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Note

Simultaneous quantification of zidovudine and its metabolites in serum and urine by high-performance liquid chromatography using a column-switching technique

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Zidovudine (AZT, 3′-azido-3′-deoxythymidine, Retrovir®), an antiviral agent with a thymidine-derived structure, is used therapeutically on account of its inhibiting effects on the human immunodeficiency virus (HIV) [1,2]. AZT is eliminated in urine partly (14%) in unchanged form but principally (75%) after conjugation as the 5′-O-glucuronide (GAZT, 3′-azido-3′-deoxy-5′-β-D-glucopyranuronosylthymidine) [3,4].

The current analytical techniques for AZT use radioimmunoassay [5] or high-performance liquid chromatography (HPLC) [6–9]. However, for a complete pharmacokinetic study, it is necessary to have a simultaneous assay for AZT and of its metabolite, as described by Good et al. [10].

This paper describes a direct switching method that reduces the management of samples to a minimum, and hence shortens the assay time considerably.

EXPERIMENTAL

Reagents

AZT and GAZT were kindly supplied by Wellcome Labs. (Research Triangle Park, NC, U.S.A.). HPLC-grade acetonitrile, trichloroacetic acid and tri-

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fluoroacetic acid were obtained from Merck (Darmstadt, F.R.G.). For stock solutions, AZT and GAZT were dissolved in methanol and stored at 4°C.

Mobile phases

Mobile phases A and B were water–acetonitrile (A, 96:4; B, 90:10). Trifluoroacetic acid was added to mobile phases A (200 μl/l) and B (15 μl/l). Both phases were subsequently filtered through a 0.2-μm membrane filter.

Sample preparation

Serum samples (200 μl) were added to 200 μl of trichloroacetic acid (diluted 10% in distilled water). The tube was vortex-mixed for 30 s and centrifuged at 5000 *g* for 5 min, and 200 μl of the supernatant were chromatographed. Standards were prepared from normal human serum spiked with different amounts of AZT and GAZT. Urine samples were diluted at 1:50 or 1:100 in distilled water and compared with aqueous standards.

High-performance liquid chromatography

The assembly was formed of two Gold systems (Beckman, Gagny, France) including two pumps (Models 116 and 126) and a spectrophotometer (Model 167). The first column (Ultrapore® RPMC, 5 μm mean particle diameter, 75 mm×4.6 mm I.D.; Altex, Berkeley, CA, U.S.A.) was connected through a six-way switching valve to the analytical column (Ultrasphère® ODS, 3 μm mean particle diameter, 75 mm×4.6 mm I.D.; Altex). The whole system, including the pumps, the spectrophotometer, the switching valve, the analysis cycle monitor and the peak integrator, was managed by a microcomputer (Tandon, Colombes, France). The absorbance of the eluent was monitored at 267 nm. Both columns were thermostatted at 25°C.

During the injection of the sample, the system was in position 1 (mobile phase A, flow-rate 1 ml/min) and the eluent from the first column was directed to waste (Fig. 1). The switching valve was activated in position 2 from 6.0 to

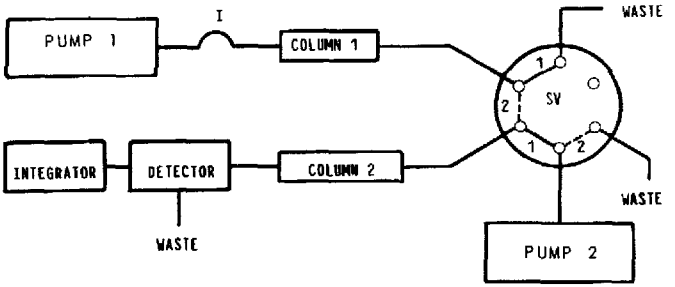

Fig. 1. Diagram of the chromatographic system. Position 1: mobile phase A (water–acetonitrile, 96:4, with trifluoroacetic acid, 200 μl/l); position 2: mobile phase B (water–acetonitrile, 90:10, with trifluoroacetic acid, 15 μl/l).

8.5 min, and the eluent was then directed toward the analytical column. Then, 8.5 min after the injection, the valve was inactivated in position 1 and the captured fraction was chromatographed on column 2 using mobile phase B and pump 2 (flow-rate 1 ml/min).

Since the supernatant was deproteinized by trichloroacetic acid, it was not necessary to regenerate column 1 after each injection.

RESULTS AND DISCUSSION

Typical elution profiles from human serum and from urine are shown in Fig. 2. Retention times of GAZT and AZT are 10.0 and 13.2 min, respectively.

The very good separation of the solutes from normal human serum and from urine yields a high sensitivity of detection. The composition of the mobile phase in pump 1 is selected in such a way that AZT and GAZT are simultaneously eluted; consequently, the time required to activate the second column can be reduced. The acid deproteinization does not induce any loss of AZT or GAZT in the protein precipitate. The recovery is determined from the comparison of peak areas obtained after injection of aqueous standard and of deproteinized serums of equivalent concentrations. The results range from 98.8 to 101.4%. The concentrations studied were 20, 50, 250, 500, 1000, 2000 and 5000 ng/ml. Each determination was made in duplicate.

The linearity, as evaluated from 20 to 5000 ng/ml, yields the following equations: $y=3.36\cdot10^{-3}x-6.12\cdot10^{-3}$ ($r=0.9997$) for AZT and $y=2.23\cdot10^{-3}x-7.39\cdot10^{-3}$ ($r=0.9998$) for GAZT, where y is the peak area and x is the serum concentration. Each point was determined from three assays.

The within-day reproducibility (Table I) is very good: the highest coeffi-

TABLE I

WITHIN-DAY AND DAY-TO-DAY COEFFICIENTS OF VARIATION OF THE ASSAYS AS DETERMINED IN HUMAN SERUM SPIKED WITH DIFFERENT AMOUNTS OF AZT AND GAZT

Concentration (ng/ml)	Coefficient of variation (%)			
	Within-day ($n=6$)		Day-to-day ($n=8$)	
	AZT	GAZT	AZT	GAZT
2500	3.7	3.8	-	-
1870	-	-	12	12.5
1250	3.7	3.9	-	-
625	3.2	3.5	-	-
160	3.5	3.9	-	-
125	-	-	5	4.9
40	5.5	6.3	-	-

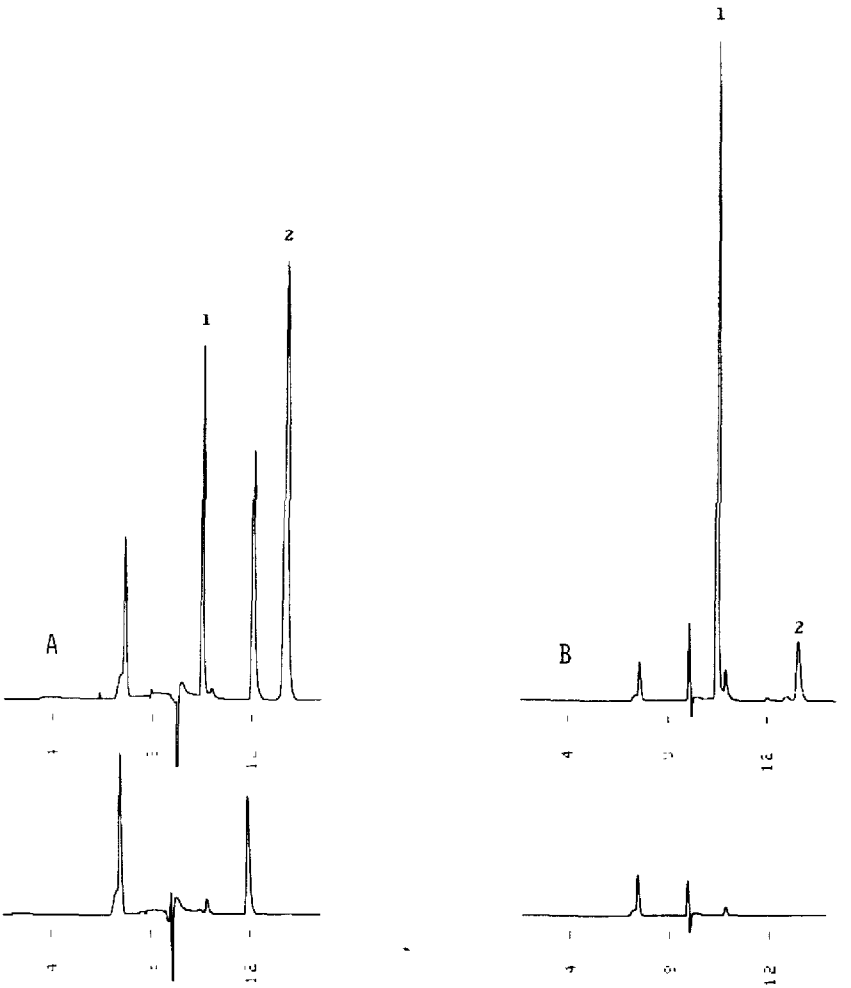

Fig. 2. (A) Chromatograms obtained from the serum of a patient treated with AZT (upper trace) (AZT 1305 ng/ml; GAZT 949 ng/ml) and from a blank serum (lower trace). (B) Chromatograms obtained from urine (diluted 1:100) (AZT 15.25 mg/l; GAZT 184 mg/l) (upper trace) and from blank urine (lower trace). Peaks: 1 = GAZT; 2 = AZT.

cients of variation (C.V.) for AZT and GAZT, respectively, are 5.5 and 6.3% ($n=6$), for a concentration of 40 ng/ml. The day-to-day C.V. have been determined in duplicate from spiked serum samples (1870 and 125 ng/ml) for eight consecutive days (Table I). At a signal-to-noise ratio of 3, the detection limits of AZT and GAZT are 25 and 20 ng/ml, respectively.

The possibility of interferences from associated medications in this type of illness (antibiotics, antifungal agents, antituberculous drugs) was considered.

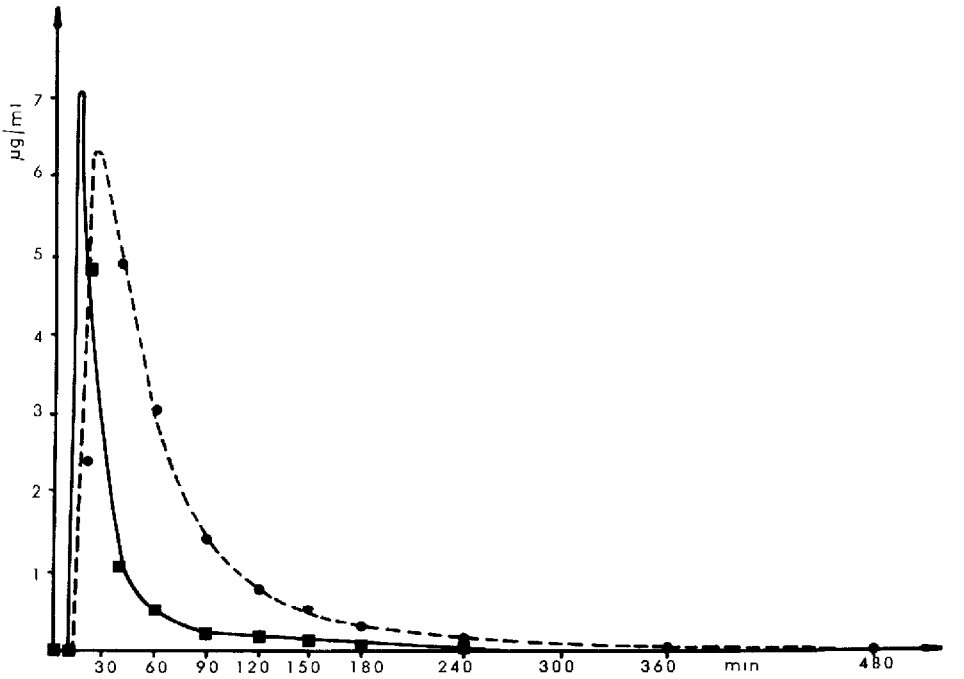

Fig. 3. Serum concentration–time curves of (■) AZT and (●) GAZT after the first oral administration of 300 mg.

The following substances were not detected: acetaminophen, amphotericin B, aztreonam, cefotaxime, cefotetan, cefsulodin, ceftazidime, clobazam, clonazepam, chloramphenicol, diazepam, 5-fluorocytosine, isoniazid, pyrazinamide and rifampicin. Caffeine and theophylline showed retention times of 12.5 and 11.5 min, respectively, which are sufficiently different from those of AZT and GAZT.

Fig. 3 shows the kinetics of administration in a patient receiving 300 mg every 6 h.

The method proposed here is simple, specific and well adapted to the pharmacokinetic study of these substances. Moreover, it is extremely rapid since the sample preparation necessitates only a deproteinization. In comparison, the technique of Good et al. [10], which also enables the simultaneous assay of AZT and GAZT, entails an analysis time 34 min since it is necessary to wash out and to equilibrate the column after each injection. Moreover, this time is increased by the preliminary extraction of the components.

This method using a column-switching system has three important advantages: (1) its very good sensitivity; (2) the reduced total analysis time; and (3) the almost total absence of sample preparation of very contaminating biological material.

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