (20 min) and centrifugation (2000 *g*, 10 min), the upper organic phase was discarded by vacuum aspiration and the remaining aqueous solution (100 μl) was quantitatively transferred into a 5-ml borosilicate glass tube. After addition of 100 μl of the neutralization buffer to lower the pH to 7.6, 100 μl (20 μg) of fluorescamine solution (0.2 mg/ml in anhydrous acetone) was added and the tube was immediately vortex-mixed. Subsequently, the acetone portion was evaporated under a gentle air flow for 2 min. All samples were simultaneously derivatized and analyzed on the same day. Finally, the remaining phase was transferred into a 300-μl autosample vial, and 170 μl were injected onto the HPLC system.

2.5. High-performance liquid chromatography

The liquid chromatograph (Hewlett-Packard 1090), was equipped with an automatic injector, a diode-array detector, a Model HP1046A programmable fluorescence detector (excitation wavelength 265 nm, emission wave length 475 nm) and a Model HP79994A analytical workstation. Reversed-phase chromatography was performed on a Hypersil octadecylsilane 5 μm column, 250 × 4 mm I.D. (Jones Chromatography, Lakewood, CO, USA). Elution was carried out isocratically at a flow-rate of 1 ml/min with a mobile phase of phosphate buffer (80 m*M* potassium dihydrogenphosphate, pH adjusted to 7.8 with 5 *M* potassium hydroxide)–methanol–acetonitrile (47:48:5, v/v/v).

2.6. Calibration and calculation

Eight plasma standards prepared by mixing AMT stock solutions with normal plasma (concentrations ranging from 3 to 100 ng/ml), set for a calibration curve, were processed together with the test samples and quality control samples. Standard curve parameters were obtained from an unweighted least-squares linear regression analysis of the AMT fluorophor peak area as a function of the prepared concentrations. Unknown concentrations were calculated by interpolation using each observed AMT fluorophor peak area and the standard curve parameters.

Complete quantitative HPLC analysis procedures were validated, including AMT heat stability during HIV deactivation at 56°C for 4 h, selectivity towards endogenous interfering substances, limit of detection and quantification, linearity, extraction recovery, precision and accuracy. Finally this novel assay was used to measure AMT plasma levels in patients after oral administration of AZT.

3. Results and discussion

3.1. Extraction

The primary amine group on the 3′ position of AMT structure (Fig. 1) leads to a high degree of polarity which prevents extraction of AMT from plasma with common organic solvents. The acidic hydrogen in the pyrrolidione moiety of AMT (Fig. 1) is ionisable in alkaline solution (the pK_a of thymine is 9.94 at 25°C) [11]. This property has led us to consider a back-extraction procedure for AMT from relatively polar organic solvent into an alkaline solution. The organic solvent used was *tert.*-butyl methyl ether–1-butanol. The ratio of the two solvents depends on the polarity of the compound to be extracted. Adequate AMT recovery was obtained with a 6:4 ratio whereas other ratios have been shown to be suitable for other polar compounds [12]. When a 5-ml volume of this mixture was added to 250 μl of an aqueous phase (200 μl plasma and 50 μl water) this resulted in a single phase. After protein removal, this phase was mixed with 200 μl of potassium hydroxide (0.1 *M*). The latter saturated the organic phase, leading to the formation of a basified aqueous phase into which ionized AMT was back-extracted.

3.2. Derivatization

Fluorescamine has previously been used as a reagent for the fluorometric determination of primary amine [13]. This non-fluorescent compound reacts with primary amine to form pyrrolinone derivatives which upon excitation at 390 nm emit high fluorescence at 475 to 490 nm. This reaction proceeds rapidly at room temperature in aqueous solution. Excess reagent is readily hydrolyzed to non-fluorescent products. The optimal pH for this reaction lies between 8 and 9.5 [13]. However, for a particular compound with a primary amine group, the optimal reaction and detection conditions should be detailed. Fig. 1 shows the reaction scheme of AMT with fluorescamine. The identity of fluorescamine labeled AMT was confirmed by FAB-MS analysis. The FAB-MS spectrum demonstrated a single positive molecular ion $(M+H)^+$ at *m/z* 520. The molecular mass of this product (519) and its breakdown pattern were consistent with that of the AMT fluorophore. The 5′-OH group of AMT, a primary alcohol that could also react with fluorescamine, did not lead to detectable amounts of fluorophor under the optimal reaction conditions described below.

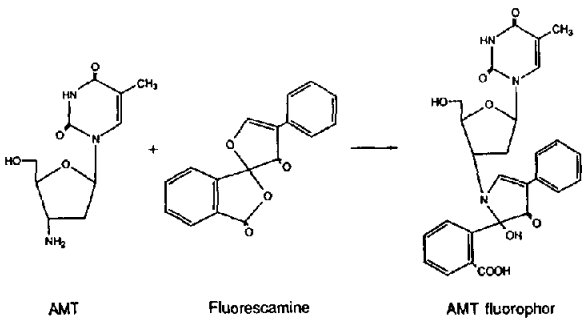

Fig. 1. Reaction scheme of AMT with fluorescamine.

The first parameters to be optimized were the excitation and emission wavelengths. These parameters largely depend on the detector of the instrument and were determined using the wavelength scan feature of the Hewlett-Packard 1046A programmable fluorescence detector. This detector gave a maximal excitation wavelength at 265 nm and a maximal emission wavelength at 475 nm for fluorescamine-labeled AMT. The fluorescence intensity of the fluorophor is very sensitive to the pH of the reaction medium. As can be seen in Fig. 2, maximal fluorescence was reached at pH 7.6–7.8. Subsequently, the amount of fluorescamine required for a complete fluorogenic reaction was determined. Results demonstrated that 20 μg of fluorescamine led to a substantial peak area with a reasonable noise and that higher amounts dramatically increased the base line and caused quenching of the fluorescence (Fig. 3). Such a decline in fluorescence induced by increasing amounts of fluorescamine has already been reported previously [12,13]. Finally, the stability of the resulting fluorophor was examined at room temperature over a 20-h period by injecting every hour an equal volume of the reaction mixture. No decrease in peak area was detected. The high

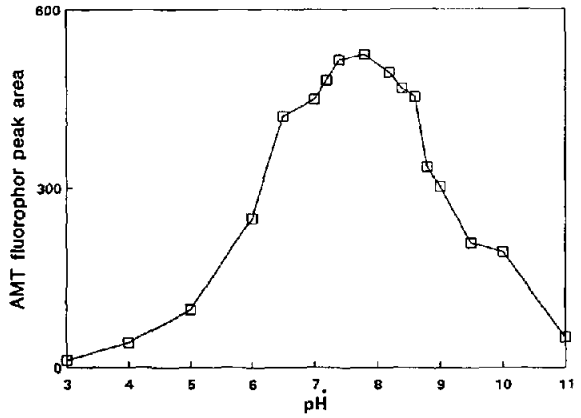

Fig. 2. Effect of pH on fluorescence intensity of AMT fluorophor.

fluorescence stability allowed overnight autosample analysis of AMT.

3.3. Chromatographic system

Both C_{18} and C_8 columns were tested as stationary phase. Only the Hypersil C_{18} columns gave satisfactory separation. The mobile phase was optimized with regard to separation and fluorescence intensity. The mobile phase of choice was a mixture of phosphate buffer (80 m*M* potassium dihydrogenphosphate, pH adjusted to 7.8 by 5 *M* potassium hydroxide)–methanol–acetonitrile (47:48:5, v/v/v). The pH of the buffer was adjusted at 7.8 to achieve good resolution between the AMT fluorophor and endogenous interferences. A lower pH resulted in an unresolved chromatographic peak and significant loss in fluorescence intensity.

Under these conditions, the retention time of the AMT fluorophor was *ca.* 5.4 min. During analyses, interferences resulting from previous injections were frequently encountered. These interferences were eliminated by a 4-min column purge using methanol–acetonitrile (95:5, v/v) followed by a 6-min postrun time allowing column reequilibration. The total run time per sample was as short as 16 min. Chromatograms of blank and spiked plasma are shown in Fig. 4.

3.4. Analyte heat stability

The stability of AMT in plasma under conditions selected for heat-inactivation of the human immunodeficiency virus (HIV) was investigated. One set of the quality control samples (5, 40 and 80 ng/ml, 6 replicates per concentration) was incubated for 4 h at 58°C while the other set was kept at 4°C. The samples were then analyzed by the HPLC method described above. Results showed no statistically significant differences in AMT concentrations between the two sets at 5%. The 90% confidence interval of AMT levels in heated samples over

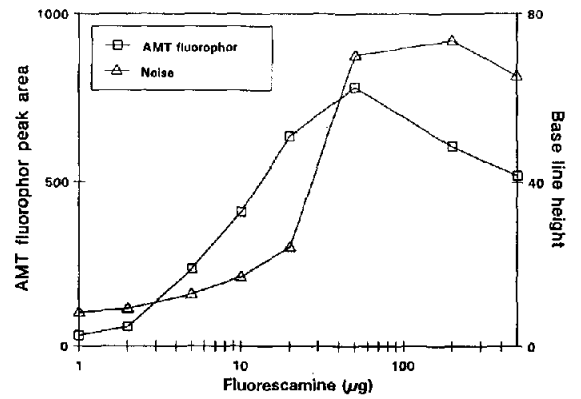

Fig. 3. Effect of fluorescamine on fluorescence intensity of the AMT fluorophor.

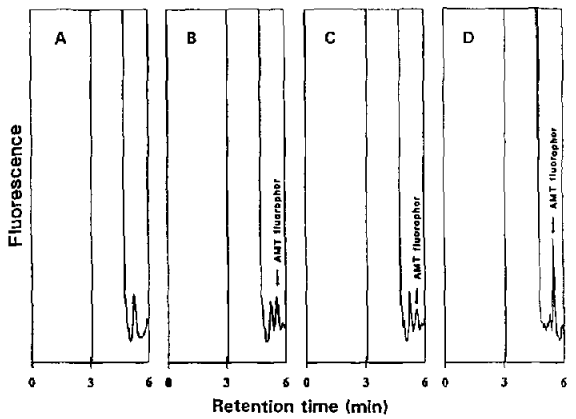

Fig. 4. Representative chromatograms. (A) Blank plasma; (B) blank plasma spiked with 5 ng/ml of AMT; (C) blank plasma spiked with 3 ng/ml of AMT (quantification limit); (D) patient's plasma sample obtained 2 h after an oral dose of 500 mg of AZT. Measured concentration: 15.6 ng/ml.

control samples, which was 90%–105%, fell within the interval of 90%–111%, indicating that the heat-inactivation procedure had no effect on AMT stability. Following heat-inactivation, AMT concentrations were 97.5% of the corresponding levels of non-heated samples.

3.5. Selectivity

Selectivity is always the major problem after extraction and derivatization of plasma samples due to the presence of various endogenous amines. These substances can also react with fluorescamine, forming fluorescent pyrrolinones which might interfere with the AMT fluorophor. However, more than fifteen human blank samples were analyzed and no interferences were observed as shown in a representative chromatogram in Fig. 4.

3.6. Limit of detection and quantification

The limit of detection, defined by a signal-to-noise ratio of 3:1, was below 2 ng/ml using a 200-μl sample. At this concentration, however, the intra-assay coefficient of variation was not acceptable (28.0%, $n = 6$). The limit of quantification was 3 ng/ml using 200 μl of plasma. The intra-assay coefficient of variation at this concentration was 9.4% ($n = 5$). A chromatogram of a sample spiked at 3 ng/ml is shown in Fig. 4C. A representative chromatogram of a patient sample is shown in Fig. 4D.

3.7. Linearity

The relationship between peak area and the concentrations of AMT was linear over the range of 3–1000 ng/ml using a 200-μl plasma sample ($r = 0.99$).

3.8. Extraction recovery

The recovery of AMT from plasma was investigated over the range 3–200 ng/ml. AMT was added, in five replicates, to blank plasma to final concentrations of 3, 10, 50, 100 and 200 ng/ml and analyzed as described above. An equal number of blank samples was simultaneously processed in a similar manner as the spiked samples and AMT was added to the final extracts of these blank samples. Recovery from the spiked extracts was considered to be 100% and that from the spiked plasma was estimated by comparing the peak areas. Results are summarized in Table 1. The mean recovery was 94.4%.

3.9. Precision and accuracy

The intra-assay precision and accuracy were measured by simultaneously assessing quality control samples (5, 40 and 80 ng/ml) in replicates of five. Results are presented in Table 2. The coefficient of variation ranged from 2.9 to 6.6%.

The inter-assay precision and accuracy were determined over a period of three months by using the quality control samples. Results are

Table 1
Recovery of AMT from human plasma

Concentration (ng/ml)	Recovery (%)		Coefficient of variation (%)
	Mean	Range	
3	94.8	82.8–106.8	12.7
10	94.4	92.5–96.3	2.0
50	93.4	90.3–96.6	3.3
100	95.6	93.2–98.0	2.5
200	93.8	92.0–95.6	2.0

Table 2
Intra-assay reproducibility from quality control samples

Concentration (ng/ml) ($n = 5$)	Measured concentration (ng/ml)		Coefficient of variation (%)
	Mean	Range	
5	5.20	5.04–5.36	3.1
40	42.7	39.9–45.6	6.6
80	84.7	82.3–87.6	2.9

Table 3
Inter-assay reproducibility from quality control samples

Concentration (ng/ml)	Replicates	Measured concentration (ng/ml)		Coefficient of variation (%)
		Mean	Range	
5	17	5.11	4.77–5.45	6.7
40	21	40.7	39.7–41.7	2.5
80	22	77.5	76.0–79.0	1.9

summarized in Table 3. The coefficient of variation varied from 1.9 to 6.7%.

3.10. Application to biological samples

Using this novel HPLC methodology, AMT plasma levels were determined following oral administration of AZT to AIDS patients. Large inter-individual variations in AMT plasma levels were observed (data not shown). An AMT plasma concentration–time curve after administration of a 500-mg oral dose of AZT to a naive patient is illustrated in Fig. 5. AMT exhibited a half-life of 1.1 h as estimated by a non-compartmental pharmacokinetic analysis of the above kinetics.

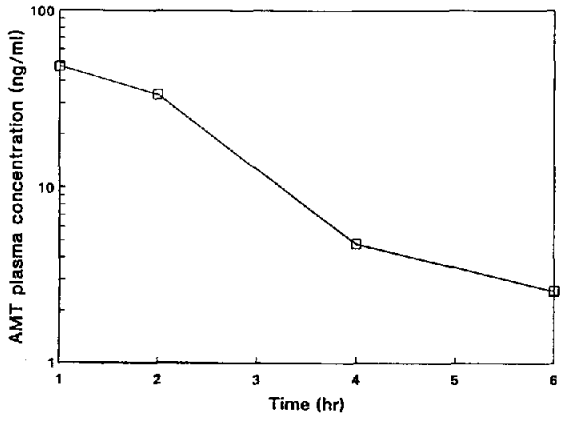

Fig. 5. Time course of plasma AMT level in a patient following an oral dose of 500 mg AZT.

4. Conclusion

A specific and highly sensitive HPLC assay for the quantification of AMT was developed using precolumn derivatization with fluorescamine and fluorescence detection. This method represents a successful application of fluorescamine as fluorogenic reagent in the determination of drug concentration in plasma. The procedure which requires only a small sample volume (200 μl) has a very low quantification limit (3 ng/ml). The short run time per sample (16 min) allows automated sampling with as many as fifty analysis being performed in one day. This assay

should permit evaluation of the pharmacodynamic consequences of AMT formation in AZT treated patients and possibly prevent drug–drug interaction which may lead to unwanted toxic effects.

5. Acknowledgments

This work was supported by Public Health Service Grant AI-32775. J.P.S. is the recipient of a Faculty Research Award from the American Cancer Society, Atlanta, Georgia.

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