Pergamon

0731-7085(95)01281-8

Journal of Pharmaceutical & Biomedical Analysis, Vol. 13, No. 6, pp. 769–776, 1995
Elsevier Science Ltd
Printed in Great Britain
0731-7085/95 $9.50 + 0.00

Simultaneous determination of the binary mixtures of cefsulodin and clavulanic acid by using first-derivative spectrophotometry

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Abstract: Two-component mixtures of cefsulodin and clavulanic acid were analysed by a first-derivative spectrophotometric method using a zero-crossing technique of measurement. The relative ease offered by this derivative technique for the quantification of these drugs, with closely overlapping spectral bands, was clearly demonstrated. As the absorption band of clavulanic acid closely overlaps with that of cefsulodin, both direct and derivative spectrophotometric methods have been investigated and evaluated by an exhaustive statistical analysis of the experimental data. The first-derivative spectrophotometric method was found to be more rapid, accurate and reproducible. The procedure does not require any separation step. The calibration graphs were linear in the range 2.0–56.0 μg ml^{-1} for cefsulodin and 2.0–28.0 μg ml^{-1} for clavulanic acid. The lower detection limits of cefsulodin and clavulinic acid (*P* 0.05 level) were calculated to be 0.16 and 0.24 μg ml^{-1}, respectively. Mixtures of cefsulodin and clavulanic acid in ratios of 1:4–7:2 were satisfactorily resolved. Both components were also determined in physiological solutions used to prepare intravenous infusions of these antibiotics.

Keywords: *Derivative spectrophotometry; first-derivative spectrophotometry; cefsulodin; clavulanic acid; simultaneous determination.*

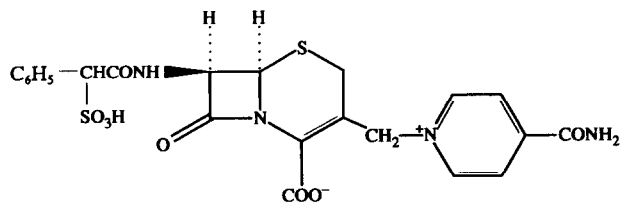
Introduction

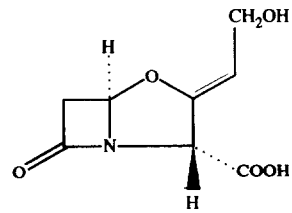
β-Lactamase inhibitors are emerging as important adjuncts in the chemotherapy of bacterial infections [1, 2]. The use of irreversible enzyme inhibitors, such as clavulanic acid, in combination with various β-lactamase-susceptible β-lactams has significantly increased the antibacterial spectrum of these drugs [3–5].

Cefsulodin is an almost "pure" antipseudomonal cephalosporin and its β-lactam ring can be hydrolysed by same transferable β-lactamases of Gram-negative bacteria [6]. Clavulanic acid has large effects on the minimum inhibitory concentrations of cefsulodin for Gram-negative anaerobes with reduced sensitivity to ampicillin and enhances the activity of this antibiotic [7]. In consequence, the joint administration of these drugs is of clinical interest as is the simultaneous determination of their mixtures.

Clavulanic acid shows an absorption spectrum that totally overlaps that of cefsulodin. Derivative spectrophotometry presents greater selectivity than does normal spectrophotometry and, therefore, offers a convenient solution to the problem of resolving spectral overlap in the analysis of multicomponent systems [8, 9].

O'Haver [10] and Fell [11] demonstrated the possibilities offered by derivative spectrophotometry for the analysis of pharmaceutical formulations. Later, this technique has been





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employed for the simulutaneous determination of substances of clinical interest, including cephalosporins in mixtures, e.g. cephapirin and cefuroxime [12], 7-aminocephalosporanic acid and 7-aminodesacetoxycephalosporanic acid [13].

The objective of this work was to demonstrate the ease with which the application of the "zero-crossing" technique to first-derivative spectrophotometry circumvents the problem of overlapping spectral bands and allows the simultaneous determination of cefsulodin and clavulanic acid in mixtures without the need for prior separation.

Experimental

Reagents

All experiments were performed with analytical-reagent grade chemicals and water filtered through a Milli-Q (Millipore) filter. A stock solution containing 200 μg ml^{-1} of cefsulodin (The Sigma Chemical Company) and a stock solution containing 100 μg ml^{-1} of clavulanic acid (Beecham Pharmaceuticals, BRL 14151) in water were prepared. These stock solutions were stored protected in the dark and below 4°C. A 0.05 M dihydrogen phosphate/hydrogen phosphate buffer solution (pH 6.8) was prepared.

Physiological solutions of 0.9% (w/v) sodium chloride were supplied by Apiroserum (Instituto de Biología y Sueroterapia, Madrid, Spain) and Grifols (Laboratorios Grifols, Madrid, Spain). A physiological solution of 5% anhydrous glucose (dextrose) was supplied by Apiroserum (Instituto de Biología y Sueroterapia, Madrid, Spain).

Apparatus

All spectral measurements and treatment of data were carried out with a Beckman DU-70 spectrophotometer connected to an IBM-PS computer with Beckman Data Leader software [14] and an Epson FX-850 printer. Among other possibilities, this system provides the capability of data manipulation for smoothing data and for generating derivatives. Moreover, special "zoom in/out" and "trace functions" allow the detailed analysis of data and interpolation values.

Procedure

Samples were prepared in 25-ml calibrated flasks containing 2.0–56.0 μg ml^{-1} of cefsulodin or 2.0–28.0 μg ml^{-1} of clavulanic acid or their binary mixtures, 5.0 ml of buffer solution (pH 6.8) and water to 25 ml.

Absorption spectra were recorded against a reagent blank using a 1.0-cm quartz cell. The first derivative was calculated and by measurement of the signal of the first-derivative spectra ($\Delta\lambda$ = 8 nm) at 280.0 nm and use of calibration graph, the cefsulodin concentration was determined. In the same way, the clavulanic acid concentration was determined by measurement of the first-derivative signal at 240.0 nm ("zero-crossing" point for cefsulodin) and use of calibration graph. Each calibration graph was constructed varying the concentration of one compound alone within the established range.

Results and Discussion

The stability of the aqueous solutions of cefsulodin and clavulanic acid was studied by recording their absorption spectra. No changes in the spectrum of each compound were observed when the solutions were stored at 4°C in the dark for 7 days.

Studies of the influence of pH on the absorption spectra indicated that the absorption of cefsulodin and clavulanic acid was maximum and constant over the pH-range 4.0–8.5. In order to avoid the possible hydrolysis of these compounds in acid and basic media, pH 6.8 was considered to be the optimum; this pH was achieved by adding a dihydrogen phosphate/hydrogen phosphate buffer.

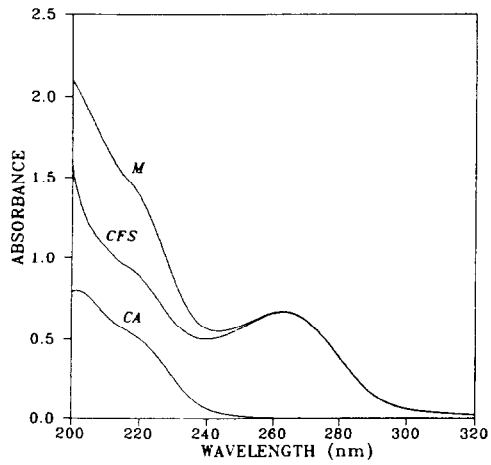

Figure 1
Absorption spectra of cefsulodin (CFS) (28.0 μg ml^{-1}), clavulanic acid (CA) (16.0 μg ml^{-1}) and a mixture (M); reference, reagent blank.

Figure 1 shows the absorption spectra of cefsulodin (CFS) (28.0 μg ml^{-1}), clavulanic acid (CA) (16.0 μg ml^{-1}) and a mixture (M) of both compounds. It can be seen that cefsulodin could be directly determined in the presence of clavulanic acid because its absorption spectrum exhibits a zone where the other component does not absorb. However, since the spectral band of clavulanic acid overlaps completely with the absorption spectrum of cefsulodin, there is no spectral feature utilizable to determine clavulanic acid directly.

The traditional Vierordt method, which involves the use of two simultaneous equations, and the modified Vierodt method [15] provide results of poor accuracy and reproducibility where the absorption spectra of the components are not sufficiently separated. To resolve the problem of closely overlapping spectra derivative spectrophotometry, particularly with digital processing, can be used. This technique involves differentiation of a normal spectrum with respect to the wavelength.

The zero-crossing method was used in this work with satisfactory results. This method involves the measurement of the absolute value of the total derivative spectrum at an abscissa value corresponding to the zero-crossing wavelengths of the derivative spectra of the individual components. Measurements made at the zero-crossing of the derivative spectrum of one of the two components are a function only of the concentration of the other component.

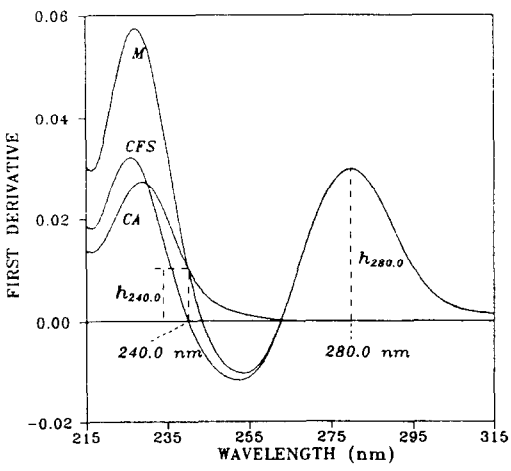

Figure 2
First-derivative spectra of cefsulodin (CFS) (28.0 μg ml^{-1}), clavulanic acid (CA) (16.0 μg ml^{-1}) and a mixture (M); reference, reagent blank.

Figure 2 shows the first-derivative spectra of cefsulodin (CFS), clavulanic acid (CA) and their mixture (M). The zero-crossing values of cefsulodin appear at 240.0 and 265.0 nm, whereas the first-derivative values of the clavulanic acid are zero at wavelengths greater than 264 nm. From these wavelengths, 240.0 and 280.0 nm were selected as optima to determine cefsulodin and clavulanic acid, respectively.

Selection of optimum instrumental conditions

The scan speed of the monochromator has virtually no effect on the derivative signal because differentiation is achieved digitally. The scan speed only determines the distance between each of the data points, which are collected every 0.05 s. A scan speed of 300 nm min^{-1} was selected; under these conditions, the instrument reads 4.0 data points per nm, which is equivalent to 0.25 nm between readings.

The main instrumental parameter affecting the shape of the derivative spectra is the wavelength increment over which the derivatives are obtained ($\Delta\lambda$). This parameter needs to be optimized to give a well resolved large peak, i.e. to give good selectivity and higher sensitivity in the determination. Savitzky and Golay [16] proposed a series of numerical tables for the smoothing of experimental data and for the computation of their derivatives. The main advantage of the method is that universal numerical functions are obtained, the convolution of which with the original vector data gives a smoothed vector as well as its successive derivative. Later, Steinier *et al.* [17] introduced a correction into Savitzky and Golay's reasoning and a check was performed by using a more general matrician formalism. The Data Leader software has a smoothing algorithm based on the method of Savitzky and Golay, which can result in an improvement in the signal-to-noise ratio.

Generally, noise decreases with an increase of $\Delta\lambda$, thus decreasing the fluctuation in a derivative spectrum. However, if the value of $\Delta\lambda$ is too large, the spectral intensity signal of the first derivative deteriorates. Various values of $\Delta\lambda$ were tested and a $\Delta\lambda = 8$ was chosen as the optimum in order to give an adequate signal-to-noise ratio. It was not necessary to use any smoothing function because of the low noise levels shown by the original and derivative spectra.

Calibration graphs and statistical analysis of experimental results

In order to test the mutual independence of the analytical signals of cefsulodin and clavulanic acid measured at 280.0 nm ($h_{280.0}$) and 240.0 nm ($h_{240.0}$), respectively, the following experiments were performed.

Four calibration graphs were constructed from the first-derivative signals from measurements at 280.0 nm for standard samples containing 2.0–56.0 μg ml^{-1} of cefsulodin, in the absence of clavulanic acid (p_0) and in the presence of 8.0 (p_1), 16.0 (p_2) and 28.0 μg ml^{-1} (p_3) of clavulanic acid. Figure 3 illustrates two of these series of first-derivative spectra where the concentration of cefsulodin is increased (from 2.0 to 56.0 μg ml^{-1}) in both cases; the first series (a) does not contain clavulanic acid but the other series (b) contains 16.0 μg ml^{-1} of clavulanic acid. The experiments showed that the height at 280.0 nm ($h_{280.0}$) was proportional to the concentration of cefsulodin.

Similarly, four calibration graphs were prepared from the first-derivative signals from measurements at 240.0 nm for standard samples containing 2.0–28.0 μg ml^{-1} of clavulanic acid, in the absence of cefsulodin (q_0) and in the presence of 12.0 (q_1), 28.0 (q_2) and 48.0 μg ml^{-1} (q_3) of cefsulodin. Figure 4 illustrates two of these series of first-derivative spectra where the concentration of clavulanic acid is increased (from 2.0 to 28.0 μg ml^{-1}) in both cases; the concentration of cefsulodin is 28.0 μg ml^{-1} in (b) but the first series (a) does not contain cefsulodin. Similarly, the experiments showed that the height at 236.75 nm ($h_{236.75}$) was proportional to the concentration of clavulanic acid.

It can be verified from Fig. 5 that all samples which contain the same concentration of clavulanic acid show a similar derivative signal value at an abscissa value corresponding to the zero-crossing wavelength of the cefsulodin (240.0 nm). Similarly, Fig. 6 indicates that all samples which contain the same concentration

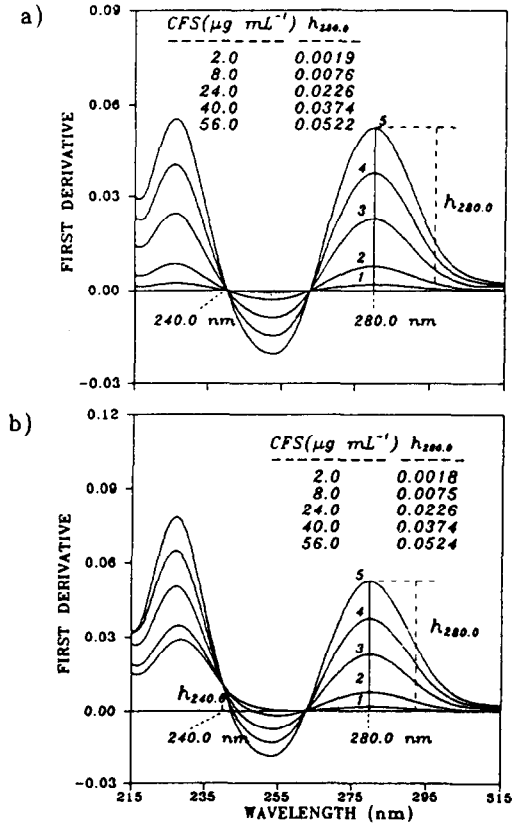

Figure 3
Two series of first-derivative spectra of: (a) concentration of cefsulodin 2.0–56.0 μg ml^{-1}; (b) 16.0 μg ml^{-1} of clavulanic acid with cefsulodin concentration 2.0–56.0 μg ml^{-1}.

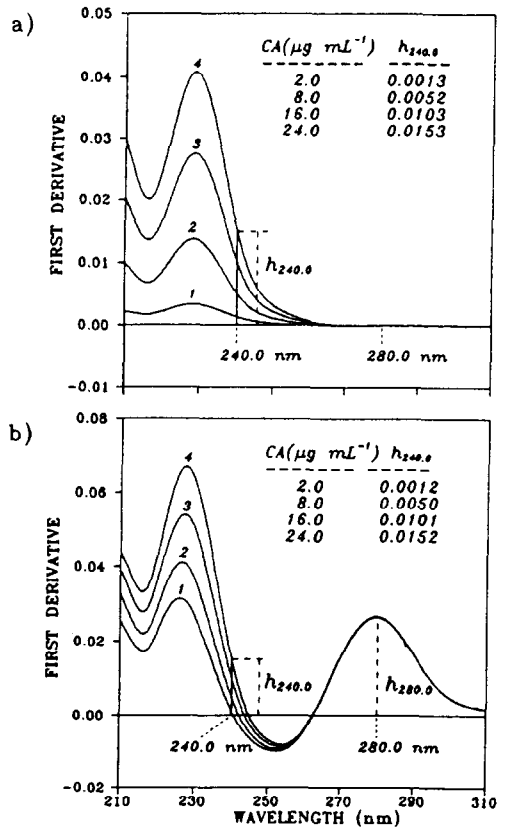

Figure 4
Two series of first-derivative spectra of: (a) concentration of calvulanic acid 2.0–28.0 μg ml^{-1}; (b) 28.0 μg ml^{-1} of cefsulodin with clavulanic acid concentration 2.0–28.0 μg ml^{-1}.

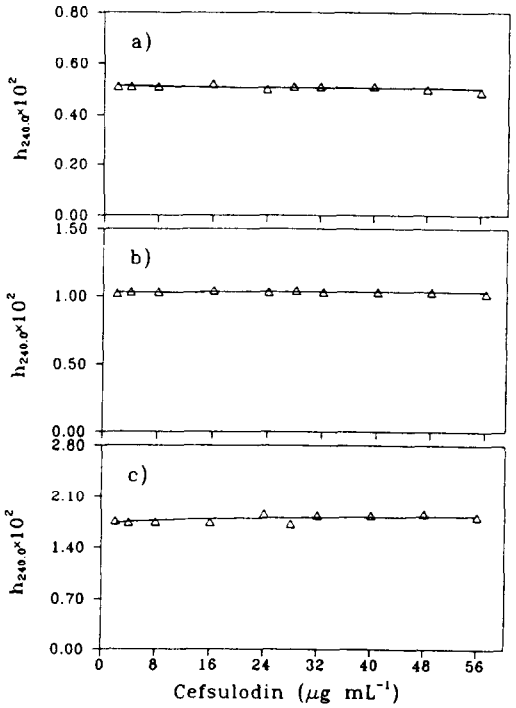

Figure 5
Derivative signal value at the zero-crossing wavelength of cefsulodin (λ = 240.0 nm), obtained for cefsulodin standards in the presence of clavulanic acid: (a) 8.0 μg ml^{-1}; (b) 16.0 μg ml^{-1}; (c) 28.0 μg ml^{-1}.

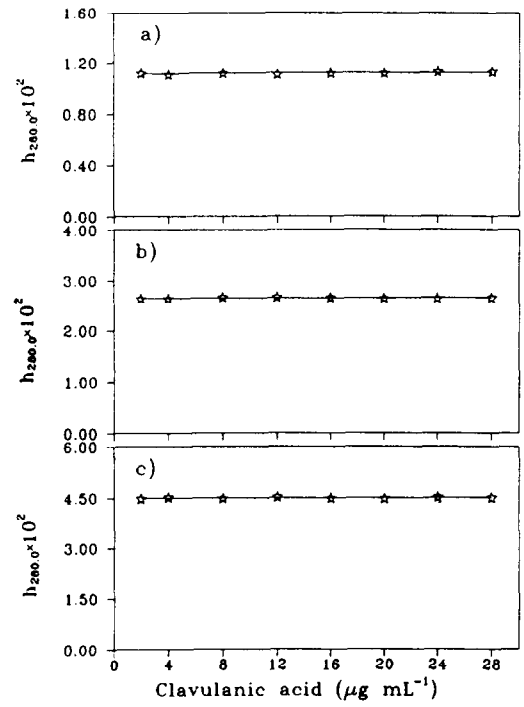

Figure 6
Derivative signal value at the zero-crossing wavelength of clavulanic acid (λ = 280.0 nm), obtained for clavulanic acid standards in the presence of cefsulodin: (a) 12.0 μg ml^{-1}; (b) 28.0 μg ml^{-1}; (c) 48.0 μg ml^{-1}.

of cefsulodin show a similar derivative signal value at the abscissa value selected to determine this antibiotic (280.0 nm) where the derivative signal of clavulanic acid is zero.

Tables 1 and 2 show the results of the statistical analysis of the experimental data for cefsulodin and clavulanic acid, respectively; the regression equations calculated from the calibration graphs are presented together with standard deviations of the slope and of the intercept on the y-axis. The linearity of the calibration graphs and the conformity of the systems to Beer's law is confirmed by the high values of the correlation coefficients of the regression equations.

The significance of the intercept on the y-axis of all regression lines was evaluated by applying Student's "t" test at the 95% confidence level with nine degrees of freedom for cefsulodin calibration graphs and seven degrees of freedom for clavulanic acid calibration graphs [18]. If the intercept on the y-axis for the regression lines calculated by the least squares method is negligible, it is necessary to fit the data according to a function whose intercept on the y-axis is zero so that the value of the slope (m_o) can be calculated. The results of this study for all the calibration graphs of cefsulodin and clavulanic acid are reported in Tables 1 and 2, respectively. It can be seen that the calculated "t" value does not exceed the theoretical value and hence the intercept on the y-axis is negligible in all of them. Consequently, the new values of the slope are calculated (Tables 1 and 2).

The precision of the proposed method was evaluated by applying the statistical technique of analysis of variance, which enables the total variation to be broken down into its components: the variation between samples; and the interaction between the calibration graphs and the sample. The validity of the analysis of variance assumes that the residual error variance does not change from one sample to another or from one calibration graph to another.

To carry out an analysis of variance, the variance ratio ($F_{\text{experimental}}$) must be calculated and compared to the theoretical value of "F" for adequate degrees of freedom at the 95% confidence level [19]. Tables 1 and 2 show the results obtained in this study. In both cefsulodin calibration graphs and clavulanic acid calibration graphs the experimental value of "F" is smaller than the theoretical value of

Table 1
Statistical analysis of calibration graphs in the determination of cefsulodin (2.0–56.0 μg ml^{-1}) in the presence of clavulanic acid by first-derivative spectrophotometry (n = 10)

	Calibration graphs of cefsulodin ($h_{280.0}$)			
		Clavulinic acid		
Cefsulodin 2.0–56.0 μg ml^{-1}	(p_0)	8.0 μg ml^{-1} (p_1)	16.0 μg ml^{-1} (p_2)	28.0 μg ml^{-1} (p_3)
Slope ($\times 10^4$)	9.34	9.37	9.37	9.37
Intercept ($\times 10^4$)	0.69	−0.07	0.06	1.03
Correlation coefficient	0.999	0.999	0.999	0.999
$\sigma^*_{(m)}$ ($\times 10^5$)	0.21	0.23	0.14	0.35
$\sigma^\dagger_{(a)}$ ($\times 10^4$)	0.64	0.73	0.42	1.08
$t_{experimental}$	1.084	0.101	0.143	0.953
$t_{theoretical}$‡	2.262	2.262	2.262	2.262
m_0 ($\times 10^4$)	9.36	9.37	9.37	9.39
$F_{experimental}$		1.82		
$F_{theoretical}$§		2.96		

*$\sigma_{(m)}$ = standard deviation of the slope.
†$\sigma_{(a)}$ = standard deviation of the intercept on the y-axis.
‡Theoretical value of t at the 95% level of confidence; nine degrees of freedom.
§Theoretical value of F at the 95% level of confidence; 27 degrees of freedom.

Table 2
Statistical analysis of calibration graphs in the determination of clavulanic acid (2.0–28.0 μg ml^{-1}) in the presence of cefsulodin by first-derivative spectrophotometry (n = 8)

	Calibration graphs of clavulanic acid ($h_{240.0}$)			
		Cefsulodin		
Clavulanic acid 2.0–28.0 μg ml^{-1}	(q_0)	12.0 μg ml^{-1} (q_1)	28.0 μg ml^{-1} (q_2)	48.0 μg ml^{-1} (q_3)
Slope ($\times 10^4$)	6.39	6.39	6.44	6.44
Intercept ($\times 10^4$)	0.43	−0.17	−1.13	−1.00
Correlation coefficient	0.999	0.999	0.999	0.999
$\sigma^*_{(m)}$ ($\times 10^5$)	0.20	0.32	0.45	0.42
$\sigma^\dagger_{(a)}$ ($\times 10^4$)	3.33	5.44	7.61	7.01
$t_{experimental}$	1.304	0.321	1.480	1.420
$t_{theoretical}$‡	2.365	2.365	2.365	2.365
m_0 ($\times 10^4$)	6.41	6.38	6.38	6.39
$F_{experimental}$		2.24		
$F_{theoretical}$§		3.07		

*$\sigma_{(m)}$ = standard deviation of the slope.
†$\sigma_{(a)}$ = standard deviation of the intercept on the y-axis.
‡Theoretical value of t at the 95% level of confidence; seven degrees of freedom.
§Theoretical value of F at the 95% level of confidence; 21 degrees of freedom.

"F"; therefore, at the 95% confidence level the source of variation is not significant. Therefore, it can be deduced that the amplitude of the derivative signal of the mixture, measured at the zero-crossing point of the derivative spectrum of one of the two components, is a function only of the concentration of the other component, in accordance with the theoretical predictions; that is, the variation of both $h_{280.0}$ and $h_{240.0}$ was not affected by the presence of clavulanic acid and cefsulodin, respectively, for any ratio of concentrations of the two components, in the full range tested.

To check the reproducibility of the method, replicate samples were measured (n = 10) containing cefsulodin (24.0 μg ml^{-1}) and clavulanic acid (20.0 μg ml^{-1}), individually. Table 3 shows the results of this study. Moreover, identification and quantification limits were calculated for each compound [20, 21].

Several binary mixtures of cefsulodin and clavulanic acid were prepared and analysed in

Table 3
Statistical parameters for the determination of cefsulodin and clavulanic acid (n = 10; P = 0.05)

	Signal measured	Standard deviation (μg ml^{-1})	Relative error (%)	Detection limit* (μg ml^{-1})	Quantification limit† (μg ml^{-1})
CFS	$h_{(280.0)}$	0.06	0.2	0.16	0.52
CA	$h_{(240.0)}$	0.10	0.4	0.24	0.81

*$C_L = 3S_B/m$; C_L = detection limit; S_B = standard deviation of blank; m = slope of calibration graph.
†$C_Q = 10S_B/m$; C_Q = quantification limit.

Table 4
Determination of cefsulodin and clavulanic acid mixtures by first-derivative spectrophotometry

	[CFS]	[CA]	[CFS]($h_{280.0}$)		[CA]($h_{240.0}$)	
Ratio [CFS]:[CA]	Theoretical (μg ml^{-1})	Theoretical (μg ml^{-1})	Found (μg ml^{-1})	Recovery (%)	Found (μg ml^{-1})	Recovery (%)
1:4	2.0	8.0	1.96	98.0	7.91	98.9
1:2	4.0	8.0	3.99	99.8	7.91	98.9
3:5	12.0	20.0	11.92	99.3	19.81	99.1
7:6	28.0	24.0	28.19	100.7	23.56	98.2
3:2	24.0	16.0	24.12	100.5	16.05	100.3
12:7	48.0	28.0	48.21	100.4	28.10	100.4
7:3	28.0	12.0	28.41	101.5	11.83	98.6
6:1	12.0	2.0	11.92	99.3	1.97	98.5
7:2	56.0	16.0	56.03	100.1	15.74	98.4

ratios of 1:4–7:2 by the proposed method. Table 4 shows the results of these tests on different mixtures. In all cases, recovery values of 98.0–101.5% were obtained. These satisfactory results demonstrate that the method is effective for the simultaneous determination of cefsulodin and clavulanic acid by first-derivative spectrophotometry.

Applications

In order to study the validity of the method, and because there are no medicines commercially available which contain both clavulanic acid and cefsulodin or cefsulodin the proposed method was applied to the determination of binary mixtures of these drugs in physiological solution of sodium chloride (0.9%) and in physiological solution of glucose (5%); these solutions were chosen because intravenous injection is the preferred route of administration in several infections. The determination of these antiobiotics was carried out with physiological solutions from different commercial companies: "Apiroserum" (Inst. de Biología y Sueroterapia, S.A.) and "Grifols" (Lab. Grifols). Three determinations were carried out for each of these solutions. Recoveries achieved were in accordance with the actual contents of cefsulodin and clavulanic acid in both physiological solutions. Recoveries varied between 98.0 and 101.8% for cefsulodin and from 98.2 to 102.2% for clavulanic acid.

Conclusions

The simplicity, precision and selectivity of derivative spectrophotometry make it particularly suitable for the simultaneous determination of binary mixtures of compounds having overlapping spectra. The experimental results of this work demonstrate that cefsulodin and clavulanic acid can be determined in their binary mixtures by first-derivative spectrophotometry using the zero-crossing technique. The measurement wavelengths were 280.0 and 240.0 nm to determine cefsulodin and clavulanic acid, respectively. Rigorous analysis of the results indicated that the presence of one component did not interfere with the determination of the other. This makes the method suitable for the routine analysis of these drugs in pharmaceutical preparations.

Acknowledgement — The authors gratefully acknowledge financial support from the "Dirección General de Investigación Científica y Técnica" (Project NO PB 88-0365).

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[Received for review 1 June 1994; revised manuscript received 27 September 1994]