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Short Communication

Quantification of zidovudine and one of its metabolites in plasma and urine by solid-phase extraction and high-performance liquid chromatography

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Abstract

A rapid, sensitive and selective method for the quantification of zidovudine (AZT) and one of its metabolites (the 5′-glucuronyl form of zidovudine, G-AZT) in biological fluids is described, based on high-performance liquid chromatography. Solid-phase extraction on-line with chromatographic separation was used. The limit of quantitation of the assay is 10 ng/ml of plasma or urine for G-AZT and 20 ng/ml of plasma or urine for AZT.

1. Introduction

Zidovudine (or 3′-azido-3′-deoxythymidine, azidothymidine, AZT) is a pyrimidine dideoxynucleoside. It is an inhibitor of reverse transcriptase and used in the treatment of human immunodeficiency virus infection. The drug is widely distributed in the body, with a volume of distribution of 1.4 l/kg, and is for approximately 20% bound to plasma proteins. The use of this drug is accompanied with serious toxic side-effects. Anemia and neutropeny are the most common toxicities associated with zidovudine therapy. The drug has a short half-life of one hour and is metabolised by conjunction in the liver to a glucuronide (75% of the dose), the 3′-azido-3′-deoxy-5′βD glucopyranosyl thymidine (G-AZT), which can be recovered in the urine and in the plasma [1]. This glucuronide has no antiviral activity.

Zidovudine has been assayed by several methods like RIA (radioimmuno assay) [2], ELISA (enzyme-linked immunosorbent assay) [3] or high-performance liquid chromatography (HPLC) [4–10].

The method presented here for the assay of zidovudine and its metabolite uses reversed-phase HPLC, with UV detection at 270 nm.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of the following components: two pumps (Isochrom, Spectra-Physics, Les Ulis, France), detector (Spectra 100, Spectra-Physics), integrator (Spectra-Station) and autosampler (360 Kontron, Kontron

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Instruments, Montigny-le-Brettoneux, France). A Novapak RP18 (300 × 3 mm I.D., end-capped 4-μm) analytical column (Millipore-Waters, St. Quentin-en-Yvelines, France) with a Bondapak C_{18} (20 × 2 mm I.D., end-capped 40-μm) guard column (Millipore-Waters) and a Perisorb RP8 (30 × 3.9 mm I.D., end-capped 40 μm) extraction column (Merck Clevenot, Nogent-sur-Marne, France) were used.

2.2. Chemicals

All reagents were of analytical reagent grade (>99% purity). They include methanol (Merck, France) and tetrahydrofuran (Rathburn, Walkerburn, UK). Potassium phosphate buffer (adjusted to pH 3.1 with orthophosphoric acid (Prolabo, France)) was made with potassium dihydrogenphosphate (Merck) and distilled water.

2.3. Chromatography

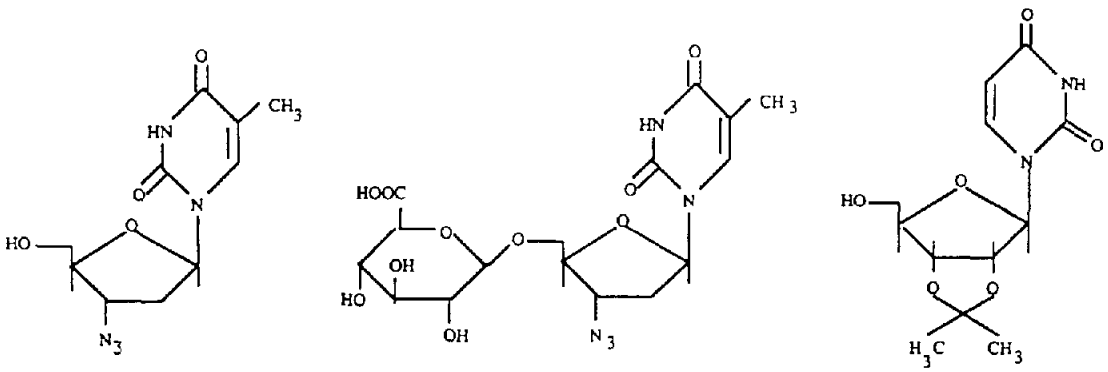
Biological samples were extracted and purified with an extraction column (2 min) (Perisorb C_8). The flow-rate of the first pump was 0.7 ml/min. The mobile phase consisted of methanol–tetrahydrofuran–potassium phosphate buffer (0.025 *M*, pH 3.1) (3.7:2.8:93.5, v/v). Analysis was performed in an isocratic mode. The mobile phase was filtered through a 0.45-μm filter and degassed by ultrasonication prior to use. The flow-rate was 1 ml/min. The absorbance of the effluent was monitored at 270 nm using a fixed-wavelength detector at 0.01 AUFS. A constant injection volume was obtained with an autosampler with a 200-μl injection loop. The assay was performed at ambient temperature.

2.4. Standards

The analytical standards were zidovudine obtained from Wellcome and Sigma and G-AZT obtained from Wellcome. The internal standard 2-O-isopropylidene uridine (IPU) was obtained from Sigma. Structures of the drugs and of the internal standard are given in Fig. 1. Standards of the compounds and internal standard were prepared at 250 mg/l in distilled water and were stored at +4°C. Dilutions of these stock solutions were used to prepare the appropriate working standard solutions (mixed standard solutions).

2.5. Sample preparation

Samples were prepared in haemolysis vials (for single use) according to their type (plasma samples or urine samples). Plasma samples (500 μl) were deproteinated by the addition of 500 μl of perchloric acid (35%). Samples were vortex-mixed for one minute and centrifuged for 5 min at 4000 *g* at +4°C. Preparation of samples used for calibration: to 200 μl of control plasma (lyotrol N, Biomerieux, France) spiked with different concentrations of AZT and G-AZT



AZT G-AZT IPU

Fig. 1. Structure of AZT, G-AZT, and IPU.

(0.05, 0.1, 0.2, 0.5, 0.75, 1 and 2 μg/ml) 800 μl of internal standard solution (0.25 μg/ml) were added.

Preparation of blank control: to 200 μl of control plasma 800 μl of buffer potassium phosphate were added. Preparation of the sample itself: prior to analysis, all samples from AIDS patients were heat-inactivated in a waterbath for one hour at 58°C and samples were stored at −20°C until analysis. To 200 μl of patient deproteinated plasma 800 μl of internal standard (0.25 μg/ml) were added. Urine was first diluted 1:50 with deionized water, because of the very high levels of G-AZT (*ca.* 50 times as high as the plasma levels).

2.6. Calibration

Calibration curves were obtained by plotting the peak-area ratios (the peak area of compounds to the peak area of the internal standard) as a function of the concentration of AZT or G-AZT per ml of plasma. The concentrations of AZT and G-AZT in a patient sample were calculated by interpolation from the standard curves.

3. Results

Fig. 2 shows a chromatogram of a lyotrol sample containing AZT and G-AZT and a chromatogram of a blank plasma. Fig. 3 shows a chromatogram of a plasma sample and Fig. 4 a chromatogram of a urine sample and a chromatogram of blank urine. Retention times were 11.7 min for G-AZT, 14 min for the internal standard, and 16.8 min for AZT. The relationship between the concentration of AZT and G-AZT and the peak-area ratio was linear for concentrations in the range of 0.02–2 μg/ml for AZT and in the range of 0.01–2 μg/ml for G-AZT. The correlation coefficients were higher than 0.999 for both compounds with slopes of $1.33 \cdot 10^{-3}$ for AZT and $7.7 \cdot 10^{-4}$ for G-AZT, respectively and the corresponding intercepts were −0.044 and −0.041. Tables 1 and 2 show the precision of the method for AZT and G-AZT, as evaluated from the intra- and inter-assay variabilities. The limit of quantification was 0.02 μg/ml of plasma or urine for AZT and 0.01 μg/ml of plasma or urine for G-AZT.

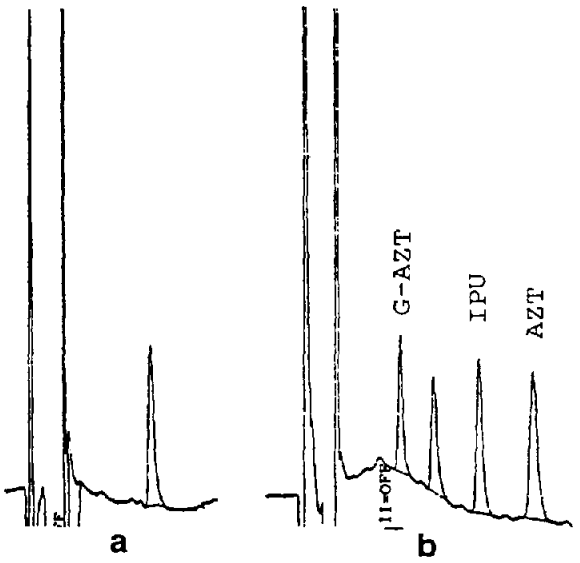


Fig. 2. (a) Chromatogram of blank plasma. (b) Chromatogram of lytotrol containing 1 μg/ml AZT, G-AZT, and IPU (internal standard).

4. Discussion

Several methods have been used to determine AZT and its metabolite (the 5'-glucuronyl form

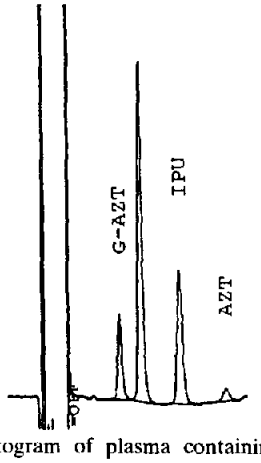


Fig. 3. Chromatogram of plasma containing 0.760 μg/ml G-AZT, 0.12 μg/ml AZT, and 1 μg/ml IPU (internal standard).

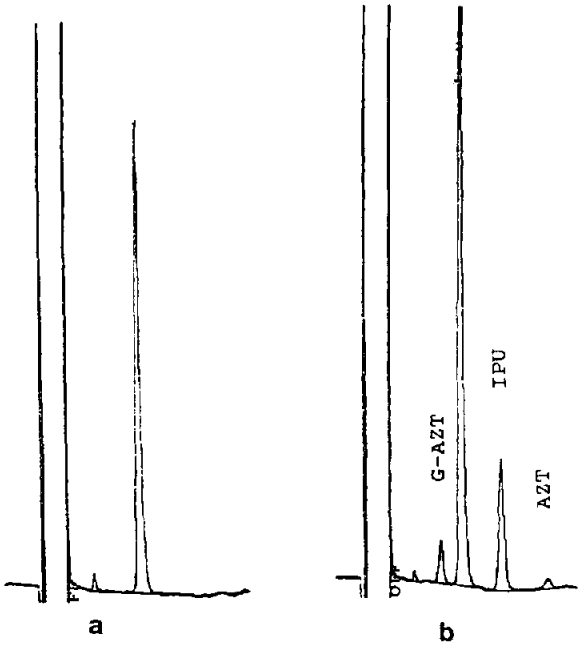


Fig. 4. (a) Chromatogram of blank urine. (b) Chromatogram of urine containing 5 μg/ml AZT, 30 μg/ml G-AZT, and 1 μg/ml IPU (internal standard). Dilution 1:50.

of zidovudine) and have been described in the literature.

All chromatographic methods described used two steps. The first being extraction (solid-phase extraction or liquid-phase extraction) and the second chromatographic analysis. Four reports [4,5,7,8] used solid-phase extraction before the analytical separation. Two studies used liquid–liquid phase extraction with ethyl acetate–diethyl ether mixture (50:50, v/v) [10] or chloroform–isopropyl alcohol (5:95, v/v) [6]. Another report [9] proposed deproteination of the plasma samples with trichloroacetic acid.

In our method, we used a solid-phase extraction on-line with the chromatographic analysis. One of the advantages of this method is its rapidity. It circumvents evaporation under nitrogen flow, necessary with solid-phase extraction with cartridges. For the analysis, the ionic strength of the potassium phosphate buffer used as the mobile phase plays an important role. The assay was first carried out at an ionic strength of 0.01 *M* in an aqueous solution. An ionic strength

Table 1
Validation of the method: intra-day assay variability ($n = 10$)

Concentration added (ng/ml)	Concentration found			
	AZT		GAZT	
	Mean ± S.D. (ng/ml)	C.V. (%)	Mean ± S.D. (ng/ml)	C.V. (%)
200	208 ± 6	2.9	199 ± 3	1.5
1000	1005 ± 7	0.7	1000 ± 7	0.7
2000	2006 ± 15	0.7	2002 ± 10	0.5

Table 2
Validation of the method: inter-day assay variability ($n = 10$)

Concentration added (ng/ml)	Concentration found			
	AZT		GAZT	
	Mean ± S.D. (ng/ml)	C.V. (%)	Mean ± S.D. (ng/ml)	C.V. (%)
200	207 ± 8.3	4	204 ± 11.4	1.5
500	504 ± 11.3	2.2	505 ± 10.1	0.7
750	745 ± 37.5	5	754 ± 21.1	0.5
1000	1006 ± 40.2	4	998 ± 22.0	0.5
2000	2008 ± 40.2	2	2010 ± 20.4	0.5

of 0.025 *M* is necessary to favour the separation of the G-AZT peak from an interfering peak present in plasma and urine. Also the pH of the mobile phase is very important. Knowledge of the pK_a of the compounds (9.68 and 3.5 for AZT and G-AZT, respectively) allow us to predict their behaviour and to choose the optimal pH for the mobile phase.

The extraction column should be replaced after *ca.* 200 analysis. After more than 200 injections, the separation deteriorates and some alterations, particularly on the AZT peak, are observed.

5. References

[1] H.D. Langtry and D.M. Campoli-Richards, *Drugs*, 37 (1989) 108–450.
[2] S, Cox, U. Ruden, K. Nagy, J. Albert, V. Holmberg, E. Sandstrom and B. Wahren, *J. Virological Meth.*, 30 (1990) 89–98.
[3] S.M. Tadapelli and R.P. Quinn, *AIDS*, 3 (1990) 19–27.
[4] S.S. Good, D.J. Reynolds and P. De Miranda, *J. Chromatogr.*, 431 (1988) 123–133.
[5] G.G. Granich, M.R. Eveland and D.J. Krogstad, *Antimicrobiol. Agents Chemother.*, 33 (1989) 1275–1279.
[6] M.A. Hedaya and R.J. Sawchuk, *Clin. Chem.*, 34 (1988) 1565–1568.
[7] R.W. Kleker, J.M. Collins, R. Yarchoan, R. Thomas, J.F. Jenkins, S. Broder and C.E. Myers, *Clin. Pharmacol. Ther.*, (1987) 407–412.
[8] R. Kupferschmidt and R.W. Schmid, *Clin. Chem.*, 35 (1989) 1313–1317.
[9] C. Lacroix, T. Phan Hoang, F. Wojciechowski, H. Duwoos, J. Nouveau and B. Diquet, *J. Chromatogr.*, 525 (1990) 381–386.
[10] J.D. Undadkat, J.R. Crosby, J.P. Wang and C.C. Hertel, *J. Chromatogr.*, 430 (1988) 420–423.