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SIMULTANEOUS QUANTIFICATION OF ZIDOVUDINE AND ITS GLUCURONIDE IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive high-performance liquid chromatography (HPLC) assay has been developed to simultaneously determine levels of the anti human immunodeficiency virus agent, zidovudine (AZT), and its major metabolite (the 5′-O-glucuronide) in serum. Samples were first mixed with an internal standard (a stereoisomer of AZT), then prepared for analysis using solid-phase extraction columns and chromatographed using a reversed-phase analytical column. Isocratic elution with a mobile phase of 15% acetonitrile, buffered to pH 2.70 with ammonium phosphate, gave good resolution of the three analytes and endogenous serum components. The HPLC analysis time required per sample was 34 min and analyte recoveries were reproducibly high (greater than 93%). Replicate analyses of prepared standards gave satisfactory precision and accuracy, with coefficients of variation less than 15% and deviations from expected concentrations less than 10%.

INTRODUCTION

The nucleoside analogue, zidovudine (AZT, 3′-azido-3′-deoxythymidine, Retrovir®, BW A509U), which has potent in vitro activity against human immunodeficiency virus (HIV) [1–3], has been studied in clinical trials [4–7] and approved for the treatment of certain patients with acquired immune deficiency syndrome (AIDS) and AIDS-related complex (ARC). In man, the drug displays a short half-life [8–10] and is metabolized extensively to the 5′-O-glucuronide (3′-azido-3′-deoxy-5′-β-D-glucopyranuronosylthymidine, GAZT) [9–11]. Shortly after intravenous or oral administration of AZT, both parent compound and metabolite can be detected in serum, with concentrations of the glucuronide usually exceeding those of AZT. Although the conjugated form of the drug is chemically and biologically stable and has no antiviral activity, it is often of interest to determine serum levels of both drug and metabolite, especially when

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investigating potential drug–drug interactions, cases of impaired hepatic or renal functions and other situations which might affect drug disposition.

A high-performance liquid chromatographic (HPLC) method for the determination of AZT in human plasma has been reported [8]. However, it does not allow for the quantification of GAZT. A recent communication from these laboratories [10] briefly describes an HPLC method for the determination of both AZT and GAZT in serum and urine. In this technique, ultrafiltered samples were eluted from a reversed-phase analytical column using an acetonitrile gradient in a mobile phase buffered to pH 7.2. The HPLC analysis time required per sample, however, was 66 min. This paper describes a faster HPLC method which has been developed for routine quantification of both AZT and GAZT in human serum.

EXPERIMENTAL

Materials and reagents

AZT and 1-(3-azido-2,3-dideoxy-β-D-*threo*-pentofuranosyl)thymine (the 3′-β-azido stereoisomer of AZT which was used as the internal standard, I.S.) were provided by Mr. B. Sickles of the Wellcome Research Labs. (Research Triangle Park, NC, U.S.A.). Both were greater than 99.5% chromatographically pure. Structures of AZT, GAZT and I.S. are presented in Fig. 1. GAZT was isolated and purified to greater than 99% from the pooled urine of two AIDS patients receiving AZT (detailed procedure to be published elsewhere). The structure and purity of GAZT was confirmed by infrared, nuclear magnetic resonance (proton and carbon-13) and mass (chemical ionization and fast atom bomdardment) spectroscopies, in addition to HPLC and carbon, hydrogen and nitrogen analyses. All analyses were consistent with the proposed structure for GAZT. The isolated GAZT was further characterized by quantitative enzymatic conversion to AZT using β-glucuronidase (EC 3.2.1.31) from either *Escherichia coli* or bovine liver (Sigma, St. Louis, MO, U.S.A.).

HPLC-grade acetonitrile, methanol and phosphoric acid were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Water was deionized and filtered through activated charcoal. All other chemicals were of reagent grade.

Preparation of standards

Stock solutions of AZT, GAZT and I.S. were prepared in water and their concentrations were determined spectrophotometrically based on their molar ab-

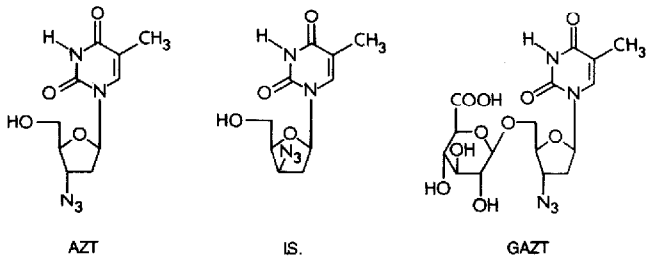

Fig. 1. Structures of AZT, its glucuronide metabolite (GAZT) and the internal standard (I.S.).

sorptivities in 0.1 *M* sodium hydroxide at 267 nm (ϵ = 7750 for AZT, ϵ = 7650 for GAZT and ϵ = 8100 for I.S.). Normal human serum standards containing known amounts of both AZT (0.1–20 μM) and GAZT (0.2–40 μM) were prepared by combining small aliquots of the two stock solutions and serially diluting the mixture with normal human serum. About 50 ml of each standard were prepared, divided into 1.1-ml replicates and stored at −20°C.

Sample preparation and extraction

Prior to analysis, all samples from AIDS or ARC patients were heat-inactivated in a water bath for 60 min at 58°C. It has been reported [12] that this procedure effectively inactivates any HIV particles present in serum samples. Samples were then stored at −20°C until analysis.

Samples and standards (0.5 ml) were individually mixed with 0.1 ml of 25 μM I.S. and allowed to equilibrate for at least 10 min while the solid-phase extraction columns were prepared. Extraction columns (high-hydrophobic octadecyl, 500 mg) (J.T. Baker, Phillipsburg, NJ, U.S.A.) were mounted on a Vac Elut manifold (Analytichem International, Harbor City, CA, U.S.A.) and, by applying reduced pressure, were prewetted with one column reservoir volume of methanol and rinsed with two reservoir volumes of phosphate-buffered saline (PBS).

Each sample mixture was drawn into an extraction column by reapplying reduced pressure. The samples were then allowed to equilibrate for at least 2 min before each column was washed with 1.0 ml PBS. Up to this point in the extraction process, care was taken not to allow the columns to become dry. This operation was simplified by inserting a one-way stopcock (Applied Separations, Bath, PA, U.S.A.) between each column and the vacuum manifold so that the flow could be stopped just as the meniscus entered the column. Following the PBS wash, the columns were dried by reapplying reduced pressure for 3–5 min. Samples were then eluted into clean test tubes with 2×1.0 ml methanol. Columns were completely drained after each elution. The samples were then evaporated to dryness in a Speed Vac (Savant Instruments, Farmingdale, NY, U.S.A.), redissolved in 0.2 ml of 15% aqueous acetonitrile, centrifuged to remove particulates (2500 *g*, 5 min) and analyzed by HPLC.

High-performance liquid chromatography

Two Constametric pumps united in parallel with a Dynamic Mixer (both from Milton Roy, Riviera Beach, FL, U.S.A.) were used to pump and mix the mobile phase at a constant flow-rate of 1.0 ml/min. Samples (100 μl) were applied with an automatic injection system (WISP 712, Waters Assoc., Milford, MA, U.S.A.) onto an Adsorbosphere™ C_{18} guard column (5 μm, 10 mm×4.6 mm I.D.) and analytical column (5 μm, 250 mm×4.6 mm I.D.) (Alltech. Assoc./Applied Science, State College, PA, U.S.A.) which were preceded by an in-line filter (0.5 μm, Scientific Systems, State College, PA, U.S.A.). The absorbance of the effluent was monitored at 267 nm using a variable-wavelength detector (SpectroMonitor III or 3000, Milton Roy). Sample elution was isocratic over 10 min, using a mobile phase (buffer A) of a 15:85 mixture of acetonitrile–ammonium phosphate buffer [0.1% (v/v) phosphoric acid, adjusted to pH 2.70 with ammonium hydroxide].

Buffer A was freshly prepared before each set of analyses. All chromatography was performed at room temperature (23–26 °C).

During analyses of samples from patients taking numerous concurrent medications, interferences from previous injections were frequently encountered. These interferences were eliminated with a 4-min column purge with 80% (v/v) aqueous acetonitrile followed by a 12-min reequilibration period in buffer A between each injection. To prolong column lifetime, 4-min linear gradients were used between the two mobile phases. Total flow-rate was always maintained at 1.0 ml/min. Thus, the total analysis time per sample was 34 min.

Analyte peak areas were digitized and integrated using a DS-80Z microcomputer (Digital Specialities, Chapel Hill, NC, U.S.A.). Standard curve parameters were obtained from an unweighted least-squares linear regression analysis of the ratios of peak areas for AZT (or GAZT) to I.S. as a function of prepared concentrations. Unknown concentrations were calculated using each observed sample/internal standard peak-area ratio and the standard curve parameters.

RESULTS

Typical elution profiles from extracts of human serum are shown in Fig. 2. The method provides good resolution of GAZT and essentially baseline resolution of

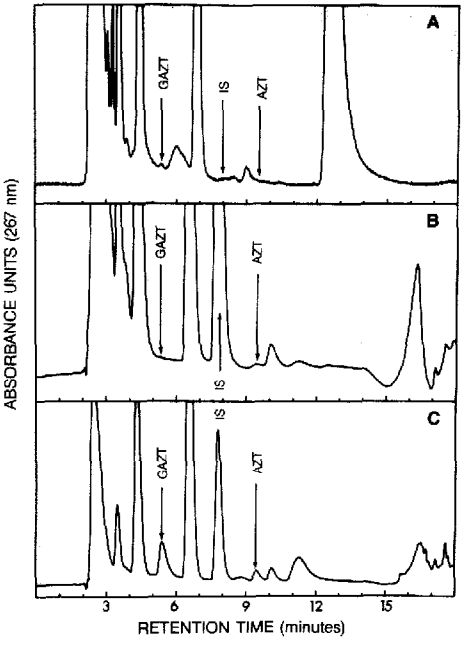

Fig. 2. Representative chromatographic profiles obtained from the analysis of extracted human sera. Panel A, normal serum pooled from five donors (0–0.01 a.u.f.s.); panel B, serum obtained from an AIDS patient prior to receiving oral AZT (0–0.01 a.u.f.s.); panel C, serum obtained 2.5 h after therapy, containing 1.53 μM GAZT and 0.45 μM AZT (0–0.03 a.u.f.s.). Retention times for GAZT, I.S. and AZT are indicated.

AZT and I.S. from normal serum constituents, even when viewed at high sensitivity.

Analyte heat stability

The stabilities of AZT and GAZT in serum under the conditions chosen for heat-inactivation of HIV were investigated. Normal serum samples containing AZT (0.44–44 μM) and GAZT (1.5–150 μM) were prepared and divided in half. One half was incubated for 60 min at 58 °C while the other was kept at 4 °C. The samples were then analyzed by HPLC as described. No breakdown of either AZT or GAZT was observed. Following heat-treatment, mean ($\pm$ S.D.) AZT concentrations were $101.0 \pm 0.6\%$ ($n=4$) of corresponding levels from non-heated samples. Mean GAZT concentrations were $101.7 \pm 1.1\%$ ($n=4$) of non-heated levels.

Recoveries from extraction columns

In order to determine recoveries of AZT, GAZT and I.S. from the solid-phase extraction columns, aqueous and normal serum samples were identically prepared to contain each compound at three different concentrations (Table I). Five replicates of each aqueous and serum standard were extracted and analyzed. Recoveries were calculated by comparing observed analyte concentrations to those obtained from the analysis of unextracted aqueous samples (six replicates each). With the exception of the serum standard containing the lowest concentration of I.S., where the recoveries averaged 93.9%, all mean recoveries were greater than 95%. The reproducibility of the recoveries was good, with all coefficients of variation (C.V.) less than 5%.

TABLE I

ANALYTE RECOVERIES AFTER EXTRACTION

Aqueous and normal serum samples were identically prepared to contain GAZT, I.S. and AZT at three different concentrations. Replicates (5) of each sample were extracted and analyzed by HPLC. Recoveries are expressed as observed analyte concentrations as a percent of the corresponding concentrations obtained from the HPLC analysis of the unextracted aqueous samples (six replicates each). Numbers in parentheses are coefficients of variation (%).

Prepared concentration (μM)	Mean recoveries after extraction (%)					
	Aqueous standards			Spiked serum standards		
	GAZT	I.S.	AZT	GAZT	I.S.	AZT
25	99.1 (0.9)	100.7 (0.2)	100.8 (0.3)	99.9 (1.3)	98.9 (1.1)	96.6 (1.1)
5	99.3 (0.9)	101.1 (1.0)	101.0 (1.3)	102.7 (3.5)	98.1 (3.9)	95.1 (4.1)
1	100.7 (4.8)	107.7 (4.1)	98.6 (1.5)	106.3 (0.6)	106.3 (4.2)	93.9 (1.8)

Standard curves

For each set of samples analyzed, a five to eight point standard curve was generated by thawing and extracting aliquots of the prepared standards (see Experimental). Before the supply of these aliquots was depleted, new standards were prepared as described and checked against the old standards in the same assay. Standards prepared in serum and stored at −20°C were shown to be stable for at least five months.

Peak-area ratio (AZT or GAZT peak area divided by I.S. peak area) was a linear function of concentration between 0.1 and 20 μM AZT and between 0.2 and 40 μM GAZT. Occasionally, the lowest concentration had to be omitted from the linear regression, usually due to the deteriorating condition of the guard column, analytical column and/or mobile phase. At concentrations between 0.03 and 0.1 μM, signal-to-noise ratios were sufficiently high (between 3 and 8) and baselines were flat enough to identify peaks corresponding to AZT but accurate quantification could not be achieved.

Precision and accuracy

The intra-assay precision was measured by simultaneously assaying six replicates of normal serum standards containing both AZT (11, 2.2 and 0.55 μM) and GAZT (10, 2.0 and 0.5 μM). Except for the determination of the lowest GAZT standard, for which the coefficient of variation was 10.6%, all coefficients of variation were below 4% (Table II).

Inter-assay accuracies were also found to be good throughout the ranges of the standard curves (Tables III and IV). Not unexpectedly, the highest variabilities were seen at the lowest concentrations. For all points, the observed mean was within 11% of the prepared value. Acceptable coefficients of variation (less than 15%) were obtained for all standards except the lowest GAZT standard, for which it was 40%. Variabilities tended to increase as analyte concentrations decreased.

All standard curves had correlation coefficients greater than 0.996 and showed low variability in their calculated slopes (Tables III and IV). The fact that the y-intercepts were close to zero (standard curves were not forced through the origin)

TABLE II

INTRA-ASSAY PRECISION FOR THE DETERMINATION OF AZT AND GAZT

Normal human serum samples were prepared to contain AZT and GAZT at three different concentrations and six replicates of each sample were individually extracted and analyzed by HPLC in a single assay.

Standard	Measured concentration (μM)		Coefficient of variation (%)
	Mean	Range	
AZT	10.8	10.7–11.2	1.5
	2.22	2.18–2.27	1.4
	0.57	0.54–0.60	3.8
GAZT	10.0	9.4–10.5	3.8
	1.91	1.84–1.99	2.9
	0.38	0.34–0.43	10.6

TABLE III

INTER-ASSAY ACCURACY AND PRECISION FOR AZT DETERMINATIONS

Normal human serum samples were carefully prepared in bulk to contain the indicated concentrations of AZT. Aliquots were extracted and analyzed by HPLC on different days using a separate standard curve for each set of aliquots.

Prepared standard concentration (μM)	Number of observations	Measured concentration (μM)		Coefficient of variation (%)	Deviation (%)
		Mean	Range		
20	14	20.2	19.7–20.7	1.4	+0.9
10	17	10.0	9.43–10.3	3.4	+0.1
5.0	16	4.92	4.86–5.02	2.4	−1.6
2.0	18	2.03	1.89–2.18	3.8	+1.7
1.0	17	0.980	0.85–1.04	4.0	−2.0
0.50	15	0.514	0.46–0.57	7.7	+2.8
0.20	14	0.259	0.18–0.31	13.0	+3.4
0.10	9	0.109	0.08–0.13	14.2	+9.7

Standard curve parameter	Number of observations	Mean	Range	Coefficient of variation (%)
Slope	18	0.1840	0.169–0.199	4.4
y-Intercept	18	−0.0005	−0.043–0.018	391
Correlation coefficient	18	0.9997	0.998–1.000	0.001

reflects the observation that there was little interference from normal serum constituents.

Interfering substances

Table V lists the therapeutic and structurally related compounds which were tested for potential interference with the quantification of AZT, GAZT and I.S. using the procedure described. The only observed interferences were with GAZT and were caused by triprolidine and dideoxycytidine.

Assay reproducibility

This HPLC method has now been used successfully by a number of different clinical centers for the quantification of AZT and GAZT in serum and plasma samples. To monitor the reproducibility of the method between centers, five normal human serum standards were prepared to contain different concentrations of both AZT and GAZT (0–13.1 μM for AZT and 0–18.4 μM for GAZT). These standards were divided into replicates, coded and shipped frozen to the various centers for analysis. The replicates were assayed for AZT and GAZT using this method (or minor modifications thereof); six centers have reported results. Mean AZT determinations were all within 7% of the expected concentrations, with coefficients of variation ranging from 4.6% for the highest concentration to 13.5% for the lowest concentration. Mean GAZT determinations were 5–14% above the

TABLE IV

INTER-ASSAY ACCURACY AND PRECISION FOR GAZT DETERMINATIONS

Normal human serum samples were carefully prepared in bulk to contain the indicated concentrations of GAZT, divided into aliquots and stored at −20°C. Individual aliquots were extracted and analyzed by HPLC on different days using a separate standard curve for each set of aliquots.

Prepared standard concentration (μM)	Number of observations	Measured concentration (μM)		Coefficient of variation (%)	Deviation (%)
		Mean	Range		
40	14	40.4	38.0–41.8	2.8	+1.1
20	17	19.7	17.7–20.3	4.1	−1.5
10	16	9.78	9.14–10.6	4.0	−2.2
4.0	18	4.05	3.86–4.28	4.5	+2.2
2.0	16	2.03	1.94–2.21	3.9	+1.5
1.0	18	1.06	0.90–1.44	13.6	+5.9
0.50	15	0.52	0.41–0.71	14.5	+4.2
0.20	10	0.22	0.14–0.43	40.0	+11.0

Standard curve parameter	Number of observations	Mean	Range	Coefficient of variation (%)
Slope	18	0.1772	0.167–0.192	3.9
y-Intercept	18	0.0103	−0.048–0.087	295
Correlation coefficient	18	0.9995	0.997–1.000	0.001

prepared concentrations, with coefficients of variation ranging from 7% for the highest concentration to 29% for the lowest one.

Factors affecting chromatographic resolution

An acidic mobile phase was chosen because protonation of the glucuronic acid moiety in GAZT ($pK_a \approx 3.5$) permits longer retention of the compound on a reversed-phase column. The resolution was particularly sensitive to the pH of the mobile phase. Slight deviations from pH 2.70 significantly affected not only the separation of GAZT but those of AZT and I.S. as well.

Another factor which was found to affect the separation of AZT from the final peak eluting at 13 min was the strength of the buffer. Lowering the concentration of the phosphate in buffer A from 25 mM to the current level (12.4 mM) caused an unexpected 2-min increase in retention time of the peak at 13 min, without much effect on the rest of the chromatogram. Thus, the separation of AZT from this late peak was greatly improved.

Analytical column stability

Although the pH of the mobile phase is close to the commonly suggested limit (pH 2) for use with silica-based analytical columns, reasonable column life-times were observed. Columns could be used for 2000–3000 sample injections if the

TABLE V

COMPOUNDS TESTED FOR POTENTIAL INTERFERENCE

Substance	HPLC retention time (min)
Analytes:	
GAZT	5.3
I.S.	7.5
AZT	9.1
Antibiotics:	
Chloramphenicol	17.5
Dapsone	17.0
Erythromycin	>17.0
Isoniazid	3.8
Penicillin G	12.3
Pyrimethamine	>17.0
Rifampicin	>17.0
Streptomycin	>17.0
Sulfadoxine	14.3
Sulfamethoxazole	17.3
Tetracycline	>17.0
Trimethoprim	>17.0
Antifungals:	
Amphotericin B	>17.0
Ketoconazole	>17.0
Nystatin	>17.0
Antivirals:	
Acyclovir	2.9
Ara-A	3.2
Dideoxycytidine	5.5*
Ribavirin	3.2
Others:	
Acetaminophen	4.5
3′-Amino-3′-deoxythymidine	3.2
Aspirin	17.0
Caffeine	6.1
Diazepam	>17.0
Probenecid	>17.0
Thymidine	2.5
Triprolidine	5.2*

*These substances, if present, will interfere with the quantitation of GAZT.

guard columns were changed at the earliest indication of peak tailing or unacceptable resolution and if the columns were purged with 80% aqueous acetonitrile before system shut-down.

Selection of extraction columns

A number of different sizes and types of solid-phase extraction columns were investigated for their ability to retain AZT, GAZT and I.S. as well as to give

sufficient sample purification to permit resolution of these three analytes from endogenous serum components during HPLC analysis. Of the many functionalities tested, only octadecyl (C_{18}) yielded good recoveries of all three compounds of interest. The recovery of GAZT, the most hydrophilic of the three, was the most sensitive to changes in column type and extraction procedures. Use of the 500-mg column size was necessary to maintain consistently high recoveries of GAZT when extracting 0.5-ml samples. Furthermore, inclusion of even 1% methanol in the 1-ml wash prior to sample elution decreased the recovery of the glucuronide. On the other hand, lowering the methanol concentration from 100% during sample elution decreased the recoveries of I.S. and AZT.

Octadecyl extraction columns from a variety of different manufacturers were tested for their performance in the assay. Most gave similar results, with acceptable recoveries and purifications. However, samples extracted with the high-hydrophobic columns from J.T. Baker gave elution profiles with fewer endogenous peaks, while maintaining high recoveries of GAZT. Three separate lots of this C_{18} material have been used with no apparent differences in recoveries or chromatographic results.

DISCUSSION

Using solid-phase extraction followed by isocratic HPLC analysis with a mobile phase buffered to pH 2.70, good resolution of AZT, GAZT and I.S. from normal serum components was readily achieved. The range of the assay, 0.1–20 μM for AZT and 0.5–40 μM for GAZT, spans the serum concentrations expected during AZT therapy. Although this method was developed and optimized to determine levels of AZT and GAZT in human serum, it has been used successfully for the quantification of the drug and its metabolite in the serum of rats, dogs and monkeys [11,13,14] as well as in the cerebrospinal fluid of man (data not presented).

This procedure cannot be used for the determination of AZT and GAZT in urine due to the large number of endogenous UV-absorbing components. In addition, we have occasionally been unable to use this method to analyze serum samples from patients with impaired renal function. For such samples, the method reported previously [10] for the quantification of AZT and GAZT is still useful. However, compared to this older procedure, the current method provides improved resolution for the three analytes and decreases by half the HPLC analysis time, while maintaining comparable limits of detection. Accordingly, several analytical groups are now using this method for routine determination of AZT and GAZT concentrations in samples of serum or plasma from patients receiving AZT therapy.

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spectroscopic characterizations of GAZT, to Mr. Robert Lee for technical assistance and to Dr. Thomas Krenitsky for continued guidance and support.

NOTE ADDED IN PROOF

Since submission of this manuscript, J.T. Baker has changed the manufacturing process for their high-hydrophobic octadecyl extraction columns. The new material gives reduced recoveries (75–80%) of AZT and I.S. and unacceptable recoveries of GAZT (5–10%). We are currently recommending use of Bond Elut® octadecyl extraction columns (500 mg, Analytichem International), since we have found that these columns give results essentially identical to those of the original, but no longer available, J.T. Baker columns.

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