



Determination of ternary mixtures of antibiotics, by ratio-spectra zero-crossing first- and third-derivative spectrophotometry*

BASILIO MORELLI

Universita' degli Studi Bari, Dipartimento di Chimica, Campus Universitario, Via E. Orabona 4 70126-BARI, Italy

Abstract: The ratio-spectra zero-crossing first- and third-derivative spectrophotometry have been used for determining ternary mixtures of penicillin-G sodium, penicillin-G procain and dihydrostreptomycin sulphate salts. The procedures are accurate, nondestructive and do not require resolutions of equations. In both methods, calibration graphs are linear, with zero-intercept, up to $30 \mu\text{g ml}^{-1}$ of penicillin-G sodium and penicillin-G procain, and up to $42 \mu\text{g ml}^{-1}$ of dihydrostreptomycin sulphate. $r = 0.9999$ in each instance. Working wavelengths, 218.5, 211 and 236 nm, respectively, in the first-derivative mode, and 222.5, 311.5 and 242 nm in the third-derivative mode. Detection limits for each drug at $p = 0.01$ level of significance were calculated to be 0.058, 0.010 and $0.014 \mu\text{g ml}^{-1}$ and 0.14, 0.012 and $0.34 \mu\text{g ml}^{-1}$, in the first- and third-derivative methods, respectively. Both methods apply favourably to either laboratory mixtures or commercial injections.

Keywords: Derivative spectrophotometry; ratio-spectra derivative spectrophotometry; antibiotics.

Introduction

Spectrophotometric methods of analysis have experienced a high evolution in the last 20 years. Derivative spectrophotometry has been applied extensively to the simultaneous determination of substances with overlapping spectra; in particular, in the last few years, it has been used with regard to the assay of formulated drug products [1-17].

Recently, Berzas *et al.* [5] developed a method and discussed the theory for resolving ternary mixtures based on the use of first-derivative of the ratio-spectra of mixtures, followed by measurements at the zero-crossing wavelengths of first-derivative of ratio-spectra of single components. The above method is an extension of one proposed for binary mixtures [18, 19], which followed from a method called Multi-Wavelength-Linear-Regression Analysis of Blanco *et al.* [20].

As a further development of our research of procedures for the assay of multi-components mixtures of drugs by derivative spectrophotometry, [21-30], in this paper we present the application of ratio-spectra zero-crossing first- and third-derivative spectrophotometry to the

simultaneous determination of penicillin-G sodium, penicillin-G procain and dihydrostreptomycin sulphate salts (A, B and C, respectively), antibiotics with extensively overlapping absorption spectra. The same mixture has recently been analysed by means of an original application of third-derivative spectrophotometry [29].

Briefly, this method was based on the preliminary location of the zero-crossing wavelengths of third-derivative spectra of mixtures A + B, A + C and B + C. These wavelengths were employed to obtain the calibration graphs of C, B and A, respectively, by measuring the third-derivative value of three-components standards in which the concentration of two drugs was kept constant and that of the other one variable.

The importance of the above mixture of antibiotics in therapy and the toxic effects of streptomycin and derivatives [29], induced us to test an improved method for a quality control of pharmaceuticals for these drugs.

Apart from this, this mixture was selected as it offered the opportunity of comparing a previously described procedure [29] with that presented here; conclusions and comments

* To my father.

reported later, could be extended to other ternary mixtures. Thus, the primary aim of this paper is to verify whether the theoretical principles of the ratio-spectra zero-crossing first-derivative method for ternary mixtures [5] could be successfully extended to third-derivative, by testing them in an already known experimental system [29], to test if the ratio-spectra derivative method was still valid for the present mixture (the limitations of this method, which sometimes prevent its use, have been exhaustively enounced in previous papers [5, 18, 19, 30], finally, to compare the experimental results achieved by first- and third-derivative ratio-spectra methods in order to establish advantages and/or drawbacks of each procedure.

Experimental

Reagents

Stock solutions (0.2 mg ml^{-1} in water) were prepared from pure samples of A, B and C (Farmitalia-Carlo Erba, Italy). Injections of Vettrimicina (Vetem S.p.A., Italy), labelled to contain, A — 0.727, B — 1.80, C — 3.0 g and sodium citrate as excipient 0.032 g, were assayed.

Apparatus

Perkin-Elmer Lambda 3b spectrophotometer, coupled with Epson PC computer and Graphtec Pen Plotter MP 4100. Suitable settings, slit width 1 nm, scan speed 60 nm/min, wavelength interval 0.5 nm. The computer calculated the derivative using the Savitzky-Golay method [31]; a value of $\Delta = 6$ was found optimal. Due to a certain extent of the noise level of the ratio-spectra, a smoothing function of 6 was used.

Procedure

Suitable volumes of A, B and C stock solutions, expected to contain up to $30 \text{ } \mu\text{g ml}^{-1}$ of A and B and $42 \text{ } \mu\text{g ml}^{-1}$ of C, were mixed in a 5-ml calibrated flask and diluted to volume with distilled water. According to the theory of the ratio-spectra zero-crossing first-derivative method [5], the absorption spectrum of the mixture was divided by a standard spectrum of C (C° , $12 \text{ } \mu\text{g ml}^{-1}$) for determining A and B, and by a standard spectrum of A (A° , $9 \text{ } \mu\text{g ml}^{-1}$) for determining C. Then, the first- and third-derivatives of the above ratio-spectra were recorded and the values of the derivatives

were measured at suitably selected wavelengths. In particular, in the first-derivative mode, the concentrations of A, B and C were found proportional, respectively, to the amplitudes of the signals at 218.5, 211 and 236 nm, zero-crossing wavelengths of the first-derivatives of the ratio-spectra of each component at a given concentration, i.e. B/C° , A/C° and B/A° . Analogously, in the third-derivative mode, the concentrations of A, B and C were proportional, respectively, to the values of the derivatives at 222.5, 311.5 and 242 nm, zero-crossing wavelengths of third-derivative of ratio-spectra B/C° , A/C° and B/A° .

The concentrations of A, B and C in a ternary mixture were computed from the corresponding calibration graphs for each drug.

Procedure for injections

The contents of a vial were dissolved in a 500-ml calibrated flask and diluted to volume with distilled water. The assay was completed as described under 'Procedure'. The percentage content of A, B and C was obtained from the corresponding regression equations.

Results and Discussion

Spectrophotometric measurements

The absorption spectra of A, B and C may be seen in [29], Fig. 1.

An accurate choice of standard divisors and working wavelengths are of capital importance for several reasons [18, 19, 30], hence we tested the methods with various divisor concentrations. The results of all tests are not shown for the sake of brevity and because these do not add to the scientific value of the work. For all subsequent measurements we used as standard spectra $9 \text{ } \mu\text{g ml}^{-1}$ of A (A°) and $12 \text{ } \mu\text{g ml}^{-1}$ of C (C°), which represented the best compromise in terms of sensitivity, repeatability, linearity range and signal-to-noise ratio.

In Fig. 1 are reported three series of ratio-spectra of A/C° , dashed lines, B/C° , continuous lines (left scale) and C/A° , dotted lines (right scale). A smoothing level of 6 was sufficient to minimize the noise.

In Fig. 2(a) and (b) are the first- and third-derivatives of ratio-spectra B/C° , A/C° and B/A° . The arrows indicate the zero-crossing wavelengths selected for determinations of each component of the ternary mixtures, by

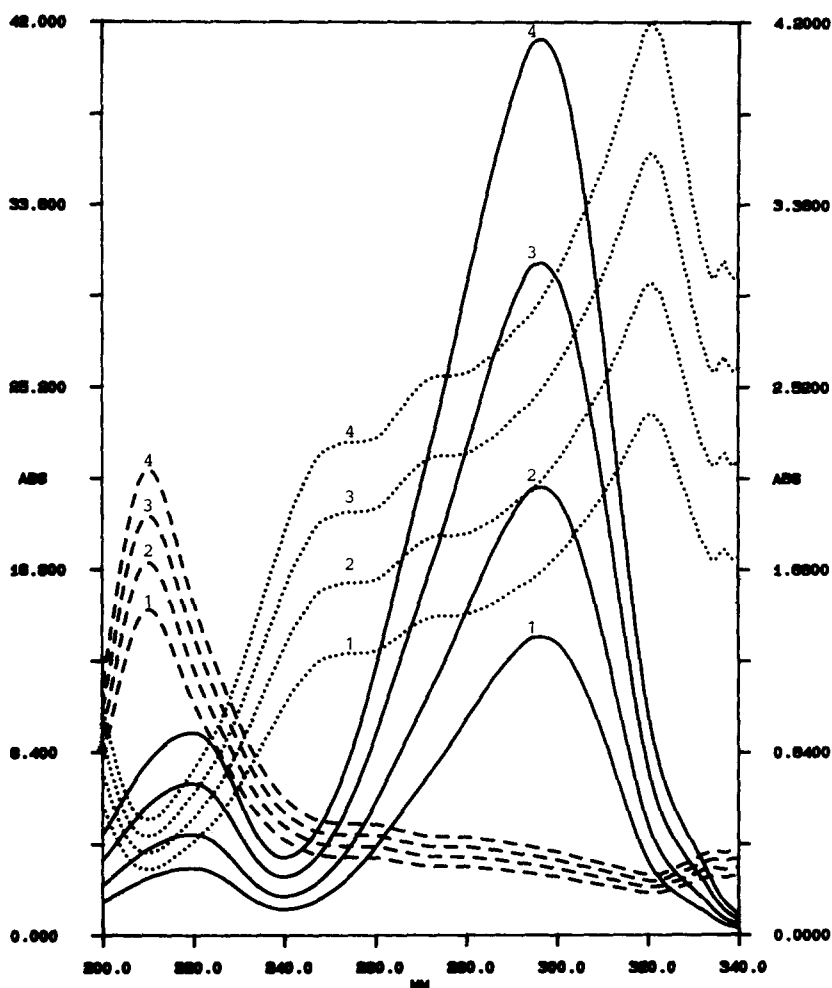


Figure 1 Ratio-spectra for different concentrations of A (21, 24, 27 and $30 \mu\text{g ml}^{-1}$, dashed curves 1 to 4, left ordinate scale, divisor C° , $12 \mu\text{g ml}^{-1}$), B (4, 6, 9 and $12 \mu\text{g ml}^{-1}$, continuous curves 1 to 4, left ordinate scale, divisor C° , $12 \mu\text{g ml}^{-1}$) and C (24, 30, 36 and $42 \mu\text{g ml}^{-1}$, dotted curves 1 to 4, right ordinate scale, divisor A° , $9 \mu\text{g ml}^{-1}$).

means of calibration graphs. Obviously, the cross-over points were independent of the concentrations utilized.

The zero-crossing wavelengths which exhibited the best linear response to the analyte concentration and were not affected by any other component, were in the first-derivative mode 218.5, 211 and 236 nm, for determining A, B and C, respectively, and in third-derivative mode, 222.5 nm for A, 311.5 nm for B and 242 nm for C.

The calibration graphs for each drug in both modes were achieved by plotting the values of first- and third-derivatives of ratio-spectra A/C° , B/C° and C/A° , with variable concentrations of A, B and C, at the above zero-crossing wavelengths, against the concentrations of A, B and C in the standards. In Fig. 3 are reported typical series of first-derivative of, (a) A/C° , (b) B/C° and (c) C/A° , with

increasing concentrations of A, B and C. The values of derivatives $D1(A)$, $D1(B)$ and $D1(C)$ are proportional to the concentration of A, B and C, respectively.

Analogously, in Fig. 4 are displayed examples of third-derivative ratio-spectra of, (a) A/C° , (b) B/C° and (c) C/A° . The amplitude of derivatives $D3(A)$, $D3(B)$ and $D3(C)$ are proportional to the concentration of A, B and C.

In Figs 3 and 4, the isosbestic points correspond, as expected, to the zero-crossing wavelengths of first- and third-derivative ratio-spectra of, (a) A/C , (b) B/C and (c) C/A .

Calibration graphs and statistical analysis

The regression equations calculated with the methods described above are presented in Table 1, together with statistical data.

Detection limits, at a $p = 0.01$ level of

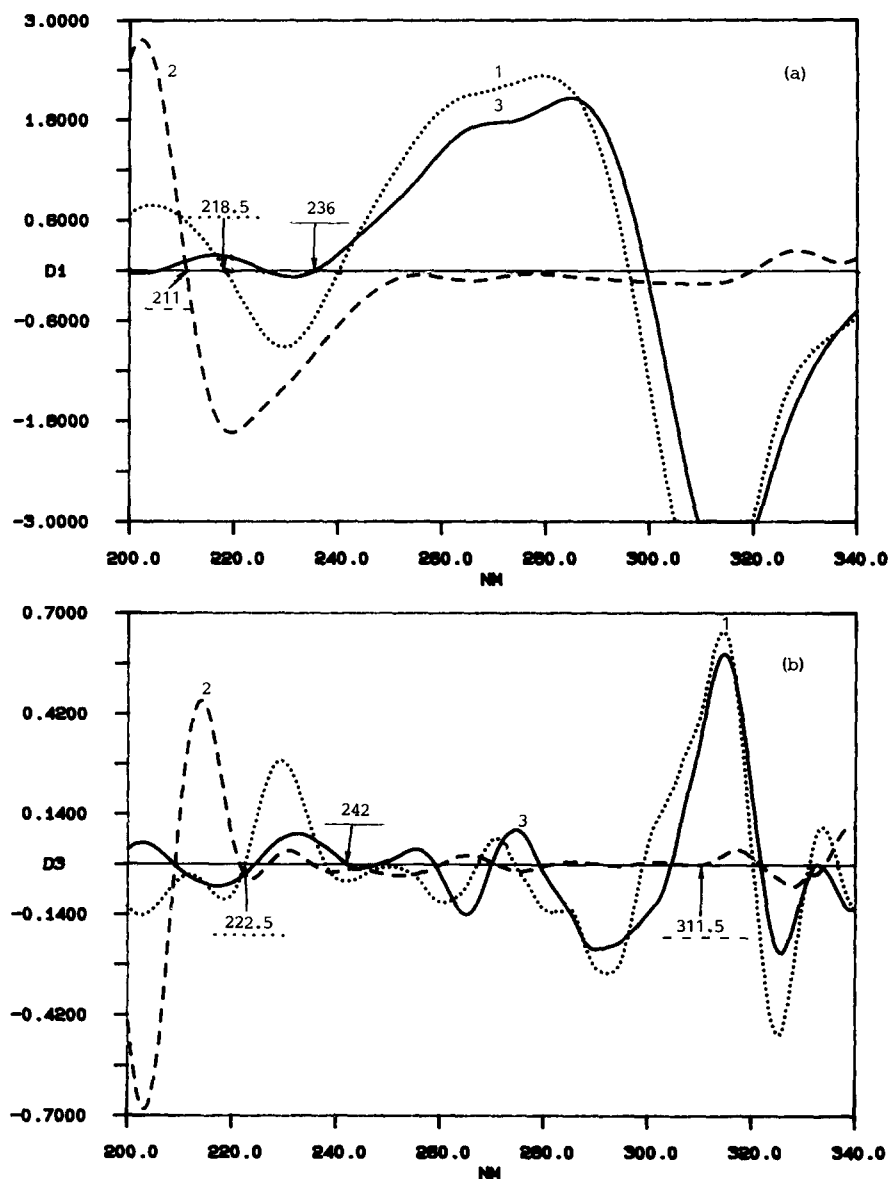


Figure 2

(a) First-derivative of ratio-spectra of, (1) B ($6 \mu\text{g ml}^{-1}$, divisor C° , $12 \mu\text{g ml}^{-1}$), (2) A ($18 \mu\text{g ml}^{-1}$, divisor C° , $12 \mu\text{g ml}^{-1}$) and (3) B ($6 \mu\text{g ml}^{-1}$, divisor A° , $9 \mu\text{g ml}^{-1}$); (b) third-derivative of ratio-spectra of, (1) B ($6 \mu\text{g ml}^{-1}$, divisor C° , $12 \mu\text{g ml}^{-1}$), (2) A ($9 \mu\text{g ml}^{-1}$, divisor C° , $12 \mu\text{g ml}^{-1}$) and (3) B ($6 \mu\text{g ml}^{-1}$, divisor A° , $9 \mu\text{g ml}^{-1}$). The arrows indicate the zero-crossing wavelengths selected for the determination of A, B and C.

significance, were calculated by a statistical treatment of calibration data [25, 29, 30, 32]. Method and equations are exhaustively reported in [25, 32]. The regression plots are linear up to $30 \mu\text{g ml}^{-1}$ of A and B, and $42 \mu\text{g ml}^{-1}$ of C, with intercepts near to zero. Tests of significance of the intercepts, a , of regression lines, showed that these did not differ significantly from the theoretical value, zero. A method of estimating the differences $a-0$, followed from the calculation of the quantities $t = a/Sa$ [25, 30, 33] and their comparison with

the tabulated data for the Student's t -distribution. The values calculated for t , shown in Table 1, never exceed the 99% criterion 3.36, which means that the intercepts of all regression lines, are not significantly different from zero, hence both first- and third-derivative methods are free from procedural errors.

As in previous papers [22–24, 26–29], the absolute error S_c [33] was calculated in the determination of a given concentration of A, B and C, by means of statistical analysis of regression equations in Table 1. The graphs S_c

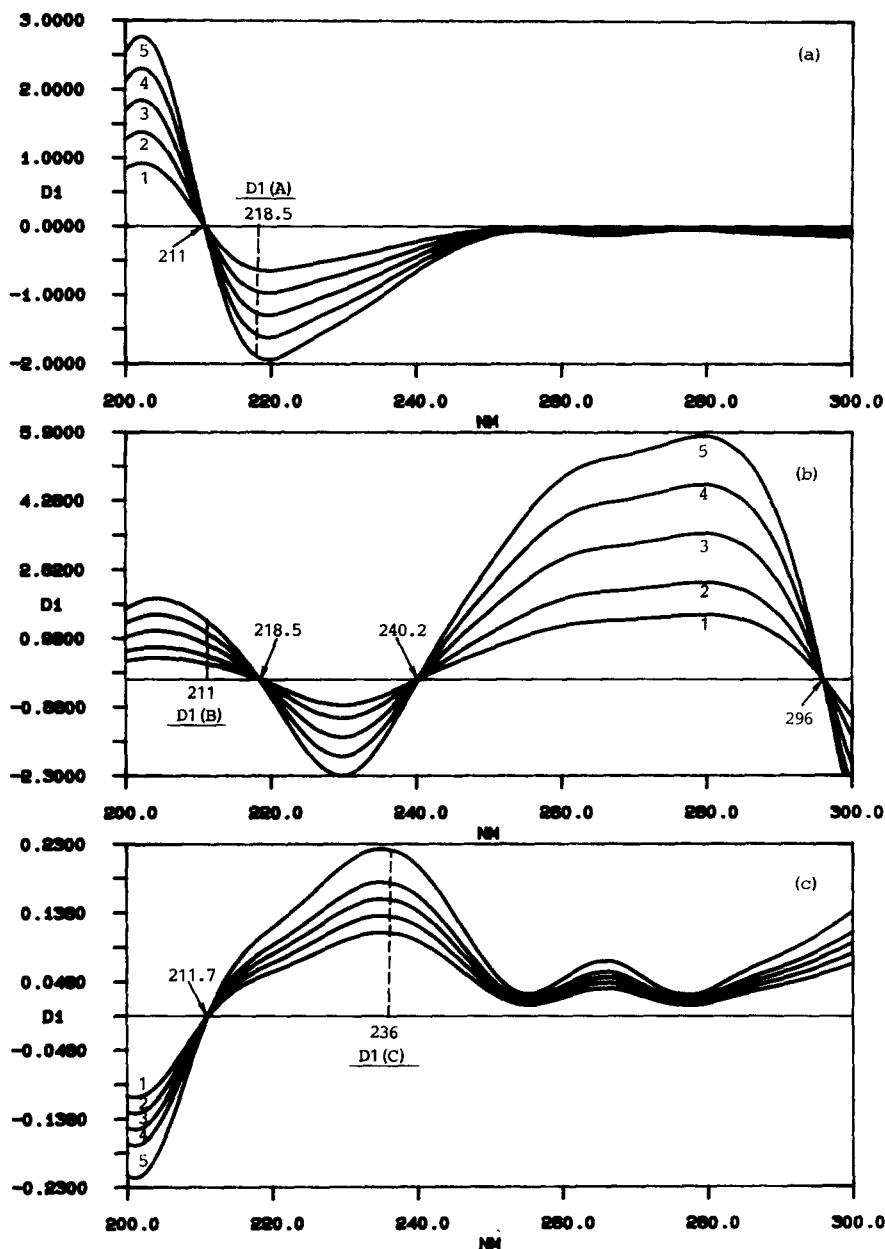


Figure 3

First-derivative ratio-spectra of A, B and C, at various concentrations. (a) A, 6, 9, 12, 15 and 18 $\mu\text{g ml}^{-1}$ (curves 1 to 5), standard divisor C° , 12 $\mu\text{g ml}^{-1}$; (b) B, 4, 6, 9, 12 and 15 $\mu\text{g ml}^{-1}$ (curves 1 to 5), standard divisor C° , 12 $\mu\text{g ml}^{-1}$; (c) C, 15, 18, 21, 24 and 30 $\mu\text{g ml}^{-1}$ (curves 1 to 5), standard divisor A° , 9 $\mu\text{g ml}^{-1}$.

vs concentration, not reported for brevity, showed that the error was at a minimum for about 16 $\mu\text{g ml}^{-1}$ of A and B and 21 $\mu\text{g ml}^{-1}$ of C, in both methods.

Accuracy and precision

Five replicate determinations of seven synthetic ternary mixtures of A, B and C, were performed to test accuracy and precision. The results reported in Table 2 are highly satisfactory.

Assay of injections

The two procedures were applied to the recovery of A, B and C in four batches of vials, containing these antibiotics in admixture (see 'Experimental', under 'Reagents'). The results, shown in Table 3, agree very well with the nominal content. The precision is very satisfactory.

It was necessary to determine if there was a statistically significant difference in the mean recoveries of A, B and C from injections,

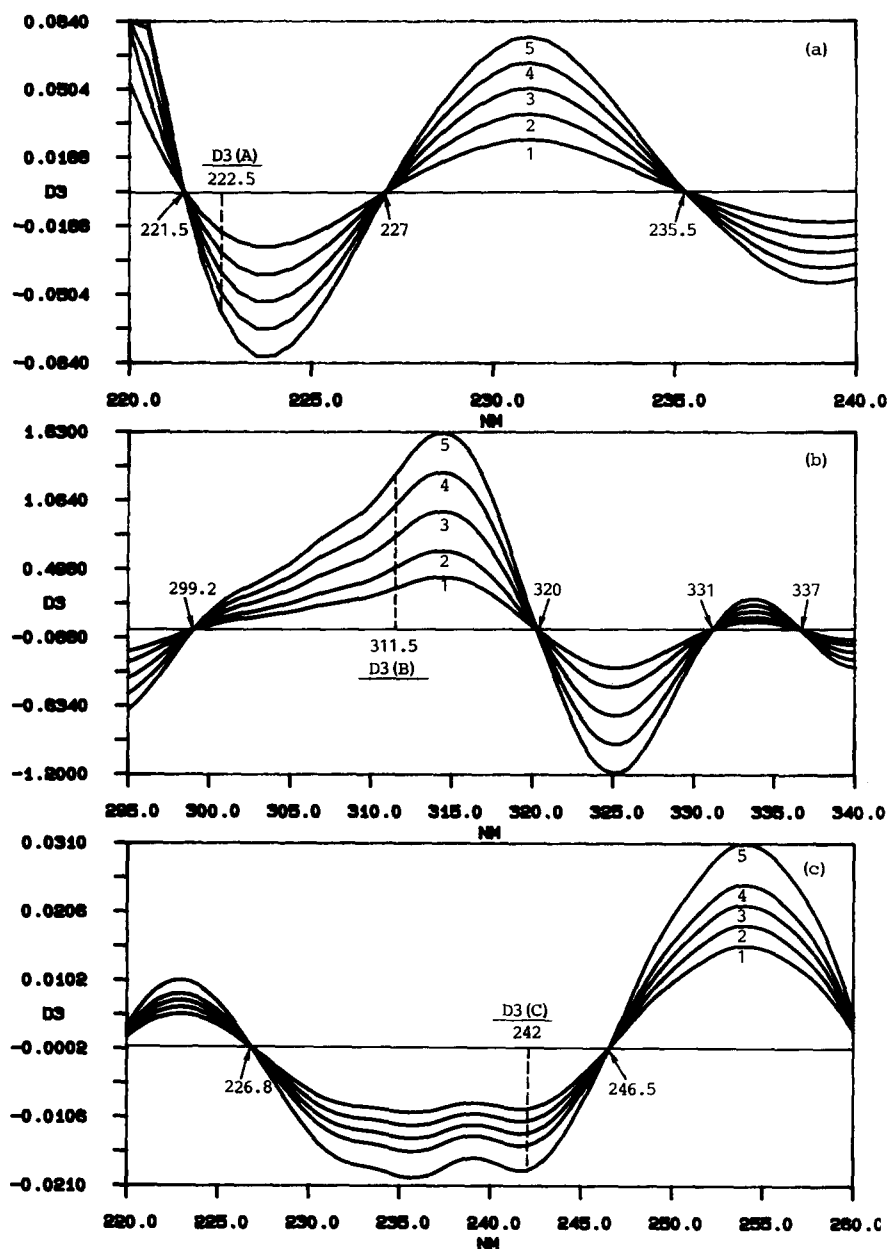


Figure 4

Third-derivative ratio-spectra of (a) A, (b) B and (c) C, at various concentrations. The concentrations of A, B and C and the standard divisors are the same as Fig. 3.

carried-out by the two ratio-spectra methods, i.e. to verify the null hypothesis $\mu_1 - \mu_2 = 0$. The problem was solved by means of the t -criterion [33]. In Table 3 are reported the t -values calculated at 0.01 level of significance, which never exceed the theoretical t -value 3.36; therefore, the null hypothesis was verified.

Conclusions

These results demonstrate that the theory on

which the first-derivative ratio-spectra zero-crossing method is based [5] can be extended to the third-derivative. Thus, the latter may represent a valid approach to quantitate ternary mixtures, and a useful alternative to the first-derivative procedure, especially when the first-derivative method gives poor results or fails completely [5, 18, 19, 30]. One of the advantages of higher derivatives is the possibility of selecting more suitable working wavelengths from a larger range of possibilities [5, 18, 19, 30].

Table 1
Statistical data for calibration graphs of A, B and C by 'ratio-spectra' first- and third-derivative spectrophotometry*

Drug	Regression equations	<i>r</i>	Variance <i>S</i> ₀ ²	Interc. <i>S</i> _a	Slope <i>S</i> _b	D.L. [†] ($\mu\text{g ml}^{-1}$)	<i>a/Sa</i> ‡
First-derivative method							
Standard divisor C ^o , 12 $\mu\text{g ml}^{-1}$							
A	D1(A) = $2.61 \times 10^{-4} - 1.07 \times 10^{-1} C_a$ (218.5 nm)	0.9999	3.8×10^{-6}	1.3×10^{-3}	7.3×10^{-5}	0.058	0.20
B	D1(B) = $7.95 \times 10^{-9} + 9.48 \times 10^{-2} C_b$ (211 nm)	0.9999	8.8×10^{-8}	2.0×10^{-4}	1.1×10^{-5}	0.010	3.90×10^{-5}
Standard divisor A ^o , 9 $\mu\text{g ml}^{-1}$							
C	D1(C) = $3.02 \times 10^{-6} + 7.42 \times 10^{-3} C_c$ (236 nm)	0.9999	1.1×10^{-9}	2.2×10^{-5}	9.3×10^{-7}	0.014	0.14
Third-derivative method							
Standard divisor C ^o , 12 $\mu\text{g ml}^{-1}$							
A	D3(A) = $8.17 \times 10^{-4} - 3.27 \times 10^{-3} C_a$ (222.5 nm)	0.9999	2.2×10^{-8}	4.1×10^{-4}	5.6×10^{-6}	0.14	1.99
B	D3(B) = $-9.87 \times 10^{-6} + 8.49 \times 10^{-2} C_b$ (311.5 nm)	0.9999	1.0×10^{-7}	2.2×10^{-4}	1.2×10^{-5}	0.012	0.044
Standard divisor A ^o , 9 $\mu\text{g ml}^{-1}$							
C	D3(C) = $4.12 \times 10^{-5} - 6.23 \times 10^{-4} C_c$ (242 nm)	0.9999	4.3×10^{-9}	4.6×10^{-5}	1.9×10^{-6}	0.34	0.89

^o A, penicillin-G sodium; B, penicillin-G procain; C, dihydrostreptomycin sulphate. Ca, Cb, Cc, concentrations of drugs, $\mu\text{g ml}^{-1}$. Number of samples, *n* = 10.

[†] Level of significance, *p* = 0.01 (99% probability).

[‡] Theoretical value of *t* at *p* = 0.01 level of significance, for *f* = *n* - 2 = 8 degrees of freedom, 3.36.

Table 2
Replicate determinations on synthetic mixtures of A, B and C by 'ratio-spectra' first- and third-derivative methods*

Nominal value ($\mu\text{g ml}^{-1}$)		First-deriv. method			Third-deriv. method		
		218.5 (nm) A	211 (nm) B	236 (nm) C	222.5 (nm) A	311.5 (nm) B	242 (nm) C
(1)	6 6 12	6.1 \pm 0.01 (0.16)	5.9 \pm 0.03 (0.51)	12.1 \pm 0.05 (0.41)	6.0 \pm 0.02 (0.33)	6.0 \pm 0.02 (0.33)	12.0 \pm 0.4 (0.33)
(2)	12 4 24	12.2 \pm 0.06 (0.49)	4.0 \pm 0.03 (0.75)	24.1 \pm 0.08 (0.33)	11.9 \pm 0.05 (0.42)	4.0 \pm 0.02 (0.51)	24.6 \pm 0.09 (0.36)
(3)	4 15 30	4.1 \pm 0.02 (0.49)	14.9 \pm 0.06 (0.40)	30.3 \pm 0.04 (0.13)	4.1 \pm 0.01 (0.24)	15.0 \pm 0.03 (0.20)	29.8 \pm 0.05 (0.17)
(4)	15 4 9	15.1 \pm 0.07 (0.46)	4.0 \pm 0.02 (0.50)	9.1 \pm 0.03 (0.33)	15.2 \pm 0.08 (0.53)	4.0 \pm 0.02 (0.50)	9.1 \pm 0.02 (0.22)
(5)	6 18 6	6.1 \pm 0.03 (0.49)	17.9 \pm 0.08 (0.45)	5.9 \pm 0.03 (0.51)	6.0 \pm 0.01 (0.17)	18.0 \pm 0.08 (0.44)	5.9 \pm 0.02 (0.34)
(6)	18 9 15	18.2 \pm 0.08 (0.44)	8.9 \pm 0.05 (0.56)	14.9 \pm 0.05 (0.33)	18.3 \pm 0.07 (0.38)	9.1 \pm 0.05 (0.55)	15.1 \pm 0.05 (0.33)
(7)	24 27 42	24.3 \pm 0.09 (0.37)	26.9 \pm 0.08 (0.30)	41.8 \pm 0.05 (0.12)	24.2 \pm 0.08 (0.33)	27.1 \pm 0.07 (0.26)	42.1 \pm 0.04 (0.09)

* A, penicillin-G sodium; B, penicillin-G procain; C, dihydrostreptomycin sulphate.

† Mean \pm Standard Deviation ($\mu\text{g ml}^{-1}$) for five determinations, with RSD % in parentheses.

Table 3
Recovery of A, B and C from injections by 'ratio-spectra' first and third-derivative methods and comparisons of the two means with the *t*-criterion*

Injections (Vettramicina)	Recovery (%)†						<i>t</i> -calc.‡
	First-deriv. method			Third-deriv. method			
	A	B	C	A	B	C	
Batch No. 1	218.5 (nm)	211 (nm)	236 (nm)	222.5 (nm)	311.5 (nm)	242 (nm)	218.5 211 236
Batch No. 2	101.3 \pm 0.91	99.3 \pm 0.89	99.9 \pm 0.78	100.1 \pm 0.32	99.6 \pm 0.41	99.9 \pm 0.79	222.5 311.5 242
Batch No. 3	99.9 \pm 0.48	100.7 \pm 0.40	99.9 \pm 0.37	100.1 \pm 0.38	100.2 \pm 0.40	99.2 \pm 0.36	278 0.72
Batch No. 4	100.5 \pm 0.68	101.5 \pm 0.33	99.2 \pm 0.51	100.7 \pm 0.58	100.9 \pm 0.23	100.4 \pm 0.85	0.73 1.97
	100.4 \pm 0.60	99.1 \pm 0.90	99.1 \pm 0.64	99.8 \pm 0.60	99.7 \pm 0.77	99.0 \pm 0.41	0.50 2.22
							1.58 1.13
							2.70 0.29

* A, penicillin-G sodium; B, penicillin-G procain; C, dihydrostreptomycin sulphate.

† Mean and standard deviation for five determinations, given as percentage of the nominal content (the label claim of Vettramicina and the firm are reported under 'Experimental' section).

‡ Number of degrees of freedom, $f = n_1 + n_2 - 2 = 8$ ($n_1 = n_2 = 5$, number of samples); theoretical value of *t* at $p = 0.01$ level of significance (99% probability), 3.36.

In the present instance, no substantial differences between the two methods were observed, although a slight advantage of the first-derivative method followed from an examination of detection limits (Table 1), which can be correlated to the slopes of calibration graphs which are generally greater in the first- than in the third-derivative, at the selected zero-crossing wavelengths. In this case, slightly more accurate and precise results were obtained by third-derivative in the assay of laboratory mixtures and, in general, in the recovery from injections.

From a comparison with the previous method [29], the present procedures offer a distinct improvement. This is particularly true for detection limits (ranging in the previous paper from 0.25 to 0.66 $\mu\text{g ml}^{-1}$) and recovery of A, B and C from injections (ranging from a minimum of 101.6 to a maximum of 103.1).

These advantages could be partially counterbalanced by the following. The requirement of the ratio-spectra method for an accurate selection of both concentration of standard divisors and working wavelengths. A greater number of measurements is also necessary for recording first the ratio-spectra and then the derivatives, making the ratio-spectra method a little more lengthy. Finally and less important, the ratio-spectra procedure can be easily performed by using computerized spectrophotometers to carry out the required mathematical operations.

In conclusion, both present and previous procedures, enable the quantitation of ternary mixtures of drugs with good accuracy and precision, both in laboratory samples and pharmaceutical products. In the present instance, better results were achieved by the ratio-spectra approach.

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