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Derivative-differential UV spectrophotometry and compensation technique for the simultaneous determination of zidovudine and lamivudine in human serum

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Three new simple, precise, rapid and selective determination methods are described for zidovudine (ZID) and lamivudine (LAM) in human serum and in pharmaceutical formulations. The first method, based on the compensation technique is presented for the derivative spectrophotometric determination of binary mixtures with overlapping spectra. Using ratios of the derivative maxima or the derivative minimum, the exact compensation of either component in the binary mixture and human serum can be achieved, followed by its determination. The second method, differential derivative spectrophotometry, comprised of measurement of the difference absorptivities derivatized in the first order (ΔD_1) of a tablet extract in 0.1 N NaOH relative to that of an equimolar solution in methanol at wavelengths of 246 nm and 263 nm, respectively. Neither sample pretreatment nor separation were required. The third method is based on HPLC on a reversed-phase column using a mobile phase of 0.01M sodium dihydrogen phosphate : methanol : acetonitrile (4 : 2 : 3 v/v/v), with detection at 285 nm. Repeatability and reproducibility studies for each compound showed no significant differences at 95% confidence level. The proposed methods were used for the simultaneous determination of the drugs in human serum samples and binary mixtures with good recoveries.

1. Introduction

Zidovudine (ZID), 3′-azido-3′-deoxythymidine, commonly referred to as AZT, is a thymidine analog with antiviral activity against HIV-1, HIV-2, human T lymphotropic virus (HTLV)-I and other retroviruses (Hardman et al. 1996; Cavert et al. 1997). Lamivudine (LAM), the (−) enantiomer of 2′-deoxy-3′-thiacytidine, is a nucleoside analog with activity against the human immunodeficiency virus (HIV). The use of ZID plus LAM in a combination therapy causes a decrease of HIV-I in blood plasma (Matheron et al. 2003).

Various methods have been used for the quantitative determination of these compounds individually such as HPLC with tandem mass spectrometric detection (Morris and Selinger 1994; Harker et al. 1994; Fan and Stewart 2001; Caufield and Stewart 2001; Zheng et al. 2001; Aymard et al. 2000; Kenny et al. 2000; Pereira et al. 2000; Fung et al. 2001), immunoassay (Wring et al. 1994; Tadepalli et al. 1990; Schrive and Plasse 1994; Granich et al. 1989) and capillary zone electrophoresis (Cahours et al. 2002; Fan and Stewart 2002).

The compensation method (Wahbi et al. 1992) is a non-mathematical method for the detection and elimination of unwanted absorption during spectrophotometric analysis. In the binary mixture analysis, the compensation method involves a comparison of several difference spectra (mixture-reference) using different concentrations of a reference solution in the reference cell. Hence, if A_m and A_r refer to the absorbances of the relevant cells against air at the same wavelength λ, then $\Delta A_\lambda = A_{m\lambda} - A_{r\lambda}$, where $A_m = A_a + A_b$ at a given wavelength λ, a and b refer to components a and b, respectively, and A_r refers to A_a or A_b. If C_r for compound a is introduced into the reference cell, the absorption characteristics of the mixture gradually approach that of compound b as c_a increases and finally coincides with the absorption curve of compound b at the end-point, for which $c_r = c_a$, and by analogy c_b can be found by repeating the same steps using c_r for compound b in the reference cell. The accuracy of the method depends on the evaluation of the balance point.

Derivative spectrophotometry offers greater selectivity than normal spectrophotometry in the simultaneous determination of two or more compounds without previous chemical separation (Morelli 1995). Difference spectrophotometry based on pH changes has also been reported to be useful in the determination of binary mixtures. There are few reports on utilization of the above two combined techniques for the estimation of individual drug substances (Erk 2000) and for combined preparations (Prasad et al. 1997). The derivative-difference spectrophotometry will offer further advantages in cancelling heavy spectral interferences to drug analysis when the irrelevant absorption is pH and solvent dependent.

In the present study, three simple, economical, accurate and reproducible analytical methods compensation techni-

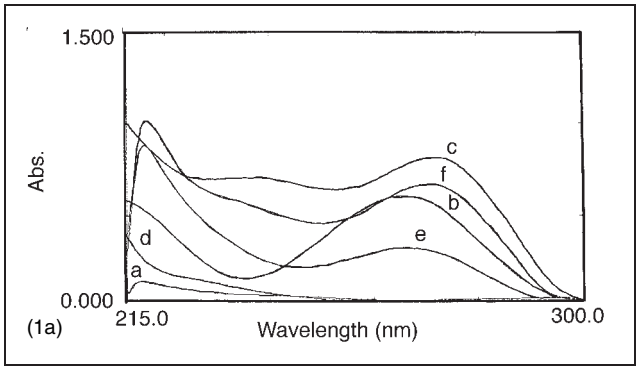

Fig. 1a: Zero-order spectra of a) blank serum with no addition of ZID and LAM, b) 45.0 $\mu g \cdot ml^{-1}$of ZID c) 25.5 $\mu g \cdot ml^{-1}$ of LAM in methanol and, d) blank serum with no addition of ZID and LAM, e) 45.0 $\mu g \cdot ml^{-1}$of ZID f) 25.5 $\mu g \cdot ml^{-1}$ of LAM in 0.1 N NaOH

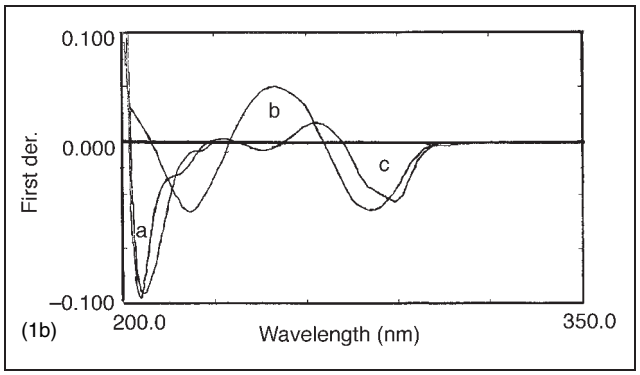

Fig. 1b: First derivative spectra of (a) blank serum with no addition of ZID and LAM, b) 45.0 $\mu g \cdot ml^{-1}$of ZID; c)25.5 $\mu g \cdot ml^{-1}$ of LAM in methanol in the presence of human serum

que, differential-derivative spectrophotometry and HPLC for the simultaneous determination of ZID and LAM in pharmaceutical formulations and in human serum were developed.

2. Investigations, results and discussion

2.1. Compensation technique

The stability of working solutions of ZID and LAM was studied by recording their absorption spectra. No changes were observed for at least three days when the solutions are stored at room temperature in the dark. For the simultaneous determination of ZID and LAM direct UV absorption measurements are not possible due to spectral overlap (Fig. 1a). Besides, a zero-order spectrum did not permit the simultaneous determination of ZID and LAM in human serum owing to lack of sensitivity and to interference from the human serum matrix. Application of the compensation technique was found to be correct for the human serum matrix interference and to enhance the sensitivity. Fig. 1b shows the first derivative spectra (^{1}D) of ZID and LAM. They were recorded for each reference solution of the components and the ratios of the ^{1}D maximum and ^{1}D minimum or ^{1}D maxima and ^{1}D minimum were calculated. Table 1 shows the mean values of the ratios calculated for ten different determinations for each standard solution. The ratios are constant, characteristic of the pure substance, independent of concentration and presence of another absorbing component. For the determination of ZID concentrations in ZID/LAM binary mixtures and in human serum, the sample cell was filled with the mixture solution and the reference cell was filled with a series of reference ZID solutions in different concentrations. The ratios of the mixture calculated are constant. The ratios of the mixture calculated from the recorded ^{1}D spectra was compared with those of LAM. At the balance point, the ratio of the mixture corresponds to that of LAM where the concentration of ZID in the mixtures in the sample cell is equal to that of the reference solution in the reference cell. For determining the other component, the same steps follow using solutions of pure compound LAM in the reference cell to determine its concentration in the mixture at the balance point. Conformity with Beer's law was evident in the concentration range from 15.0 to 45.0 $\mu g \cdot ml^{-1}$ of ZID and from 8.5 to 42.5 $\mu g \cdot ml^{-1}$ of LAM. Linear relationships between the selected amplitudes from the ^{1}D spectra and concentrations of ZID and LAM were observed in absence and presence of human serum. Under the experimental conditions described, the graphs obtained by plotting the derivative values of each drug in this mixture, in the absence and presence of human serum, versus concentration, in the range stated in Table 1, show linear relationships. The relative standard deviation values of the slope and intercepts of the calibration graphs indicated the high reproducibility of the proposed method. In this method, the synthetic mixtures were prepared by adding known amounts of ZID and LAM, in absence and presence of human serum. The selectivity of the proposed method for the estimation of the drugs in the presence of various tablet excipients such as starch, lactose, talc and magnesium strearate was investigated. A placebo, comprising starch 10%, lactose 40%, talc 2% and magnesium strearate 1% was prepared, just as a 1 : 1 blend of drug and placebo. Recoveries and relative standard deviations were found to be 99.4, 99.9 and 0.96, 0.81% for ZID and 101.8, 100.2 and 1.42, 0.97% for LAM in their binary mixture, in absence and presence of human serum (Table 2). The accuracy of measurements, expressed in terms of relative errors was about 1.4% even less, thus indicating negligible influence of serum proteins. The developed method was applied to the recovery of ZID and LAM in three batches of commercial formulations, respectively. The results presented in Table 3 are in good agreement with the labelled content. Low values of relative standard deviation indicate very good reproducibility of the measurement. The proposed procedure was successfully

Table 1: Parameters for the simultaneous determination of ZID and LAM in binary mixture by the compensation technique

Method	With human serum		Without human serum	
	ZID	LAM	ZID	LAM
Linearity range ($\mu g \cdot ml^{-1}$)	15.0–45.0	8.5–45.0	15.0–45.0	8.5–45.0
Ratio	^{1}D (249)/^{1}D(280)	^{1}D(263)/^{1}D(289)	^{1}D (249)/^{1}D(280)	^{1}D(263.5)/^{1}D(289.2)
Mean*	0.968	1.21	1.12	1.41
RSD (%)	0.8	0.04	1.1	0.08

* Mean of ten separate determinations

Table 2: Assay results of ZID and LAM in laboratory-made mixtures

Sample	Recovery (mean ± sd)%[a]					
	ZID			LAM		
	Compen. tech.	Diff. der. spectr.	HPLC	Compen. tech.	Diff. der. spectr.	HPLC
With human serum	99.4 ± 0.96 t = 0.9 (2.26)[b]	100.1 ± 1.26 1.3	97.1 ± 2.34	101.8 ± 1.42 t = 1.1	100.4 ± 1.45 0.9	97.2 ± 1.78
Without human serum	99.9 ± 0.81 t = 0.8	98.1 ± 0.91 1.1	99.1 ± 0.9	100.2 ± 0.97 t = 0.8	97.9 ± 1.28 1.0	99.8 ± 2.1

[a] Mean and relative standard deviation for ten determinations; percentage recovery from the label claim amount.
[b] Values in parentheses are the theoretical values at p = 0.95. Theoretical values at % 95 confidence limits; t = 2.26

applied to the determination of the studied compounds in human serum and pharmaceutical dosage forms.

2.2. Differential derivative spectrophotometry

The zero-order UV spectra of ZID and LAM in 0.1 N NaOH and in methanol are shown in Fig. 1a. The difference spectra of ZID and LAM in binary mixtures and human serum of ZID − LAM are set out in Fig. 2a. Fig. 2b shows the first derivative difference spectrum. The spectra of both the drugs in Fig. 2b offer an advantage for their simultaneous determination by having zero crossing points. In particular the absorbances at 246 nm and 263 nm in the ZID − LAM mixture were considered as the optimum working wavelengths for their determination. In addition, application of the differential first derivative spectrophotometry was found to be correct for the human serum matrix interference and to enhance the sensitivity. By measuring the values of the ΔD_1 amplitudes at 246 nm for ZID and 263 nm for LAM, the concentration of each drug can be directly calculated since the differential first derivative measurement cancels the irrelevant absorbance due to the human serum matrix at these wavelengths. Under the experimental conditions described, the graphs obtained by plotting the derivative values of each drug in this mixture, in the absence and presence of human serum, versus concentration, in the range stated in Table 4, show linear relationships. The relative standard deviations of the slopes and intercepts of the calibration graphs indicated the high reproducibility of the proposed method. Conformity with Beer's law was evident in the concentration range of the final dilution cited in Table 4. The correlation coefficients were 0.9981, 0.9990, 0.9985 and 0.9982 indicating good linearity. The relative standard deviations were found to be less than 1.5%, indicating reasonable repeatability of the proposed method. The detection limits (LOD) were 1.08 $\mu g \cdot ml^{-1}$ for ZID and 1.62 $\mu g \cdot ml^{-1}$ for LAM; while the quantification limits (LOQ) (Miller and Miller 1993) were 2.89 $\mu g \cdot ml^{-1}$ for ZID and 2.14 $\mu g \cdot ml^{-1}$ for LAM. The selected method were successfully applied to the determination of these drugs in laboratory-prepared mixtures in absence and presence of human serum. Excipients (starch 10%, lactose 40%, talc 2% and magnesium strearate 1%) were added to the drug for recovery studies according to manufacturer's batch formula for tablets. The results are summarized in Table 2. The method was applied to the recovery of ZID and LAM in three batches of commercial formulations, respectively. The results presented in Table 3 are in good agreement with the labelled content. The values of relative standard deviation indicate very good reproducibility.
The proposed procedure was successfully applied to the determination of the studied compounds in human serum and pharmaceutical dosage forms.

2.3. HPLC

The procedure for the simultaneous analysis of ZID and LAM was developed. A chromatogram of serum spiked with ZID and LAM is shown in Fig. 3. It can be seen that serum proteins do not interfere with the elution of ZID and LAM. The retention times were 5.8 min for ZID and 4.5 min for LAM in combined pharmaceutical dosage forms and human serum. To find the appropiate HPLC conditions for separation of the examined drugs, various reversed phase columns, isocratic and gradient mobile phase systems were tested. Successful attempts were performed with a reversed phase Supelcocil C_{18} column. The mobile phases used were 0.01M sodium dihydrogen phosphate : methanol:acetonitrile. The optimum wavelength for detection was 285 nm at which much better detector responses for the drugs were obtained. Under the described HPLC parameters, the respective compounds were clearly separated at reasonable retention times. For the quantitative analysis, the analytical data for the calibration graphs are listed in Table 5. The linearity of the detector response

Table 3: Determination of ZID and LAM in pharmaceutical dosage forms[a]

Method	Compensation technique Mean (mg) ± SD[b]		Differential derivative spect. Mean (mg) ± SD[b]		HPLC Mean (mg) ± SD[b]	
	ZID	LAM	ZID	LAM	ZID	LAM
Batch no 1	300.4 ± 1.05 t = 1.7 (2.26)[c]	149.1 ± 0.78 t = 1.0	298.7 ± 1.11 t = 1.4	147.9 ± 0.74 t = 1.8	300.2 ± 0.87	151.6 ± 0.45
Batch no 2	299.1 ± 1.42 t = 0.9	150.5 ± 0.61 t = 1.4	298.8 ± 0.09 t = 1.8	149.9 ± 0.97 t = 1.6	299.9 ± 0.68	150.9 ± 0.77
Batch no 3	299.8 ± 1.64 t = 1.1	149.7 ± 1.40 t = 1.3	299.6 ± 0.27 t = 0.7	151.7 ± 0.97 t = 1.0	299.7 ± 0.59	151.3 ± 1.24

[a] Combivir® film tablets were labeled to contain 300.0 mg ZID, 150.0 mg LAM per tablets respectively
[b] Each value is the mean of ten experiments; SD = Standard deviation
[c] Values in parentheses are the theoretical values at p = 0.95. Theoretical values at % 95 confidence limits; t = 2.26

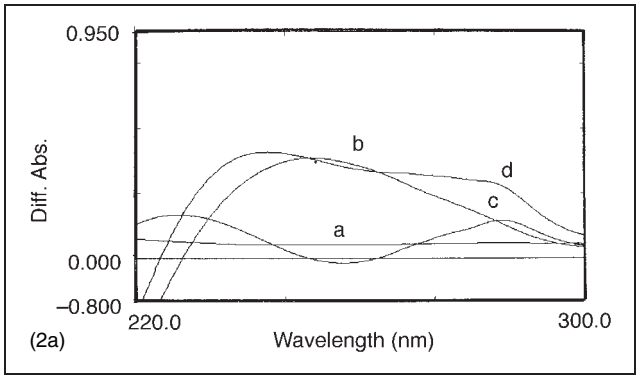

Fig. 2a: Differential spectra of a) blank serum with no addition of ZID and LAM, b) 45.0 $\mu g \cdot ml^{-1}$of ZID; c) 25.5 $\mu g \cdot ml^{-1}$ of LAM and d) mixture (45.0 $\mu g \cdot ml^{-1}$of ZID, 25.5 $\mu g \cdot ml^{-1}$ of LAM and human serum) in methanol versus 0.1 N NaOH

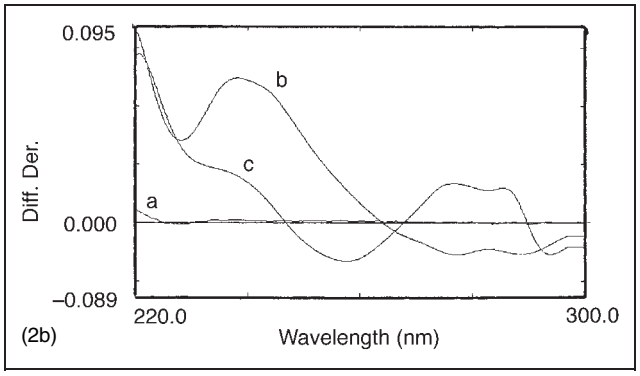

Fig. 2b: Differential derivative spectra of a) blank serum with no addition of ZID and LAM, b) 45.0 $\mu g \cdot ml^{-1}$of ZID; c) 25.5 $\mu g \cdot ml^{-1}$ of LAM and in methanol versus 0.1 N NaOH

for both drugs was determined by plotting peak area ratios vs concentration. The detection limits calculated as the intercept of the calibration graphs are listed in Table 5. The relative standard deviations (RSD) were found to be less than 2.1%. The selected method was successfully applied to the determination of these drugs in laboratory-prepared mixtures in absence and presence of human serum. The excipients (starch 10%, lactose 40%, talc 2% and magnesium strearate 1%) were added to the drug for recovery studies according to manufacturer's batch formula for tablets. The results are summarized in Table 2. The data shown indicate good accuracy and precision of the proposed procedure. The lower limits of detection, using a signal-to-noise ratio of three, were 0.18 $\mu g \cdot ml^{-1}$ for ZID and 0.43 $\mu g \cdot ml^{-1}$ for LAM, while the quantification limits (LOQ) 1.02 $\mu g \cdot ml^{-1}$ for ZID and 1.55 $\mu g \cdot ml^{-1}$ for LAM respectively.

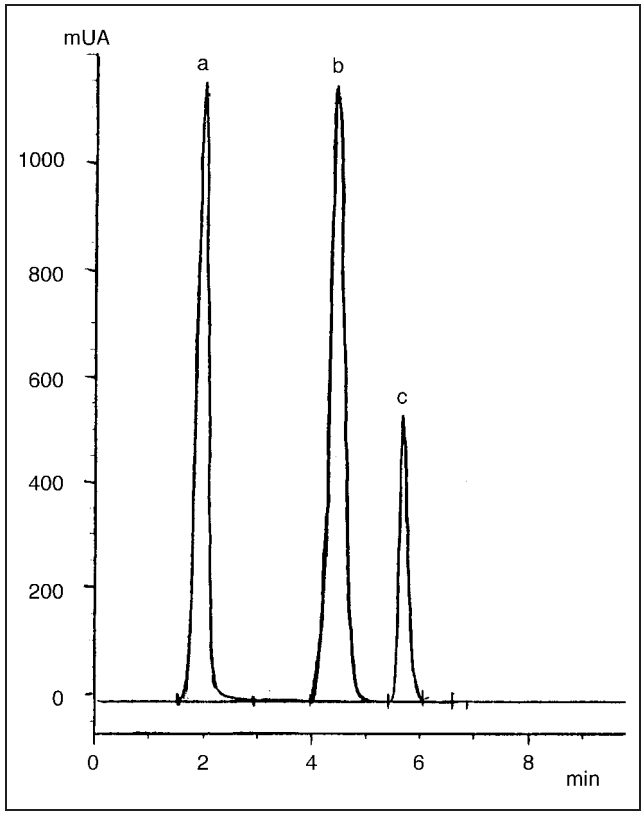

Fig. 3: HPLC Chromatogram of a 20 µl injection containing a) human serum; b) 25.0 $\mu g \cdot ml^{-1}$ of LAM; c) 42.5 $\mu g \cdot ml^{-1}$ of ZID

In order to assess the validity and applicability of the proposed methods (compensation technique, differential derivative spectrophotometry and HPLC), recovery studies were performed by analyzing synthetic mixtures with different proportions of the two drugs in the absence and presence of human serum. HPLC was chosen as the analytical reference method. The results obtained were summarized in Table 3. No significant differences were found between the results obtained by the HPLC method, the compensation technique and differential derivative spectrophotometry.

Table 4: Statistical analysis of calibration graphs of ZID and LAM in mixtures by use of differential derivative spectrophotometry

Method	With human serum		Without human serum	
Parameters	ZID	LAM	ZID	LAM
Wavelengths (nm)	246	263	246	263
Range ($\mu g \cdot ml^{-1}$)	15.0–45.0	8.5–42.5	15.0–45.0	8.5–42.5
Detection limits ($\mu g \cdot ml^{-1}$)	1.08	1.62	1.08	1.62
Regression equation (Y)[a]				
Slope (b)	3.78×10^{-5}	5.87×10^{-5}	2.91×10^{-5}	3.12×10^{-5}
Std. dev. on slope (S_b)	7.86×10^{-5}	2.84×10^{-6}	5.71×10^{-4}	8.24×10^{-7}
Intercept (a)	4.12×10^{-5}	8.78×10^{-5}	7.79×10^{-5}	1.66×10^{-5}
Std. dev. on intercept (S_a)	6.28×10^{-5}	4.84×10^{-7}	3.88×10^{-4}	9.62×10^{-6}
Std. error of estimation (S_e)	2.26×10^{-5}	8.73×10^{-5}	4.81×10^{-6}	8.88×10^{-5}
Correlation coefficient (r)	0.9981	0.9990	0.9985	0.9982
Rel. std. dev. (%)[b]	1.44	0.63	0.46	1.55
% Range of error[b] (% 95 confidence limit)	1.82	1.98	0.94	1.39

[a] Y = a+bC where C is concentration in $\mu g \cdot ml^{-1}$ and Y in absorbance units. [b] Five replicate samples

Table 5: Statistical analysis of calibration graphs of ZID and LAM in mixtures by analyzed HPLC

Method	With human serum		Without human serum	
Parameters	ZID	LAM	ZID	LAM
Wavelengths (nm)	285	285	285	285
Range ($\mu g \cdot ml^{-1}$)	10.5–52.5	6.4–55.8	10.5–52.5	6.4–55.8
Detection limits ($\mu g \cdot ml^{-1}$)	0.18	0.43	0.18	0.43
Regression equation (Y)[a]				
Slope (b)	1.98×10^{-1}	2.98×10^{-1}	1.48×10^{-1}	3.00×10^{-1}
Std. dev. on slope (S_b)	4.89×10^{-6}	3.74×10^{-6}	4.22×10^{-7}	2.79×10^{-6}
Intercept (a)	5.17×10^{-2}	1.42×10^{-2}	5.40×10^{-2}	1.15×10^{-2}
Std. dev. on intercept (S_a)	4.76×10^{-6}	1.23×10^{-7}	4.99×10^{-5}	2.47×10^{-7}
Std. error of estimation (S_e)	8.72×10^{-6}	3.95×10^{-5}	8.17×10^{-5}	6.32×10^{-5}
Correlation coefficient (r)	0.9994	0.9979	0.9998	0.9999
Rel. std. dev. (%)[b]	1.31	2.05	1.39	2.17
% Range of error[b] (% 95 confidence limit)	0.98	0.76	0.99	2.08

[a] $Y = a + bC$ where C is concentration in $\mu g \cdot ml^{-1}$ and Y in absorbance units. [b] Five replicate samples

The HPLC and the spectrophotometric methods are suitable techniques for the simultaneous determination of ZID and LAM in multi- component formulations and in human serum without interference of each other. The compensation technique, and differential derivative spectrophotometric method are rapid, simple and sensitive, and work without solving equations or separation steps. In the ratio spectra derivative spectrophotometry separate peaks and higher values of measurements can be obtained owing to the advantages of the selectivity of divisor concentration. The HPLC method gives a good resolution between ZID and LAM within a short analysis time (< 5.9 min). The HPLC method may be considered more specific than other methods, but also more expensive, requiring sophisticated chromatographic instrumentation for its performance. All the developed methods may be recommended for routine and quality control analysis of the investigated drugs in human serum and two-component pharmaceutical preparations.

3. Experimental

3.1. Apparatus

Spectrophotometric analysis was carried out on a Shimadzu 1601 double beam spectrophotometer with a fixed slit width (2 nm) connected to an IBM – PC and a HP LaserJet 1100 printer. The chromatographic system consisted of a HP 1100 series mode quaternary pump with a HP 1100 series manual injector 20 µl fixed loop, equipped with a VWD variable wavelength UV/VIS detector. The detector was set at 285 nm (0.02 a.u.f.s.). Peak areas were integrated automatically by a computer using Hewlett-Packard Chem Station software programme. Other apparatus used included a Radiometer NEL pH 890 pH meter digital equipped with a combined glass- calomel electrode and an ultrasound generator.

3.2. Chemicals

ZID and LAM were kindly supplied by Glaxo Wellcome Pharm.Ind. and were used without purification. Analytical grade phosphoric acid, HPLC grade methanol, and acetonitrile were purchased from Merck Chem. Ind.

3.3. Pharmaceutical preparation

Combivir® film tablets (Glaxo Wellcome Pharm. Ind. Turkey) were assayed. Its declared content was as follows: Zidovudine 300.0 mg, lamivudine 150.0 mg/film tablet

3.4. Procedures

3.4.1. Compensation technique

3.4.1.1. Determination of Standard Ratios

The first derivative spectra for each set of reference solutions using the appropriate solution were recorded. The first derivative maxima and minima ($^1D_{\lambda 1}/^1D_{\lambda 2}$), where appropriate at the specified wavelengths (λ_1 and λ_2) as indicated in parentheses in Table 1.

3.4.1.2. Spectrophotometric measurements

Prepare a series of solutions containing different concentrations of pure ZID above and below that present in the binary mixture solution and place them in succession in the reference cell. Place the solution of the mixture (containing ZID – LAM) in the sample cell. The first (^1D) absorption spectra of the solutions prepared were recorded and calculated the corresponding ratio (Table 1) in each instance and follow the calculated ratio for pure LAM. Determine the exact balance point (the ratio of the sample is equal to that of pure compound LAM) at which the concentration of compound ZID in the sample solution is equal to that in the reference solution. Follow the same steps using solutions of pure LAM in the reference cell to determine its concentration in the binary mixture at the balance point.

3.4.1.3. Standards solutions and calibration graphs

Stock solutions were prepared by dissolving ZID and LAM in methanol to obtain a concentration of 1.0 $mg \cdot ml^{-1}$ for each compound. The standard solutions were prepared by dilution of stock solutions in methanol to reach concentration ranges of 15.0 to 45.0 and 8.5 to 42.5 $\mu g \cdot ml^{-1}$ for ZID and LAM, respectively.

3.4.1.4. Assay procedure for dosage forms

An accurately weighed amount of powdered tablets in methanol equivalent to about one tablet was transferred into a 100 ml conical flask. After 30 min of mechanical shaking, the solution was filtered in a 100 ml calibrated flask through Whatman No 42 filter paper. The residue was washed three times with 10 ml of solvent and then the volume was completed to 100 ml with the same solvent. Appropriate solutions were prepared by taking suitable aliquots of the clear filtrates and diluting them with methanol.

3.4.1.5. Recovery experiments

Known amounts of the binary mixtures were added to the different preanalysed formulations of ZID, LAM. The binary mixtures were analysed by the proposed methods. The recoveries obtained after five repeated experiments were calculated.

3.4.1.6. Sample treatment

Blood was obtained from fasting healthy men. The samples were centrifuged for 20 min 6000 rpm and filtered through a cellulose acetate filter. The filtrates were collected in glass containers that had been carefully cleaned with hydrochloric acid and washed with deionised water and stored at 4 °C until analysis was performed. Aliquots of these filtrates (1.0 ml serum sample) were taken and treated as the described proposed methods.

3.4.1.7. Procedure for human serum samples

1 ml human serum was mixed with acetonitrile (2 ml) and the known amounts of ZID and LAM in laboratory-prepared mixtures, to give drug concentrations of 15.0 to 45.0 and 8.5 to 42.5 $\mu g \cdot ml^{-1}$. The mixtures were vortexed for 5 min. After deproteinization and centrifugation (20 min at 6000 rpm) the prepared solutions were filtered through a 0.45 µm membrane filter. The compensation technique and differential derivative spectra were taken against the serum blank, prepared as described above but without addition of the drugs.

3.4.1.8. Analytical recovery

Ten different concentrations of these drugs (ZID and LAM) in laboratory prepared mixtures were added to human serum to get concentrations of 15.0 to

45.0 and 8.5 to 42.5 $\mu g \cdot ml^{-1}$ for ZID and LAM, respectively. These serum samples were treated in the same way as for the calibration graph.

3.4.2. Differential derivative spectrophotometry

3.4.2.1. Standard solutions and calibration graph

Stock solutions were freshly prepared by dissolving ZID and LAM in methanol to obtain a concentration of 1.0 $mg.ml^{-1}$ for each compound. Appropriate volume aliquots of the stock solution were transferred to 25 ml calibrated flasks. Accurate volumes were transferred into two sets of 25 ml calibrated flasks. One set was diluted to volume with 0.1 N NaOH and the other one was diluted to volume with methanol. The first series contained a constant concentration of ZID (25.0 $\mu g \cdot ml$) and a varying concentration of LAM (15.0–45.0 $\mu g \cdot ml$). The second series contained a constant concentration of LAM (15.0 $\mu g \cdot ml$) and a varying concentration of ZID (8.5–42.5 $\mu g \cdot ml$), respectively. The solutions were protected from light throughout the study.

3.4.2.2. Assay procedure for dosage forms

Ten tablets (Combivir® film tablet) were accurately weighed and finely powdered. Quantities of the powdered tablets equivalent to 300.0 mg ZID and 150.0 mg LAM (one tablet) were weighed accurately, taken and dissolved in methanol in 100 ml calibrated flasks. After 30 min of mechanically shaking, the solution was filtered in a 100 ml calibrated flask through Whatman no 42 filter paper. The residue was washed three times with 10 ml of solvent. Then the volume was completed to 100 ml with methanol. The solution was diluted 1 : 50 with 0.1 N NaOH and methanol, separately. The difference spectra between the methanolic solution and equimolar 0.1 N NaOH solution of pure drugs and sample were recorded by placing the methanolic solution in the reference compartment and the 0.1 N NaOH solutions in the sample compartment. A first derivative spectrum of each of the differential curves was subsequently recorded. The solutions were measured at 246 nm and 263 nm for ZID and LAM, respectively.

3.4.3. HPLC

3.4.3.1. Chromatographic conditions

Solutions and mobile phases were prepared in the moment of use. The mobile phases used were 0.01 M sodium dihydrogen phosphate : methanol : acetonitrile (4 : 2 : 3 v/v/v). The analytical column was a Supelcocil C_{18} (5 µm, 150 × 4.6 mm) column. All analyses were done under isocratic conditions at a flow rate of 0.5 $ml \cdot min^{-1}$ and at room temperature.

3.4.3.2. Calibration

An external standard method was used for quantitative determinations. Calibration graphs were recorded from authentic samples of ZID and LAM in the mobile phase. Triplicate 20 µl injections were made for each solution. The final concentrations of ZID and LAM in the samples were calculated by comparison of sample and standard peak area obtained with the average of three injections of standard solutions.

3.4.3.3. Analysis of tablets

Ten commercial tablets and the contents of 10 tablet ingredients were separately weighed and powdered in different mortars. A portion of the powder equivalent to about one tablet and the content of one tablet was accurately weighed, transfered to a 100 ml calibrated flask and suspended in mobile phase for HPLC method. The flasks were completed to volume with the same solvent. The samples were filtered through a 0.45-µm membrane filter, then further diluted to suit the calibration graphs.

3.4.3.4. Procedure for human serum samples

1 ml human serum was mixed with acetonitrile (2 ml) and known amounts of ZID and LAM in laboratory-prepared mixtures, to give drugs concentrations of 10.5–52.5 $\mu g \cdot ml^{-1}$ for ZID and 6.4–55.8 $\mu g \cdot ml^{-1}$ for LAM, respectively. These mixtures were vortexed for 5 min. After deproteinization and centrifugation (20 min at 6000 rpm) the prepared solutions were filtered through a 0.45 µm membrane filter. The prepared solutions were transferred to chromatographic vials for analysis. 20 µl of these solutions were injected onto the chromatographic system.

3.4.3.5. Analytical recovery

Ten different concentrations in laboratory prepared mixtures of ZID were added to human serum to get concentrations of 10.5–52.5 $\mu g \cdot ml^{-1}$ for ZID and 6.4–55.8 $\mu g \cdot ml^{-1}$ for LAM, respectively. These serum samples were treated in the same ways as for the calibration graph.

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