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Rapid and sensitive determination of zidovudine and zidovudine glucuronide in human plasma by ion-pair high-performance liquid chromatography☆

Teresa Nadal*, Jordi Ortuño, José A. Pascual

Department of Pharmacology and Toxicology, Institut Municipal d'Investigació Mèdica (IMIM), Autonomous University of Barcelona, Dr. Aiguader 80, 08003 Barcelona, Spain

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Abstract

A rapid, simple and sensitive method is described for the simultaneous determination in human plasma of the well known antiviral agent zidovudine (AZT) and its main metabolite, zidovudine-5′-O-glucuronide (G-AZT), using a solid-phase extraction method for sample preparation and a rapid ion-pair HPLC separation method with diode-array ultraviolet detection. The method overcomes the problems experienced in other procedures because of the large difference in polarity of the two compounds and the pH-sensitive retention of G-AZT by using *n*-octylamine to increase the retention of the glucuronide and improve the overall chromatographic behaviour. AZT and G-AZT are eluted at 3.6 and 5 min, respectively, with 7-ethyltheophylline used as internal standard eluting at 4.2 min. Caffeine, a good marker for the elution of related compounds present in plasma, appears well before, at 2.6 min. Quantification limits were established at 0.025 and 0.050 μg/ml for AZT and G-AZT, respectively. The improvement in method reproducibility due to the late elution of G-AZT could be observed even at the quantification limit at which an inter-assay relative standard deviation of only 6.4% was found after 3 months of routine use of the method.

1. Introduction

The antiviral agent zidovudine (3′-azido-3′-deoxythymidine, AZT) is probably the substance most widely used in acquired immunodeficiency syndrome (AIDS) treatment owing to its potent inhibitory effect on human immunodeficiency virus (HIV) [1–7]. It is rapidly metabolized to its 5′-O-glucuronide (G-AZT) form, in which 75% of the dose is eliminated from the body [8–10]. Because of its dose-dependent toxic effects [11,12] and the differences in bioavailability described, it is convenient to monitor the plasma concentration of AZT to individualize dosage regimens and reduce toxic side-effects. Moreover, the simultaneous determination of both AZT and its metabolite G-AZT can be of great interest in bioavailability studies and especially when dealing with patients with hepatic or renal function impairment or when drug interaction is likely to occur.

Some HPLC methods have been published for

* Corresponding author.

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the determination of AZT in plasma from both animals and humans [13–18], but only a few are able to determine AZT and G-AZT simultaneously. In 1988, Good et al. [19] published a method in which a solid-phase extraction procedure was used followed by an isocratic HPLC separation at acidic pH. However, the system was very sensitive to small changes in pH and G-AZT eluted too fast, resulting in poor reproducibility at low concentrations; 34 min were needed for a complete chromatographic run. An attempt to shorten the chromatographic run time was published by Qian et al. [20] for application to monkey samples. The quantification limits were comparably high and great variability was observed. Other approaches used a more complicated set-up such as the column-switching system described by Lacroix et al. [21].

The HPLC method presented here allows the determination of AZT and G-AZT with rapid elution of the compounds (3.6 and 5 min for AZT and G-AZT, respectively). Using *n*-octylamine as an ion-pairing reagent, the retention of G-AZT increases, avoiding all plasma interferences. Under these conditions, very low detection limits are reached and good reproducibilities are achieved even at the low quantification limits of the method.

2. Experimental

2.1. Materials and reagents

Zidovudine (3′-azido-3′-deoxythymidine, AZT) and zidovudine-5′-Oglucuronide (G-AZT) was purchased from Sigma (St. Louis, MO, USA). 7-Ethyltheophylline, used as internal standard (ISTD), was synthesized from theophylline (Sigma, St. Louis, MO, USA) and ethyl iodide (Merck, Darmstadt, Germany) in alkaline solution. Structures of the compounds and their UV spectra are given in Fig. 1.

Ultra-pure water was obtained using a Milli-Q purification system (Millipore, Molsheim, France). Methanol (Carlo Erba, Milan, Italy), acetonitrile (Merck) and glacial acetic acid (Scharlau, Barcelona, Spain) were of HPLC grade. Octylamine (Panreac, Barcelona, Spain) was of GC grade. Other reagents were of analytical-reagent grade. A 25 m*M* phosphate buffer (pH 6.55) was prepared by diluting 1.44 ml of concentrated phosphoric acid to 1 l with water and adjusting the pH to 6.55 adding concentrated ammonia dropwise.

Bond Elut C_{18} solid-phase extraction columns (3 ml/200 mg) were purchased from Varian (Harbor City, CA, USA) and mounted on a Vac Elut vacuum manifold (Supelco, Bellefonte, PA, USA).

2.2. Preparation of standards

Stock standard solutions of AZT, G-AZT and the ISTD were prepared in methanol (1 mg/ml). Separate working standard solutions of 1, 10 and 100 μg/ml of AZT and G-AZT were prepared by dilution of the stock standard solutions with methanol. All solutions were stored at −20°C until used. A working standard solution of the ISTD (40 μg/ml) was prepared by dilution of the stock standard solution with water. This solution was stored at 4°C. All working standard solutions were checked chromatographically for purity on a routine basis.

2.3. Preparation of calibration graphs and quality control samples

Calibration graphs containing AZT and G-AZT together were prepared daily for each analytical batch in checked drug-free (blank) plasma. Suitable amounts of methanolic working solutions of AZT and G-AZT (Table 1) were placed in 15-ml screw-capped disposable glass tubes and evaporated to dryness under a stream of nitrogen before adding 1 ml of blank plasma. The tubes were gently vortex mixed before use.

Pooled quality control (QC) samples containing AZT and G-AZT were prepared, at two different concentrations, at the beginning of the study (Table 1). They were divided into 1-ml aliquots in glass tubes and kept at −20°C until used. Quality control samples were included in each analytical batch to check for method repro-

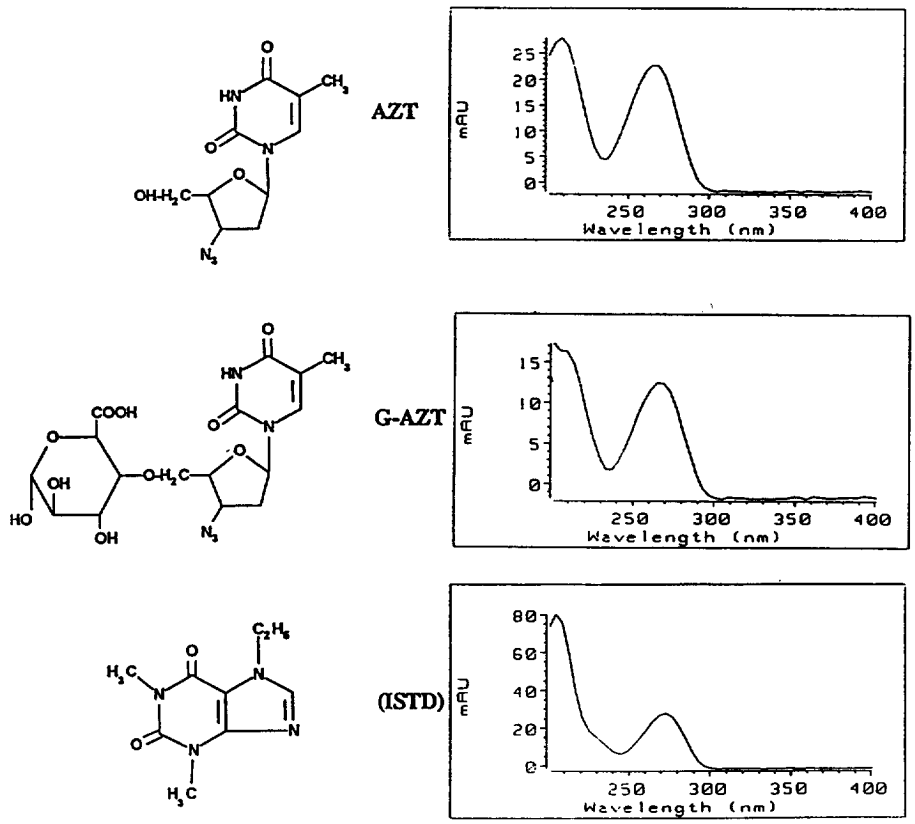

Fig. 1. Structures and UV spectra of zidovudine (AZT), zidovudine-5′-O-glucuronide (G-AZT) and 7-ethyltheophylline (ISTD).

Table 1
Preparation of calibration graphs and quality control samples containing AZT and G-AZT

Plasma concentration (μg/ml)		Standard solution concentration (μg/ml)		Spiked volume (μl)		Sample volume (ml)
AZT	G-AZT	AZT	G-AZT	AZT	G-AZT	
Calibration graphs						
0.025	0.05	1	1	25	50	1
0.1	0.25	10	10	10	25	1
0.25	1.0	10	100	25	10	1
0.5	5.0	10	100	50	50	1
2.5	10.0	100	100	25	100	1
Quality control samples						
0.1	0.1	100	100	25	25	25
1	4	1000	1000	25	100	25

ducibility and stability of samples under storage conditions.

2.4. Sample extraction

Real samples together with QC samples were allowed to thaw in a water-bath at 40°C. In parallel, a calibration graph was prepared following the procedure described above. Aliquots of 1 ml of the samples were pipetted into 15-ml screw-capped glass tubes and 25 μl of the aqueous ISTD working solution were added. The tubes were vortex mixed and the contents were poured into Bond Elut C_{18} extraction columns and forced to pass through at not more than 0.5 ml/min by applying reduced pressure. The columns had previously been activated and conditioned with 3 ml of methanol and 2 ml of 25 m*M* phosphate buffer (pH 6.55), avoiding running dry. After application of the samples, the columns were washed with 1 ml of 25 m*M* phosphate buffer (pH 6.55) and dried by passing a steam of air for 3 min. The analytes were then eluted with 2 ml of methanol and the eluates were evaporated to dryness under a stream of nitrogen in a water-bath at 50°C. The dry extracts were reconstituted in 100 μl of 5% acetic acid–acetonitrile (90:10, v/v) by vigorous vortex mixing, transferred into 200-μl injection vials and centrifuged at 10 000 *g* for 5 min. Volumes of 20 μl of the supernatant were injected into the chromatographic system.

2.5. High-performance liquid chromatography

Chromatographic analysis was carried out using a Series II 1090L liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode-array detector. The instrument was linked to an HP 9000/300 workstation (Hewlett-Packard). The column used was an Ultrasphere ODS, 3 μm particle size, 7.5 × 0.46 cm I.D. (Beckman, San Ramon, CA, USA).

The mobile phase was 25 m*M* phosphate buffer containing 0.4% of *n*-octylamine and adjusted to pH 6.55 with ammonia (bottle A) and acetonitrile (bottle B). The aqueous component of the mobile phase was filtered through a 0.45-μm filter (Scharlau) before use. Linear gradient elution from 5% to 30% acetonitrile in 7 min was applied, followed by a rapid increase to 80% acetonitrile in 1.5 min to clean the column. The system was allowed to return to the initial conditions in 1 min and stabilized for 2.5 min. The total analytical time was 12 min for the whole run. The flow-rate was 1 ml/min.

The diode-array detector was set to monitor the signal at 266 nm corresponding to the absorbance maximum for AZT and G-AZT. In addition, the full UV spectra between 200 and 400 nm of the detected peaks were stored in the data system to check for peak purity.

Peak areas were measured and the ratios with the internal standard were used for all calculations.

3. Results

After 3 months of routine use, the method proved to be reliable and reproducible even when column changes were needed.

Fig. 2 shows typical chromatograms obtained when analysing real samples from volunteers who had received a single 250-mg oral dose of AZT. UV spectra corresponding to the peak purity test are also presented.

Diode-array detectors may show lower sensitivity than other variable-wavelength detectors, but the possibility of obtaining the whole UV spectrum of every peak detected in routine analysis proved to be very useful in checking for peak purity, so ensuring proper identification and correct quantification. The peak purity test could be applied with good results even at concentrations as low as the quantification limit of the method.

Although, prior to the application of the method to real samples, a complete intra- and inter-assay validation protocol was followed, an on-going long-term validation scheme after long routine use was also applied and the results were as follows.

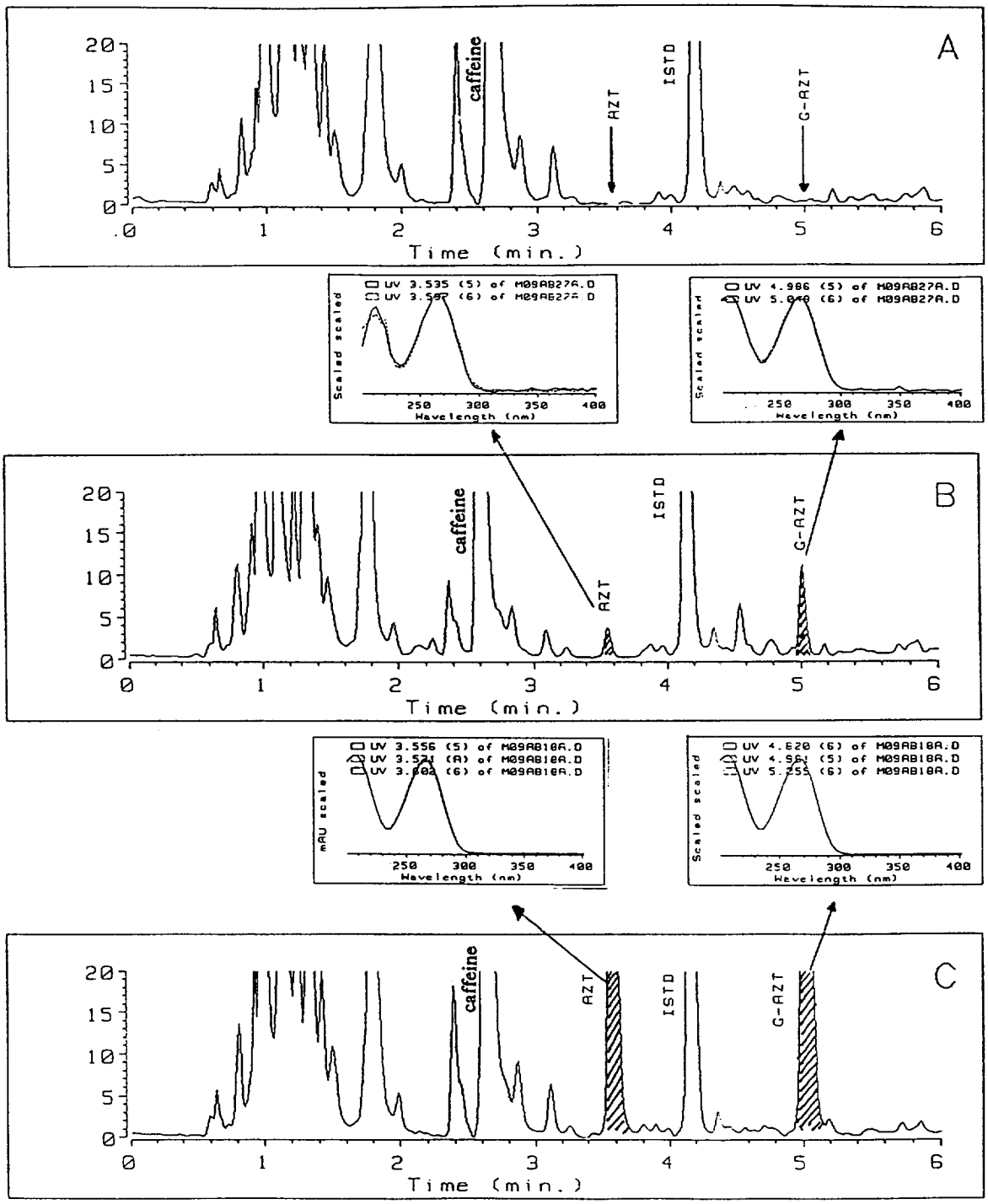

Fig. 2. Typical chromatographic profiles corresponding to real samples. (A) Chromatogram corresponding to a plasma sample prior to the administration of AZT. (B) Chromatogram corresponding to a plasma sample obtained 5 h after the administration of a single oral dose of 250 mg of AZT; 0.032 μg/ml of AZT and 0.159 μg/ml of G-AZT were found. (C) Chromatogram corresponding to a plasma sample obtained 40 min after the administration of a single oral dose of 250 mg of AZT; 1.121 μg/ml of AZT and 6.108 μg/ml of G-AZT were found. UV spectra obtained along the peaks are shown as peak purity tests in all cases.

3.1. Selectivity and chromatographic behaviour

The selectivity of the method was studied analysing a series of different plasmas and checking for the presence of any interfering substances at the retention times of the compounds of interest. At the retention time of the substances of interest no interferences could be found. An a posteriori assessment of the selectivity of the method after its routine use strengthened those results.

The use of *n*-octylamine in the mobile phase as an ion-pairing reagent did not significantly affect the retention of AZT or the internal standard (7-ethyltheophylline), but drastically changed that of G-AZT, eluting later in the chromatogram in a zone free of interferences. Further, the addition of such an amine improved the chromatographic behaviour of all three compounds, because of the blocking effect on the silanol groups, so preventing them from tailing, especially with column deterioration.

7-Ethyltheophylline was chosen as the internal standard because of its chromatographic behaviour, eluting at a retention time between those of the two compounds of interest and free from interferences. The UV spectrum is very similar and other physico-chemical properties were shown to be compatible (stability, recovery, etc.).

The use of a fast chromatographic column (7.5 cm long, 3 μm particle size) together with the ion-pairing reagent allows the compounds to be eluted in 5 min (the retention times for AZT, ISTD and G-AZT being 3.6, 4.2 and 5.0 min, respectively) but nevertheless very well separated from other structurally related plasma interferences (as indicated by the position of the peak of caffeine in Fig. 2).

3.2. Recovery

The recoveries of AZT, G-AZT and the ISTD were calculated by comparing the peak areas obtained when calibration samples were analysed with the reference substances added prior and after extraction.

For the ISTD, ten aliquots of the same blank plasma were extracted. After extraction, 25 μl of the ISTD working solution (a total of 1 μg) were added to the organic phase and taken through the analytical procedure. In parallel, ten additional aliquots of the same blank plasma were spiked with the same amount of the ISTD and extracted following the complete analytical procedure. The recovery obtained was 91% [relative standard deviation (R.S.D.) (4.40%)].

The same experiment was carried out for AZT and G-AZT but in this instance with concentrations of the analytes (in duplicate) identical with those used for the calibration graph. Table 2 shows the results obtained.

G-AZT was found, as described elsewhere [19], to be very sensitive to the presence of small amounts of methanol in the samples. For this reason, the spiked calibration graphs were prepared by evaporating the methanolic standard solutions of AZT and G-AZT prior to the addition of the blank plasma.

3.3. Linearity

For the study of linearity, a calibration graph was prepared covering the whole expected concentration range. Values of the concentrations studied both for AZT and G-AZT are shown, together with the preparation scheme, in Table 1. Following the validation protocol, six replicates were analysed for low, medium and high concentrations. The rest were analysed in dupli-

Table 2
Recovery of AZT and G-AZT from human plasma

Concentration (μg/ml)		Recovery (%)	
AZT	G-AZT	AZT	G-AZT
0.025	0.050	89.3	91.6
0.100	0.250	85.8	82.1
0.250	1.000	82.0	84.3
0.500	5.000	80.8	80.6
2.500	10.000	88.6	87.5
Mean		85.3	85.2
S.D.		3.81	4.43
R.S.D. (%)		4.47	5.20

cate. Peak-area ratios (between AZT or G-AZT and the ISTD) were used for calculations. The results obtained clearly showed the non-constant variance of data (heteroscedasticity). To illustrate this point, the correlation between the concentrations and the standard deviation (S.D.) of the peak-area ratios obtained was calculated. A linear correlation was found, clearly showing the heteroscedasticity of the method. Fig. 3 shows the results of such calculations for the data corresponding to a long-term validation (after twenty experiments had been performed). In such cases the general least-squares regression analysis cannot be applied since larger fitting errors occur as we approach lower concentrations. To overcome this problem, data transformation (log–log) or the use of weighted least squares (with $1/s^2$ as weighing factor) is required [22]. The latter was used in this case. The spreadsheet program CALWER21, available from S.M. Gort and R. Hoogerbrugge (RIVM, Bilthoven, Netherlands), was used to perform all the calculations for weighted least squares.

The calibration graphs obtained showed good determination coefficients (r^2) with a mean value of 0.9911 for AZT and 0.9941 for G-AZT, and the slopes showed an R.S.D. below 1% for both substances.

3.4. Limits of detection and quantification

Assuming that variance becomes constant on approaching the detection limit, six replicate analyses were performed with a spiked sample containing 0.025 μg/ml of AZT and 0.050 μg/ml of G-AZT. The S.D. of the values obtained was used as a measure of the "noise" for the calculation of the limits of detection (three times the S.D.) and quantification (ten times the S.D.).

The detection limits for AZT and G-AZT calculated following this method were 0.007 and 0.014 μg/ml, respectively, and the quantification limits were 0.022 and 0.045 μg/ml, respectively.

3.5. Precision and accuracy

Six replicates of blank plasmas spiked with 0.025, 0.25 and 2.5 μg/ml of AZT and 0.05, 1.0 and 10.0 μg/ml of G-AZT were used to determine the intra-assay precision and accuracy. The inter-assay precision and accuracy were determined using all calibration points analysed during 3 months of study.

Precision is expressed as the R.S.D. of the concentrations calculated using the calibration graphs. Accuracy is expressed as the relative error of the estimated concentrations.

The inter-assay assay precision varied from 3.1 to 9.80% (at the quantification limit used) over the whole concentrations range for AZT and from 3.1 to a 6.4% for G-AZT. The accuracy (relative error) was always below 10% for AZT with normal values of ca. 4–6%. For G-AZT the values were always below 7%. Tables 3 and 4 show the results obtained.

3.6. Quality control samples

Whereas calibration graphs were prepared daily, quality control samples were prepared once from bulk blank samples aliquoted and kept at −20°C. Samples at two different concentrations were prepared, one containing 0.1 μg/ml of AZT and G-AZT ("low" control) and the other containing 1 μg/ml of AZT and 4 μg/ml of G-AZT ("high" control).

One quality control sample of each concentration was included in each analytical batch. The mean calculated concentrations together with their R.S.D.s and relative errors obtained during a 3-month period are shown in Table 5.

The results show the stability of the samples under the storage conditions and the reproducibility and accuracy of the determinations fit those values found for daily calibration graphs.

4. Discussion

The combination of rapid liquid chromatography (particle size 3 μm, column length 7.5 cm) and the use of the primary amine *n*-octylamine as an ion-pairing reagent dramatically increased the performance of the method, especially when the simultaneous determination of both AZT and G-AZT is needed. Other ion-pairing re-

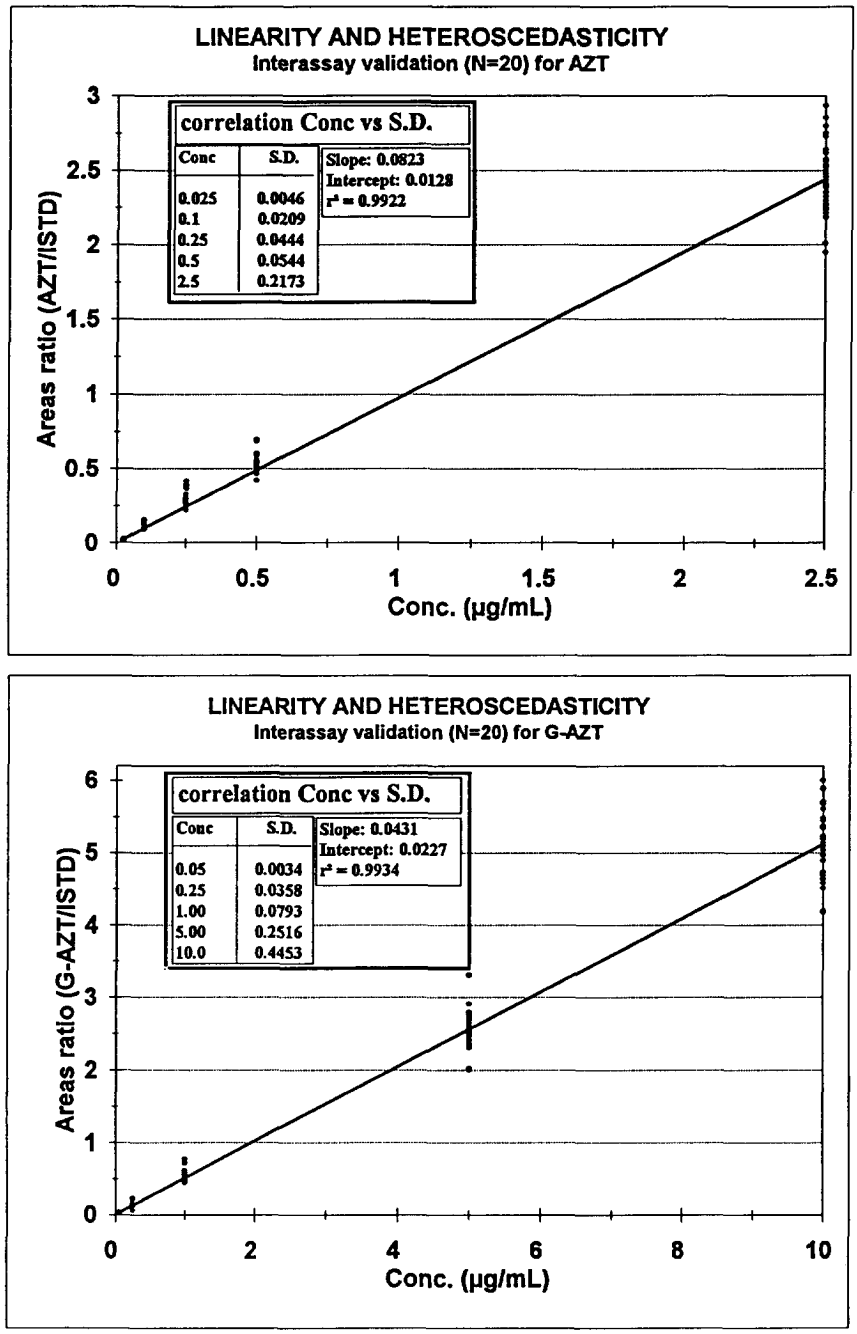

Fig. 3. Plot of all values obtained from twenty different calibration graphs during 3 months of application of the method showing the heteroscedasticity of the data. The correlation between concentration and standard deviation of the response obtained is also shown.

Table 3
Intra-assay precision and accuracy obtained for the determination of AZT and G-AZT

Analyte	Concentration (μg/ml)	Number of observations	Estimated concentration ±S.D. (μg/ml)	Precision [R.S.D. (%)]	Accuracy [Relative error (%)]
AZT	0.025	6	0.0247 ±0.0022	8.90	−1.12
	0.250	6	0.2638 ±0.0295	5.86	5.53
	2.500	6	2.3086 ±0.0428	3.98	−7.66
G-AZT	0.05	6	0.0498 ±0.0045	8.95	−0.50
	1.000	6	1.0111 ±0.0815	5.58	1.11
	10.000	6	9.6743 ±0.1126	4.38	−3.26

Spiked samples were prepared individually rather than from a bulk plasma preparation.

Table 4
Inter-assay precision and accuracy obtained for the determination of AZT and G-AZT

Analyte	Concentration (μg/ml)	Number of observations	Estimated concentration ±S.D. (μg/ml)	Precision [R.S.D. (%)]	Accuracy [Relative error (%)]
AZT	0.025	40	0.0247 ±0.0024	9.80	−1.22
	0.100	33	0.1021 ±0.0048	4.67	2.12
	0.250	38	0.2675 ±0.0177	6.62	7.01
	0.500	40	0.4941 ±0.0154	3.12	−1.19
	2.500	39	2.2777 ±0.1013	4.45	−8.89
G-AZT	0.05	40	0.0497 ±0.0032	6.44	−0.67
	0.250	32	0.2568 ±0.0147	5.72	2.73
	1.000	39	1.0591 ±0.0678	6.40	5.91
	5.000	40	4.8149 ±0.1490	3.10	−3.70
	10.000	39	9.6138 ±0.3353	3.49	−3.86

Spiked samples were prepared fresh individually on each day of analysis.

Table 5
Results obtained from the analysis of quality control samples in a 3-months period

Parameter	Low Control		High Control	
	AZT	G-AZT	AZT	G-AZT
No. of observations	14	14	20	20
Mean (μg/ml)	0.098	0.098	0.946	3.746
S.D. (μg/ml)	0.0084	0.0118	0.0731	0.2882
R.S.D. (%)	8.71	12.00	7.73	7.69
Relative error (%)	−1.55	−2.06	−5.41	−6.34

agents such as tetrabutylammonium chloride and amines (triethylamine) were tested during method development to achieve the necessary selectivity but without much success. Octylamine produced the appropriate shift in the retention time of G-AZT without affecting AZT so that both could be eluted with just a 1.5-min difference and far from other polar plasma interferences shown in other methods [19]. Different internal standards structurally related to the analytes were also tested (azido-ddU and 7-propyltheophylline) for appropriate chromatographic behaviour. Under the described conditions, 7-ethyltheophylline elutes between AZT and G-AZT, being ideal for the quantification of both compounds.

The method avoids the problem of the very pH-sensitive elution of G-AZT, so improving the stability of the conditions and the reproducibility. The lack of critical points positively affected the robustness of the method, which, although not specifically studied, was found to be very good as no significant variations were found when different analysts applied it or when the analytical columns (same batch) were changed during the period of study.

The use of a diode-array detector was very useful for certifying the peak purity for all samples, although sacrificing some sensitivity.

The extraction method is a modification of other reported approaches in which different solid-phase columns were used. Bond-Elut C_{18} cartridges of 200 mg were sufficient to treat up to 1 ml of plasma.

The use of a Prospekt on-line automatic sample processor (Spark Holland, Emmen, Netherlands) will be the next logical step for a fully automated, rapid analytical method able to analyse more than four samples per hour in a completely unattended operation.

References

[1] H. Nakashima, T. Matsui, S. Harada, N. Kobayashi, A. Matsuda, T. Ueda and N. Yamamoto, Antimicrob. Agents Chemother., 30 (1986) 933.
[2] R.E. Ghaisson, J.P. Allain and P.A. Volberding, N. Engl. J. Med., 315 (1986) 1610.
[3] R. Yarchoan R.W. Klecker, K.J. Weinhold, et al., Lancet, i (1986) 575.
[4] R. Yarchoan, G. Berg, P. Brouwers, et al., Lancet, i (1987) 132.
[5] M.A. Fischl, D.D. Richman, M.H. Grieco, et al., N. Engl. J. Med., 317 (1987) 185.
[6] M.S. Hirsch and J.C. Kaplan, Antimicrob. Agents Chemother., 31 (1987) 839.
[7] H.C. Lane, H. Masur, J.A. Kovacs, R. Walker, S. Carleton, T. Folks and A.S. Fauci, Clin. Res., 35 (1987) 480A.
[8] S.S. Good, D.T. Durack and P. de Miranda, Fed. Proc. Fed. Am. Soc. Exp. Biol., 45 (1986) 444.
[9] R.W. Klecker, J.M. Collins, R. Yarchoan, R. Thomas, J.F. Jenkins, S. Broder and C.E. Meyers, Clin. Pharmacol. Ther., 41 (1987) 407.
[10] M.R. Blum, S.H.T. Liao, S.S. Good and P. de Miranda, Am. J. Med., 85 (1988) 189.
[11] P.S. Gill, M. Rarick, R.K. Brynes, D. Causey, C. Loureiro and A. Levine, Ann. Intern. Med. 107 (1987) 502.
[12] D.D. Richman, M.A. Fischl, M.H. Grieco, et al., N. Engl. J. Med., 317 (1987) 192.
[13] J.D. Unadkat, S.S. Crosby, J.P. Wang and C.C. Hertel, J. Chromatogr., 430 (1988) 420.
[14] M.A. Hedaya and R.J. Sawchuk, Clin. Chem., 34 (1988) 1565.

[15] R.M. Ruprecht, A.H. Sharpe, R. Jaenisch and D. Trites, J. Chromatogr., 528 (1990) 371.
[16] N. Frijus-Plessen, H.C. Michaelis, H. Foth and G.F. Kahl, J. Chromatogr., 554 (1990) 101.
[17] R. Kupferschmidt and R.W. Schmid, Clin. Chem., 35 (1989) 1313.
[18] C.M. Riley and J.M. Ault, Jr., J. Chromatogr., 531 (1990) 295.
[19] S.S. Good, D.J. Reynolds and P. de Miranda, J. Chromatogr., 431 (1988) 123.
[20] M. Qian, T.S. Finco and J.M. Gallo, J. Pharm. Biomed. Anal., 9 (1991) 275.
[21] C. Lacroix, T. Phan Hoang, F. Wojciechowski, H. Duwoos and J. Nouveau, J. Chromatogr., 525 (1990) 240.
[22] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte and L. Kaufman, Chemometrics: a Textbook, Elsevier, Amsterdam, 1988, p. 84.