

## Original article

**Chemometrics-assisted spectrophotometric  
determination of certain  $\beta$ -lactam antibiotics combinations****Abd El-Maboud I. Mohamed<sup>1</sup>, Hesham Salem<sup>2\*</sup> and Eman Maher<sup>2</sup>**<sup>1</sup>Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Assiut University, Assiut, Egypt.<sup>2</sup>Analytical Chemistry Department, Faculty of Pharmacy, Minia University, Minia, Egypt.

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**Abstract:**

Multivariate and derivative spectrophotometric techniques (first derivative and derivative ratio) were developed for the determination of four  $\beta$ -lactam antibiotic binary mixtures; ampicillin with flucloxacillin (mix I), ampicillin with dicloxacillin (mix II), amoxicillin with flucloxacillin (mix III) and amoxicillin with dicloxacillin (mix IV) in pharmaceutical combinations containing these compounds. The simultaneous determination of these compounds was accomplished by first derivative ( $dA/d\lambda$ ) spectrophotometric technique, applying zero-crossing technique and first derivative of the ratio spectrum. The influence of  $\Delta\lambda$  for obtaining the first derivative of the ratio spectra and the effect of the divisor concentration on the calibration graphs were studied. Lastly by multivariate methods; (classical least squares (CLS) and principle component regression (PCR)). Absorption spectra of compounds were used to optimize the spectral data set performs the calibration by CLS and PCR. These calibration models were evaluated by internal validation (prediction of compounds in its own designed training set of calibration), by cross-validation (obtaining statistical parameters that show the efficiency for a calibration fit model) and by external validation over synthetic and pharmaceutical mixtures. The four described procedures were successfully applied to the determination of these compounds in synthetic mixtures and in pharmaceutical preparations with high percentage of recovery, accuracy and precision. The procedures do not require any separation step.

**Keywords:**  $\beta$ -lactam antibiotics; Classical least squares; Derivative ratio spectrum; First derivative spectrophotometry; Principle component regression

## Introduction

Ampicillin is one of extended-spectrum penicillins, it is acid stable and moderately well absorbed when taken orally. It has a spectrum broader than that of benzyl penicillin and it is bactericidal against gram-negative bacteria including *H. influenzae*, *Salmonella*, *Shigellae*, *E. Coli*, and some proteus strains [1]. The recent methods for determination of ampicillin include, electrochemical [2], liquid chromatographic [3] and spectrophotometric methods [4, 5].

Amoxicillin is better absorbed from the gut, and it is more effective against *Salmonella*, *Strep. faecalis* and penicillin-resistant pneumococci. It is used in empiric treatment (with or without clavulanate) of bite wound infections, otitis media, sinusitis and urinary tract infections and in prevention of endocarditis in persons at risk with an oral dose of 250 mg 8 hourly [1]. The recent methods for determination of amoxicillin include, spectrofluorometric methods [6] and liquid chromatographic [7].

Dicloxacillin is one of penicillinase-resistant penicillins. It resists degradation by gastric acid and is absorbed from gut. The substitution of chlorine atoms on both carbons *ortho* to the position of attachment of the phenyl ring to the isoxazole ring is presumed to enhance further the stability of this oxacillin congener and to produce high plasma concentrations of it. Dicloxacillin is used in treatment of skin and soft tissue infections with an oral dose of 500 mg 6 hourly [1]. The literature presents spectrophotometric methods [4] and liquid chromatographic [8, 9] and for simultaneous determination of dicloxacillin.

While flucloxacillin is one of penicillinase-resistant penicillins. It is more fully absorbed and so gives higher blood levels. Also it is strongly protein bound and used for benzyl penicillin-resistant staphylococcal infections, with an orally or I.M. dose of 250 mg 6 hourly [1]. The literature presents liquid chromatographic [3] and spectrophotometric methods [3, 10] for simultaneous determination of flucloxacillin.

All studied combinations are broad-spectrum antibiotic combination widely used in the treatment of wide range of gram-negative and gram-positive organisms. They are acid stable and well absorbed producing good serum and urine concentration [1]. Derivative spectrophotometry is an analytical technique of great utility for resolving some mixtures of compounds with overlapping spectra [11-13]. Multivariate calibration methods applied to spectral data are being increasingly used for pharmaceutical analysis. Classical least squares (CLS) and principal components regression (PCR) analysis are the most simplest multivariate methods that can be performed with easily accessible statistical software [13, 14]. This CLS method is intuitively appealing since it is based on some generally assumed relationship, e.g. Beer's law, and it can be used for moderately complex composition of the calibration mixtures, i.e. the concentration of each absorbing species.

Principal component regression (PCR) is a two-step procedure. In the first step, one determines principal components that are linear combinations of the original variables. They can be considered as new variables that summarize in an optimal way the variation present in the spectra. In the second step, CLS is applied to the newly obtained latent variables.

In this work, new chemometric methods were used to develop spectrophotometric methods for the simultaneous determination of the components of these binary mixtures without prior separation.

## Experimental

### Apparatus

Spectrophotometric measurements were carried out on a computerized Spectronic Gensys 2PC, UV/visible spectrophotometer (Milton Roy, USA), using 1.00 cm quartz cells. The obtained spectral data were saved in PC apparatus program and the subsequent statistical manipulation was performed by transferring

the spectral data to Microsoft excel XP program and processing them with the standard curve fit package and matrix calculations. Sonicator (Bransonic 220, Bender-Hobein) was used. Curve Expert version 1.37 Copyright©1995-2001 by Daniel Hyams. GraphPad Instat version 3.05.32 bit for win 95/NT created Sep. 27, 2000 Copyright©1992-2000 by GraphPad software.

## **Materials**

### **Pharmaceutical compounds**

All materials and reagents used were of analytical grade. Amoxicillin and ampicillin were supplied by EIPCO Egypt, dicloxacillin was supplied by Memphis Co., Egypt and flucloxacillin was supplied by CID Co. Egypt. All drugs were used as working standards without further purification and analyzed to one of the official methods or reported methods to determine their purity and compliance with the requirements.

### **Formulations**

Ampiflux<sup>®</sup> capsules with batch number 270903 Pharco Co. Egypt, labeled to contain 250 mg of ampicillin and 250 mg of flucloxacillin per capsule, Dipenacid<sup>®</sup> vial with batch number 1203111 CID Co. Egypt. labeled to contain 250 mg of ampicillin and 250 mg dicloxacillin per vial, Flumox capsules with batch number 056767 EIPICO Egypt and Amoclox<sup>®</sup> capsules with batch number 304177 Memphis Co., Egypt as commercial pharmaceutical preparations were purchased from the local market and subjected to analysis by the proposed methods.

## **Solvents and solutions**

### **Solvents**

Distilled water was used through the all procedures in mixture I. Absolute ethanol was used through the all procedures in mixture II. Sodium hydroxide solution was used through the all procedures in mixture III and IV.

## **Preparation of stock and working standard solutions**

Stock solutions of authentic were prepared by dissolving an accurately weighed amount (50 mg) of the studied drugs in about 40 ml of the suitable solvent in 50 ml volumetric flask. The solution is then made up to the volume with the same solvent. Suitable aliquots of the stock solutions were completed quantitatively with the solvent to obtain the suitable working standard solutions according to the linear calibration range for each drug.

### **Preparation of dosage form samples**

The contents of twenty capsules were evacuated, thoroughly mixed and finely powdered if necessary. An accurately weighed amount equivalent to 50 mg of both drugs was transferred quantitatively to 50 mL volumetric flask and then about 40 ml with the previously specified solvent was added. The mixture was shaken well for about 10 minutes, diluted to the mark with the same solvent and then filtered. The first portion of the filtrate was discarded. The clear solution obtained was used as stock sample solution. A suitable aliquot of the stock solution was then diluted quantitatively with the used solvent to obtain the suitable working sample solution for UV measurements at the specified range.

## **General procedures**

### **Procedures for determination of linearity range of standard solutions**

In order to obtain the calibration curve for applying quantitative analysis six solutions of each of the pure components of each mixture were prepared with concentrations in the calibration range. These ranges were previously verified to obey Beer's law for each of the studied drugs.

### **Procedures for preparation of laboratory prepared mixture solutions**

Laboratory prepared mixtures were prepared by mixing known amounts of working solution of one of the mixture components with known amounts of working

solution of the other component in different proportions (ratios) in order to verify the precision of the method for analysis of such mixtures and matching the commercial formulations with those having comparable concentrations

#### **Procedures for preparation of dosage form solutions**

Different dilutions of dosage form working solutions were assayed for its studied drugs content as a procedure for prediction step.

#### **Procedures for standard addition technique**

Portion of dosage form working solution was quantitatively transferred to six volumetric flasks, then serial portions of authentic working solutions of both drugs were added to each flask and the solution was completed with the used solvent and measured at the specified wavelength.

#### **Optimization**

##### **Data processing**

Data were processed on an Intel Pentium III 750 MHz PC-compatible computer. For CLS calculations, the spectral data were transferred to Microsoft excel XP program and processing them with the standard curve fit package and matrix calculations. The MVSP version 3.13g (1985-2003), and VISTA 6 version 6.4.3436-EWU (May 10, 2001) software were used for the principal component regression applications.

##### **Selection of wavelength range for CLS and PCR calculations**

By trials and errors wavelength ranges were selected to give the most accurate and precise results.

##### **Degree of spectral overlapping**

The absorption spectra for the studied drugs showed a considerable degree of spectral overlapping.

The degree of spectral overlapping can be conveniently given by  $(D_i)^{0.5}$  [12], where  $D_i$  is the magnitude of the dependency which can be calculated for a two component mixture from the equation:

$$D_i = \frac{\sum (k_1 k_2^t)^2}{\sum k_1 k_1^t \sum k_2 k_2^t}$$

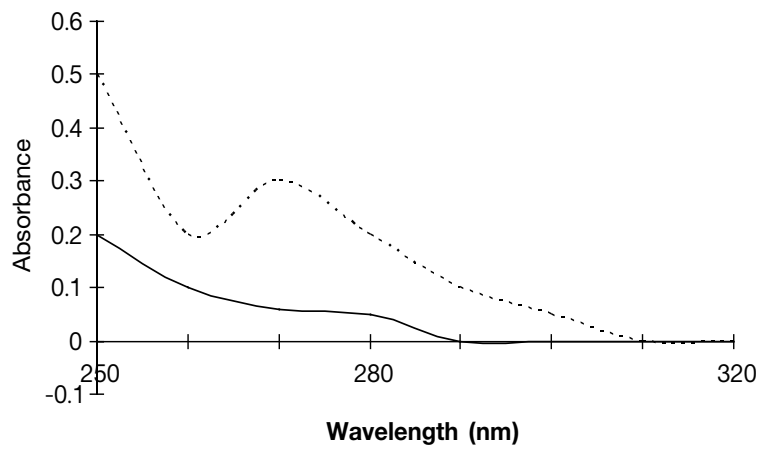
; where  $k_1$  and  $k_2$  are the  $l \times n$  matrices of regression coefficients for studied drugs and  $k^t$  is the transposed  $k$  matrix.

## **Results and Discussion**

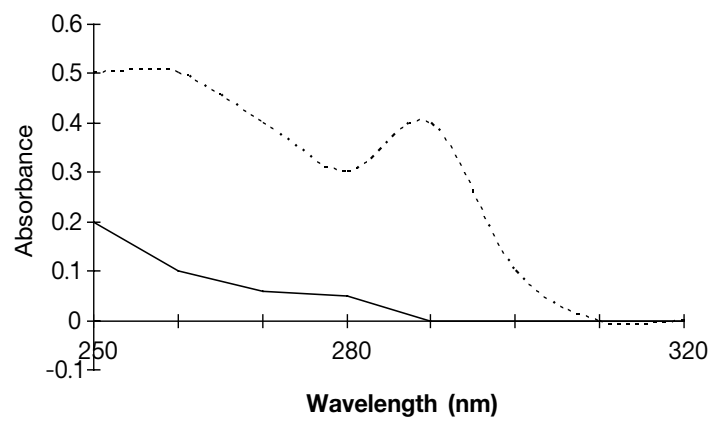
### **Derivative spectrophotometric analysis**

#### **Zero-crossing technique**

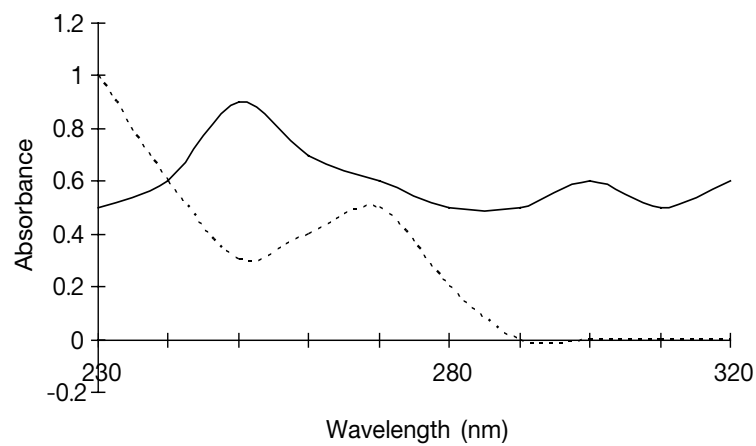
Figures 1-4 show the degree of absorption spectra overlapping for both drugs in each mixture. In the corresponding  $^1D$  curves (Figures 5-8), ampicillin shows a well absorption  $^1D$  value at ( $\lambda = 262.5$  nm) while flucloxacillin have no contribution. On the other hand, flucloxacillin exhibited an absorption  $^1D$  value at ( $\lambda = 281.5$  nm) where ampicillin absorbance was nil (Figure 5). In Figure 6, ampicillin shows a well absorption  $^1D$  value at ( $\lambda = 267.5$  nm) while dicloxacillin have no contribution. Dicloxacillin exhibited an absorption maximum  $^1D$  value at ( $\lambda = 285.5$  nm) where ampicillin absorbance was nil. In Figure 7, amoxicillin shows a well absorption  $^1D$  value at ( $\lambda = 262.5$  and  $268.5$  nm) while flucloxacillin have no contribution. Flucloxacillin exhibited an absorption maximum  $^1D$  value at ( $\lambda = 277.5$  nm) where amoxicillin absorbance was nil. In Figure 8, amoxicillin shows a well absorption  $^1D$  value at ( $\lambda = 267.5$  nm) while dicloxacillin have no contribution. Dicloxacillin exhibited an absorption maximum  $^1D$  value at ( $\lambda = 273.5$  nm) where amoxicillin absorbance was nil. The analytical parameters for the assay of binary mixtures of the studied drugs are presented in Table 1.



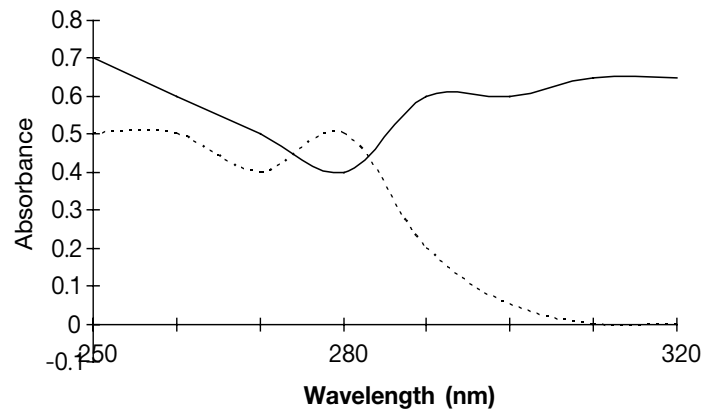
**Figure 1.** Absorption spectra of 200 µg/mL ampicillin sodium (–) and 200 µg/mL flucloxacillin sodium (---).



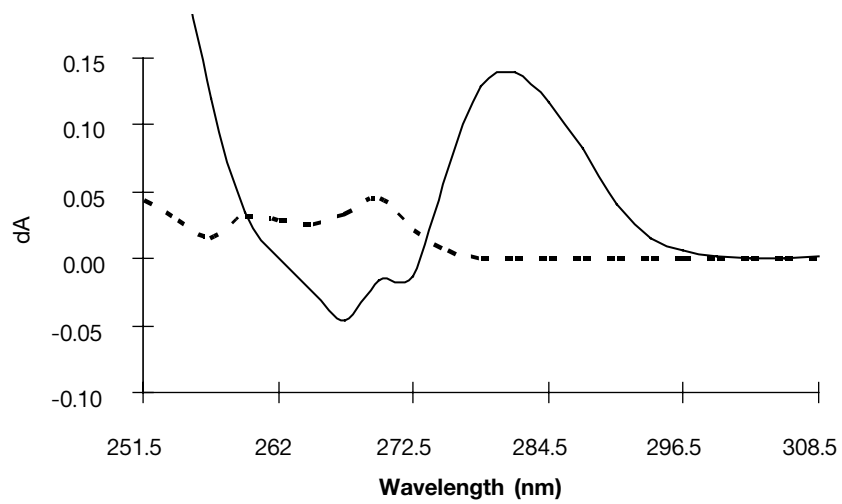
**Figure 2.** Absorption spectra of 200 µg/mL ampicillin sodium (–) and 200 µg/mL dicloxacillin sodium (---).



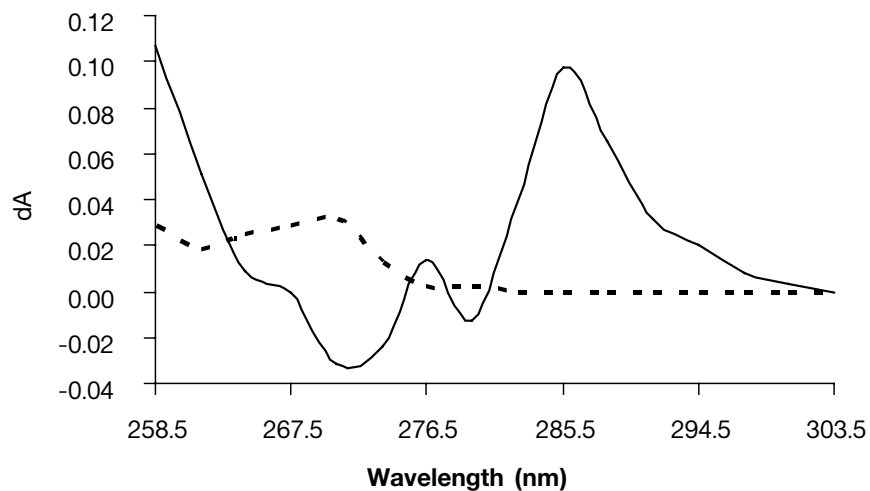
**Figure 3.** Absorption spectra of 30 µg/mL amoxicillin sodium (–) and 60 µg/mL flucloxacillin sodium (---).



**Figure 4.** Absorption spectra of 100 µg/mL amoxicillin (—) and 600 µg/mL dicloxacillin sodium (---).



**Figure 5.** First derivative spectra of ampicillin sodium (---) at 262.5 nm and flucloxacillin sodium (—) at 281.5 nm.



**Figure 6.** First derivative spectra of ampicillin sodium (---) at 267.5 nm, dicloxacillin sodium (—) at 285.5 nm (zero-crossing) and at 279.5 & 285.5 nm (peak-to-peak) spectrophotometric techniques.

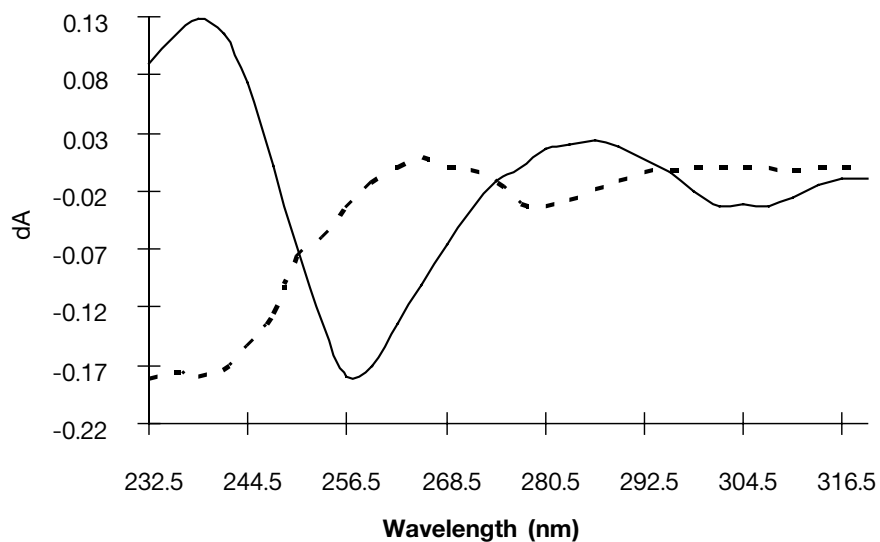


Figure 7. First derivative spectra of amoxicillin sodium (–) at 262.5, 268.5 nm and flucloxacillin sodium (–) at 277.5 nm

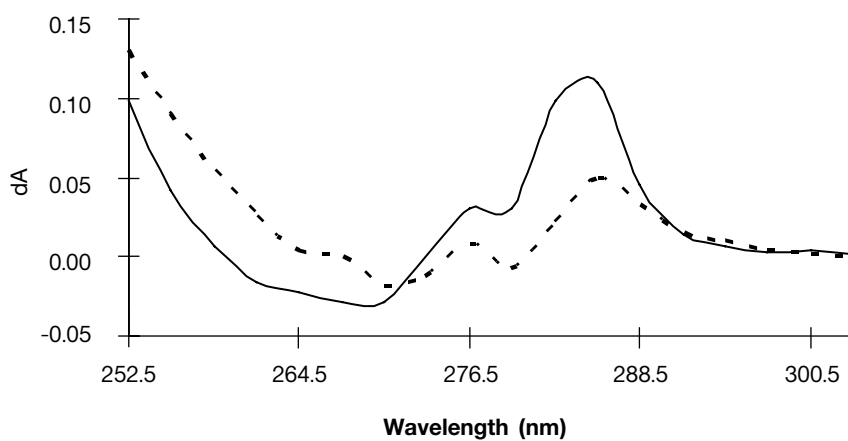


Figure 8. First derivative spectra of amoxicillin trihydrate (–) at 267.5 nm and dicloxacillin sodium (–) at 273.5 nm.

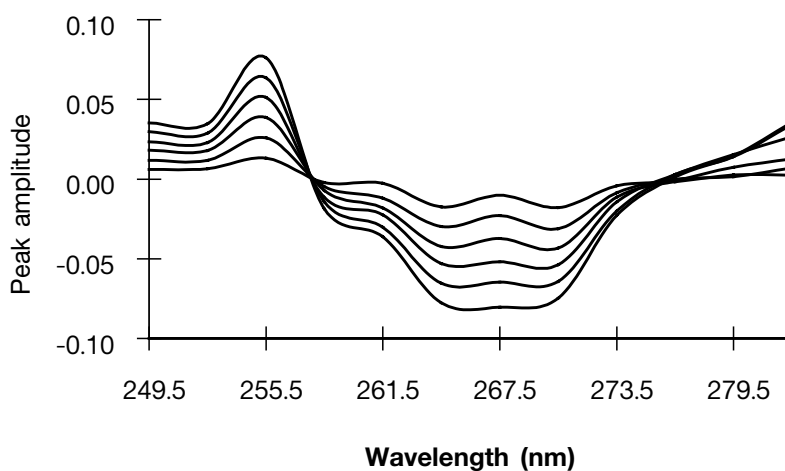


Figure 9. First derivative ratio spectra of ampicillin sodium (50 - 300 µg/mL). Divisor is 100 µg/mL flucloxacillin sodium.

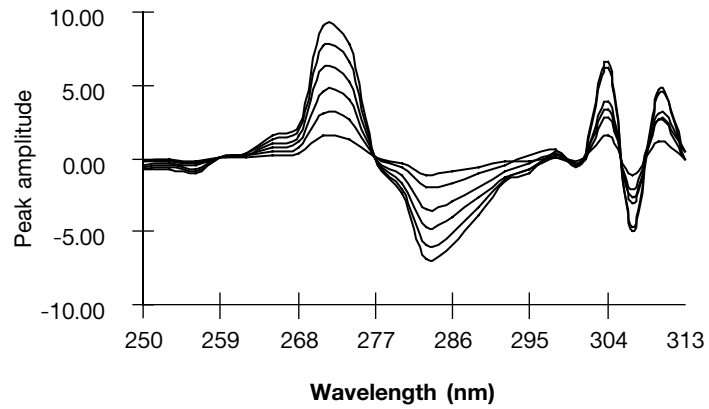


Figure 10. First derivative ratio spectra of flucloxacillin sodium (50 - 300  $\mu\text{g/mL}$ ). Divisor is 70.70  $\mu\text{g/mL}$  ampicillin sodium.

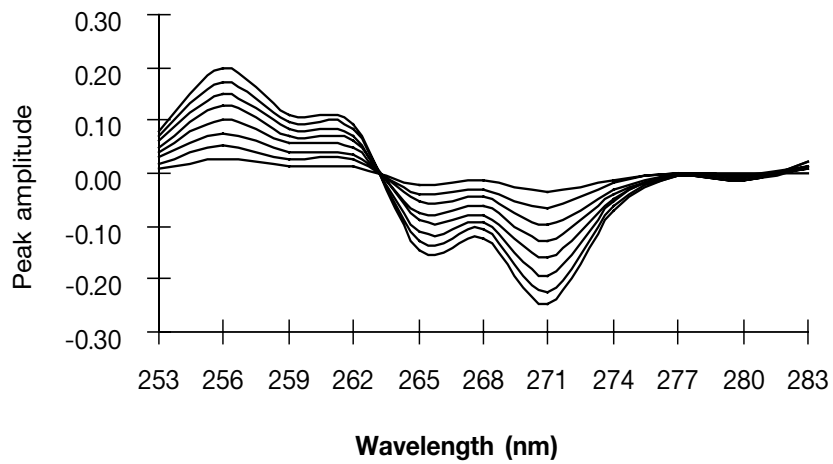


Figure 11. First derivative ratio spectra of ampicillin sodium (50 - 400  $\mu\text{g/mL}$ ). Divisor is 100  $\mu\text{g/mL}$  dicloxacillin sodium.

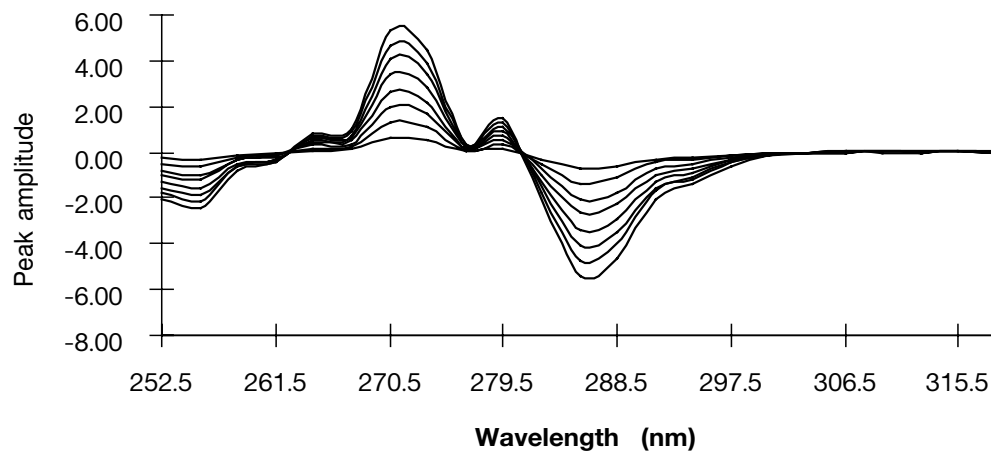
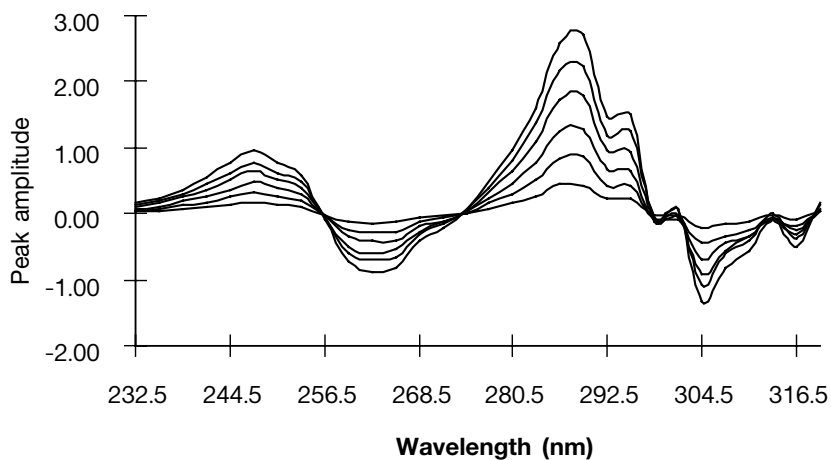
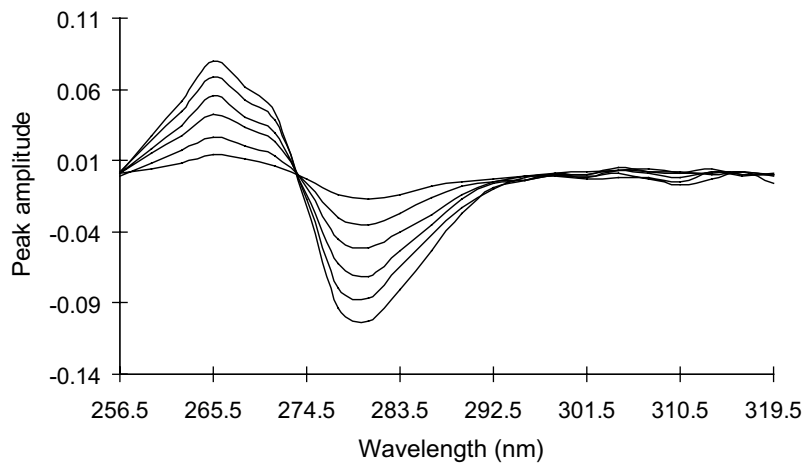


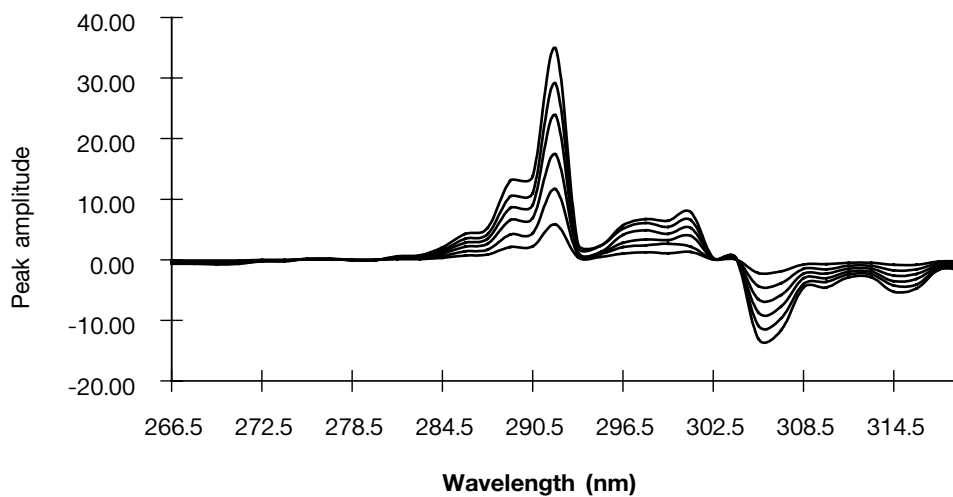
Figure 12. First derivative ratio spectra of dicloxacillin sodium (50 - 400  $\mu\text{g/mL}$ ). Divisor is 68.47  $\mu\text{g/mL}$  ampicillin sodium.



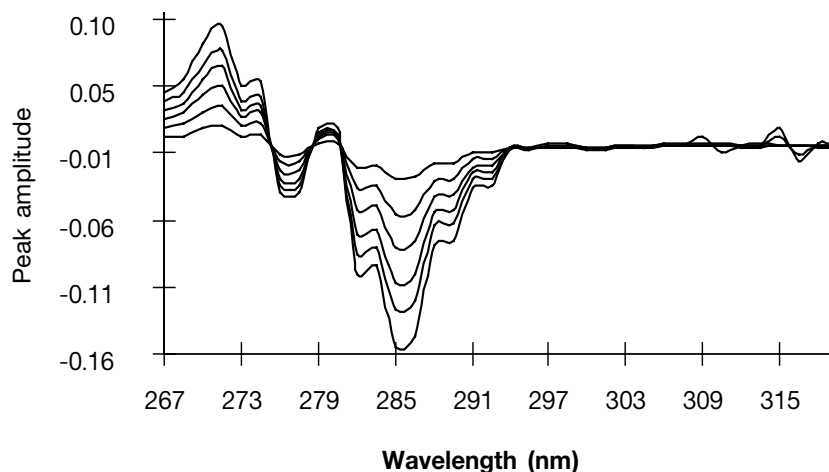
**Figure 13.** First derivative ratio spectra of amoxicillin sodium (10 - 60 µg/mL). Divisor is 58.90 µg/mL flucloxacillin sodium.



**Figure 14.** First derivative ratio spectra of flucloxacillin sodium (15 - 90 µg/mL). Divisor is 50 µg/mL amoxicillin sodium.



**Figure 15.** First derivative ratio spectra of amoxicillin sodium (20 - 120 µg/mL). Divisor is 121.64 µg/mL dicloxacillin sodium.



**Figure 16.** First derivative ratio spectra of dicloxacillin sodium (100 - 600 µg/mL). Divisor is 100 µg/mL amoxicillin sodium.

**Table 1.** Analytical parameters for determination of the studied  $\beta$ -lactam antibiotics with the proposed derivative spectrophotometric techniques.

Standard solution of	Technique	Conc. range (µg/mL)	$\lambda$ (nm)	Linear regression equation parameters					
				a	b	r	$r^2$	LOD (µg/mL)	LOQ (µg/mL)
<b>Mix I</b>									
Ampicillin	<sup>1</sup> D (zero crossing)	100-350	281.5	-0.005	0.001	0.9999	0.9999	18.73	62.44
	<sup>1</sup> D (derivative ratio)	50-300	255.5	0.001	0.002	0.9999	0.9999	7.18	23.99
Flucloxacillin	<sup>1</sup> D (zero crossing)	50-300	262.5	-0.004	0.001	0.9999	0.9999	8.50	28.33
	<sup>1</sup> D (derivative ratio)	50-300	273.5	0.043	0.025	0.9999	0.9999	5.82	19.40
<b>Mix II</b>									
Ampicillin	<sup>1</sup> D (zero crossing)	50-400	267.5	-0.0001	0.00016	0.9999	0.9998	5.73	19.12
	<sup>1</sup> D (derivative ratio)	50-400	255.5	-0.0039	0.00050	0.9999	0.9998	5.51	18.40
Dicloxacillin	<sup>1</sup> D (zero crossing)	50-400	285.5	-0.0174	-0.0062	0.9998	0.9996	5.11	17.02
	<sup>1</sup> D (derivative ratio)	50-400	273.5	0.0161	0.01120	0.9999	0.9996	6.00	20.00
<b>Mix III</b>									
Amoxicillin	<sup>1</sup> D (zero crossing)	10-60	262.5	0.00119	-0.00226	0.9999	0.9998	3.00	10.0
	<sup>1</sup> D (derivative ratio)	10-60	289.5	-0.06635	0.04603	0.9999	0.9998	2.00	6.60
Flucloxacillin	<sup>1</sup> D (zero crossing)	15-90	277.5	0.00064	-0.00035	0.9999	0.9998	2.83	9.43
	<sup>1</sup> D (derivative ratio)	15-90	280.5	-0.00124	-0.00113	0.9999	0.9998	3.09	10.30
<b>Mix IV</b>									
Amoxicillin	<sup>1</sup> D (zero crossing)	20-120	267.5	-0.0011	-0.00022	0.9999	0.9998	2.00	1.33
	<sup>1</sup> D (derivative ratio)	20-120	307.5	0.0459	0.2915	0.9998	0.9996	0.63	2.08
Dicloxacillin	<sup>1</sup> D (zero crossing)	100-600	273.5	-0.0005	-0.00012	0.9999	0.9998	12.72	42.40
	<sup>1</sup> D (derivative ratio)	100-600	284.5	-0.01237	-0.00025	0.9998	0.9996	24.00	80.00

a; intercept; b: slope; r: correlation coefficient;  $r^2$ : coefficient of determination; LOD: limit of detection; LOQ: limit of quantitation.

### Derivative ratio technique

The influence of  $\Delta\lambda$  for obtaining the first derivative of the ratio spectra as well as the effect of divisor concentration on the calibration graphs for the proposed mixture were studied in order to select the best factors affecting the determination. Results indicated that  $\Delta\lambda = 3$  nm was considered the most suitable one, while the divisor concentration has no significant effect on the assay results for the studied mixtures.

For determination of ampicillin in mixture I, the absorption spectra of ampicillin were divided by that of standard solutions of flucloxacillin (100  $\mu\text{g/mL}$ ). The first derivatives of the developed ratio spectra were calculated with  $\Delta\lambda = 3$  nm. Figure 9 shows that ampicillin could be determined by measuring the amplitude at 255.5 nm where flucloxacillin has no contribution. On the other hand, for determination of flucloxacillin, an analogous procedure was followed. The absorption spectra of flucloxacillin were divided by that of a standard solution of ampicillin (70.70  $\mu\text{g/mL}$ ) and the first derivative of the developed ratio spectra were calculated (Figure 10). Flucloxacillin could be determined at many wavelengths (273.5, 285.5 and 306.5 nm) where ampicillin has no contribution, but it was found that the amplitude at 273.5 nm gives the most accurate and sensitive results.

For determination of ampicillin in mixture II, the absorption spectra of ampicillin were divided by that of standard solutions of dicloxacillin (100  $\mu\text{g/mL}$ ). The first derivative of the developed ratio spectra were calculated with  $\Delta\lambda = 3$  nm. Figure 11 shows that ampicillin could be determined by measuring the amplitude at 255.5 and 270.5 nm where dicloxacillin has no contribution. On the other hand, for determination of dicloxacillin, an analogous procedure was followed. The absorption spectra of dicloxacillin were divided by that of a standard solution of ampicillin (68.47  $\mu\text{g/mL}$ ) and the first derivatives of the developed ratio spectra were calculated (Figure 12). Dicloxacillin could be determined at 255.5 and 273.5 nm where ampicillin has no contribution.

For determination of amoxicillin in mixture III, the absorption spectra of amoxicillin were divided by that of standard solutions of flucloxacillin (58.90  $\mu\text{g/}$

mL), The first derivative of the developed ratio spectra were calculated with  $\Delta\lambda = 3$  nm. Figure 13 shows that amoxicillin could be determined by measuring the amplitude at 247.5, 262.5 and 289.5 nm where flucloxacillin has no contribution. On the other hand, for determination of flucloxacillin, an analogous procedure was followed. The absorption spectra of flucloxacillin were divided by that of a standard solution of amoxicillin (50  $\mu\text{g/mL}$ ) and the first derivative of the developed ratio spectra were calculated (Figure 14). Flucloxacillin could be determined at 280.5 nm where amoxicillin has no contribution.

For determination of amoxicillin in mixture IV, the absorption spectra of amoxicillin were divided by that of standard solutions of dicloxacillin (121.64  $\mu\text{g/mL}$ ). The first derivative of the developed ratio spectra were calculated with  $\Delta\lambda = 3$  nm. Figure 15 shows that, amoxicillin could be determined by measuring the amplitude at 292.5 nm where dicloxacillin has no contribution. On the other hand, for determination of dicloxacillin, an analogous procedure was followed. The absorption spectra of dicloxacillin were divided by that of a standard solution of amoxicillin (100  $\mu\text{g/mL}$ ) and the first derivative of the developed ratio spectra were calculated (Figure 16). Dicloxacillin could be determined at 284.5 nm where amoxicillin has no contribution.

Under the specified conditions and the specified wavelengths for each drug, regression equations for the drugs were derived using the least-squares regression analysis, Table 1 summarizes the obtained results for all the used techniques, the results include the intercepts (a), slopes (b), correlation coefficients (r), determination coefficients ( $r^2$ ), limits of detection (LOD) and limits of quantification (LOQ).

Statistical analysis of the results obtained for authentic, laboratory prepared mixtures and pharmaceutical preparations of studied drugs (Tables 2-5, respectively), using the proposed and official methods [15] showed that the proposed and reported methods were equally precise and accurate.

**Table 2.** Statistical analysis of the results obtained for assay of the pure authentic  $\beta$ -lactam antibiotics by the proposed derivative spectrophotometric methods.

	Statistical parameters	Reported methods [15]	<sup>1</sup> D (zero- crossing)	<sup>1</sup> D (derivative ratio)
<b>Mix. I</b>				
Ampicillin	X	99.74	100.09 (at 281.5 nm)	100.37 (at 255.5 nm)
	$\pm S$	0.59	0.52	0.72
	n	6	6	6
	S <sup>2</sup>	0.36	0.28	0.53
	t	-	10.7	1.64
	F	-	1.27	1.48
Flucloxacillin	X	100.09	100.41 (at 262.5 nm)	100.01 (at 273.5 nm)
	$\pm S$	0.62	0.66	0.62
	n	6	6	6
	S <sup>2</sup>	0.36	0.44	0.39
	t	-	0.88	0.23
	F	-	1.21	1.07
<b>Mix. II</b>				
Ampicillin	X	99.50	99.90 (at 267.5 nm)	99.90 (at 255.5 nm)
	$\pm S$	0.74	1.02	1.10
	n	8	8	8
	S <sup>2</sup>	0.54	1.03	1.21
	t	-	0.90	0.86
	F	-	1.91	2.23
Dicloxacillin	X	100.98	100.72 (at 285.5 nm)	100.07 (at 273.5 nm)
	$\pm S$	0.99	1.31	0.74
	n	8	8	8
	S <sup>2</sup>	0.98	1.71	0.55
	t	-	0.45	2.08
	F	-	1.74	1.79
<b>Mix. III</b>				
Amoxicillin	X	100.25	100.17 (at 262.5 nm)	100.11 (at 289.5 nm)
	$\pm S$	0.79	1.21	0.82
	n	6	6	6
	S <sup>2</sup>	0.62	1.46	0.68
	t	-	0.14	0.30
	F	-	2.34	1.09
Flucloxacillin	X	100.09	100.02 (at 277.5 nm)	99.95 (at 280.5 nm)
	$\pm S$	0.60	0.84	0.60
	n	6	6	6
	S <sup>2</sup>	0.36	0.71	0.37
	t	-	0.17	0.40
	F	-	1.97	1.01
<b>Mix. IV</b>				
Amoxicillin	X	100.26	99.77 (at 267.5 nm)	100.00 (at 307.5 nm)
	$\pm S$	0.80	0.59	0.00
	n	6	6	6
	S <sup>2</sup>	0.64	0.35	0.00
	t	-	1.21	-
	F	-	1.80	-
Dicloxacillin	X	100.6	100.23 (at 273.5 nm)	99.88 (at 284.5 nm)
	$\pm S$	0.59	1.02	1.04
	n	6	6	6
	S <sup>2</sup>	0.35	1.03	1.07
	t	-	0.77	1.48
	F	-	2.97	3.08

Theoretical values at 95% confidence limit are: t = 2.228 and F = 5.05 ( $n_1 = 6$  and  $n_2 = 6$ ); t = 2.145 and F = 30.79 ( $n_1 = 8$  and  $n_2 = 8$ ); X= mean; n = number of observations; S = standard deviation; S<sup>2</sup> = variance

**Table 3.** Statistical analysis of the results obtained for assay of the laboratory prepared mixtures of the  $\beta$ -lactam antibiotics by the proposed derivative spectrophotometric methods.

	Statistical parameters	Reported methods [15]	<sup>1</sup> D (zero- crossing)	<sup>1</sup> D (derivative ratio)
<b>Mix. I</b>				
Ampicillin	X	99.24	99.70 (at 281.5 nm)	100.46 (at 255.5 nm)
(In presence of flucloxacillin)	$\pm S$	0.75	0.90	1.41
	n	5	5	5
	S <sup>2</sup>	0.56	0.81	2.01
	t	-	0.88	1.70
	F	-	1.44	3.58
Flucloxacillin	X	100.19	99.84 (at 262.5 nm)	99.67 (at 273.5 nm)
(In presence of ampicillin)	$\pm S$	0.62	1.27	0.75
	n	5	5	5
	S <sup>2</sup>	0.38	1.61	0.57
	t	-	0.56	1.20
	F	-	4.23	1.50
<b>Mix. II</b>				
Ampicillin	X	99.24	100.05 (at 267.5 nm)	99.46 (at 255.5 nm)
(In presence of dicloxacillin)	$\pm S$	0.75	1.47	1.30
	n	5	5	5
	S <sup>2</sup>	0.56	2.16	1.69
	t	-	1.10	0.60
	F	-	3.84	3.00
Dicloxacillin	X	100.46	99.84 (at 285.5 nm)	99.96 (at 273.5 nm)
(In presence of ampicillin)	$\pm S$	0.54	0.82	0.76
	n	5	5	5
	S <sup>2</sup>	0.29	0.67	0.58
	t	-	1.41	1.20
	F	-	2.33	2.01
<b>Mix. III</b>				
Amoxicillin	X	100.10	99.15 (at 262.5 nm)	100.20 (at 289.5 nm)
(In presence of flucloxacillin)	$\pm S$	0.78	0.92	0.75
	n	5	5	5
	S <sup>2</sup>	0.61	0.85	0.57
	t	-	1.76	0.21
	F	-	1.72	1.07
Flucloxacillin	X	99.72	100.36 (at 277.5 nm)	99.00 (at 280.5 nm)
(In presence of amoxicillin)	$\pm S$	0.58	0.40	0.76
	n	4	4	4
	S <sup>2</sup>	0.34	0.16	0.58
	t	-	1.81	1.51
	F	-	2.10	1.70
<b>Mix. IV</b>				
Amoxicillin	X	100.10	99.11 (at 267.5 nm)	100.20 (at 307.5 nm)
(In presence of dicloxacillin)	$\pm S$	0.78	0.99	0.64
	n	5	5	5
	S <sup>2</sup>	0.61	0.99	0.42
	t	-	1.75	0.22
	F	-	1.62	1.47
Dicloxacillin	X	100.46	100.44 (at 273.5 nm)	99.82 (at 284.5 nm)
(In presence of amoxicillin)	$\pm S$	0.54	1.31	0.80
	n	5	5	5
	S <sup>2</sup>	0.29	1.72	0.64
	t	-	0.03	1.49
	F	-	5.97	2.21

Theoretical values at 95% confidence limit are: t = 2.447 and F = 9.28 ( $n_1 = 4$  and  $n_2 = 4$ ); t = 2.306 and F = 6.39 ( $n_1 = 5$  and  $n_2 = 5$ ); X = mean; n = number of observations; S = standard deviation; S<sup>2</sup> = variance

**Table 4.** Statistical analysis of the results obtained for assay of the pharmaceutical preparations of the  $\beta$ -lactam antibiotics by the proposed derivative spectrophotometric methods.

	Statistical parameters	Reported methods [15]	<sup>1</sup> D (zero- crossing)	<sup>1</sup> D (derivative ratio)
<b>Mix. I</b>				
Ampicillin	X	99.24	99.58 (at 281.5 nm)	98.41 (at 255.5 nm)
In Ampiflux <sup>®</sup> capsules	$\pm$ S	0.75	0.38	0.44
	n	5	5	5
	S <sup>2</sup>	0.56	0.51	0.19
	t	-	0.90	1.70
	F	-	1.44	2.14
Flucloxacillin	X	100.19	100.34 (at 262.5 nm)	101.00 (at 273.5 nm)
In Ampiflux <sup>®</sup> capsules	$\pm$ S	0.62	0.65	0.50
	n	5	5	5
	S <sup>2</sup>	0.38	0.43	0.32
	t	-	0.37	2.28
	F	-	1.12	1.52
<b>Mix. II</b>				
Ampicillin	X	100.3	99.50 (at 267.5 nm)	100.33 (at 255.5 nm)
In Dipenacid <sup>®</sup> vials	$\pm$ S	0.30	0.50	0.46
	n	3	3	3
	S <sup>2</sup>	0.09	0.25	0.21
	t	-	2.38	0.10
	F	-	2.78	2.30
Dicloxacillin	X	100.13	99.97 (at 285.5 nm)	99.55 (at 273.5 nm)
In Dipenacid <sup>®</sup> vials	$\pm$ S	0.23	0.46	0.32
	n	3	3	3
	S <sup>2</sup>	0.05	0.21	0.10
	t	-	0.54	2.53
	F	-	3.93	1.20
<b>Mix. III</b>				
Amoxicillin	X	100.10	101.00 (at 262.5 nm)	99.28 (at 289.5 nm)
In Flumox <sup>®</sup> capsules	$\pm$ S	0.78	0.73	0.83
	n	5	5	5
	S <sup>2</sup>	0.61	0.54	0.15
	t	-	1.88	2.11
	F	-	1.14	4.17
Flucloxacillin	X	100.25	100.10 (at 277.5 nm)	99.80 (at 280.5 nm)
In Flumox <sup>®</sup> capsules	$\pm$ S	0.51	0.78	0.49
	n	5	5	5
	S <sup>2</sup>	0.26	0.50	0.10
	t	-	1.92	1.37
	F	-	1.91	2.59
<b>Mix. IV</b>				
Amoxicillin	X	100.10	99.51 (at 267.5 nm)	100.31 (at 307.5 nm)
In Amoclox <sup>®</sup> capsules	$\pm$ S	0.78	1.02	0.76
	n	5	5	5
	S <sup>2</sup>	0.61	1.03	0.57
	t	-	1.03	0.43
	F	-	1.69	1.07
Dicloxacillin	X	100.46	100.14 (at 273.5 nm)	99.84 (at 284.5 nm)
In Amoclox <sup>®</sup> capsules	$\pm$ S	0.54	1.15	1.33
	n	5	5	5
	S <sup>2</sup>	0.29	1.32	1.77
	t	-	0.56	0.97
	F	-	4.59	6.15

Theoretical values at 95% confidence limit are:  $t = 2.776$  and  $F = 19.0$  ( $n_1 = 3$  and  $n_2 = 3$ );  $t = 2.306$  and  $F = 6.39$  ( $n_1 = 5$  and  $n_2 = 5$ ); X = mean; n = number of observations; S = standard deviation; S<sup>2</sup> = variance

**Table 5.** Results obtained by applying CLS and PCR analysis to the pure form of the studied  $\beta$ -lactam antibiotics.

Mix	Component	Real ( $\mu\text{g/mL}$ )	CLS			PCR				
			Found ( $\mu\text{g/mL}$ )	Found (%)	RRMSE* (%)	Found ( $\mu\text{g/mL}$ )	Found (%)	RRMSE* (%)		
Mix. I	Ampicillin	50	50.0	100.0	0.0	49.7	99.4	0.6		
		100	100.4	100.4	0.4	99.2	99.2	0.8		
		150	151.0	100.7	0.7	149.2	99.4	0.5		
		200	201.1	100.6	0.6	200.3	100.2	0.2		
		250	250.0	100.0	0.0	249.1	99.6	0.4		
		300	298.6	99.5	0.5	298.1	99.4	0.6		
	Flucloxacillin	50	50.6	101.2	1.2	49.8	99.6	0.4		
		100	99.4	99.4	0.6	99.0	99.0	1.0		
		150	151.3	100.9	0.9	149.0	99.3	0.7		
		200	201.9	100.9	0.9	199.2	99.6	0.4		
		250	251.1	100.4	0.4	248.9	99.6	0.4		
		300	297.3	99.1	0.9	298.1	99.4	0.6		
		Mix. II	Ampicillin	50	50.5	101.0	1.0	49.4	98.8	0.6
				100	100.7	100.7	0.7	98.9	98.9	0.8
150	150.3			100.2	0.2	148.9	99.3	0.5		
200	201.0			100.5	0.5	198.2	99.1	0.2		
250	253.3			100.3	0.3	248.4	99.4	0.4		
300	300.3			100.0	0.0	297.4	99.1	0.6		
350	350.0			100.0	0.0	350.1	100.0	0.0		
400	396.9			99.2	0.8	395.7	98.9	1.1		
Dicloxacillin	50		50.5	101.0	1.0	49.2	98.4	1.6		
	100		100.2	100.2	0.2	99.1	99.1	0.9		
	150		150.7	100.5	0.5	148.6	99.0	0.9		
	200		196.9	98.5	1.5	198.0	99.0	1.0		
	250		252.5	101.0	1.0	248.2	99.3	0.7		
	300		300.4	100.1	0.1	297.1	99.0	1.0		
Mix. III	Amoxicillin	10	10.03	100.3	0.3	9.9	99.0	1.0		
		20	19.9	99.5	0.5	19.6	98.0	2.0		
		30	29.7	99.0	1.0	29.2	97.3	2.6		
		40	40.3	100.8	0.8	39.4	98.5	1.5		
		50	49.4	98.8	1.2	49.2	98.4	1.6		
		60	60.5	100.8	0.8	59.2	98.6	1.4		
	Flucloxacillin	15	14.9	199.6	0.4	14.9	99.3	0.7		
		30	30.4	101.3	1.3	29.3	97.7	2.3		
		45	45.1	100.2	0.2	44.2	98.2	1.8		
		60	60.9	101.5	1.5	59.3	98.8	1.2		
		75	74.2	98.9	1.0	74.3	99.1	0.9		
		90	89.9	99.9	0.1	89.0	98.9	1.1		
		Mix. IV	Amoxicillin	20	20.0	100.0	0.0	19.7	98.5	1.5
				40	40.0	100.0	0.0	39.7	99.3	0.7
60	60.1			100.2	0.2	59.5	99.2	0.8		
80	79.8			99.8	0.2	79.1	98.9	1.1		
100	99.9			99.9	0.1	98.8	98.8	1.2		
120	120.2			100.2	0.2	119.0	99.2	0.8		
Dicloxacillin	100		99.6	99.6	0.4	99.1	99.1	0.9		
	200		199.9	100.0	0.0	198.3	99.2	0.8		
	300		301.0	100.3	0.3	297.7	99.2	0.8		
	400		403.8	101.0	1.0	396.4	99.1	0.9		
	500		494.1	98.8	1.2	495.2	99.0	0.9		
	600		602.1	100.4	0.4	592.8	98.8	1.2		

\*RRMSE = Relative Root Mean Squared Error

**Table 6.** Results obtained by applying CLS and PCR analysis to the laboratory prepared mixtures of the studied  $\beta$ -lactam antibiotics.

Mix	Component	Real ( $\mu\text{g/mL}$ )	CLS			PCR		
			Found ( $\mu\text{g/mL}$ )	Found (%)	RRMSE* (%)	Found ( $\mu\text{g/mL}$ )	Found (%)	RRMSE* (%)
<b>Mix. I</b>								
1	Ampicillin	150	151.9	101.3	1.3	148.3	98.9	1.1
	Flucloxacillin	300	297.4	99.1	0.9	298.6	99.5	0.5
2	Ampicillin	300	298.2	99.4	0.6	298.0	99.3	0.7
	Flucloxacillin	150	147.9	98.6	1.4	148.6	99.1	0.9
3	Ampicillin	100	101.7	101.7	1.7	99.1	99.1	0.9
	Flucloxacillin	300	295.4	98.5	1.5	296.9	99.0	1.0
4	Ampicillin	250	253.6	101.4	1.4	248.2	99.3	0.7
	Flucloxacillin	250	249.4	99.5	0.1	247.3	98.9	1.1
<b>Mix. II</b>								
1	Ampicillin	150	151.9	101.3	1.3	148.3	98.9	1.1
	Dicloxacillin	300	297.4	99.1	0.9	298.6	99.5	0.5
2	Ampicillin	300	298.2	99.4	0.6	298.0	99.3	0.7
	Dicloxacillin	150	147.9	98.6	1.4	148.6	99.1	0.9
3	Ampicillin	100	101.7	101.7	1.7	99.1	99.1	0.9
	Dicloxacillin	300	295.4	98.5	1.5	296.9	99.0	1.0
4	Ampicillin	250	253.6	101.4	1.4	248.2	99.3	0.7
	Dicloxacillin	250	249.5	99.5	0.1	247.3	98.9	1.1
<b>Mix. III</b>								
1	Amoxicillin	30	29.9	99.8	0.2	29.8	99.2	0.8
	Flucloxacillin	60	59.8	99.7	0.3	59.1	98.6	1.4
2	Amoxicillin	60	59.0	98.3	1.7	59.1	98.5	1.5
	Flucloxacillin	60	59.0	98.3	1.7	59.6	99.3	0.7
3	Amoxicillin	20	19.6	98.2	1.8	19.8	98.9	1.1
	Flucloxacillin	60	60.8	101.3	1.3	59.2	98.7	1.3
4	Amoxicillin	15	14.8	98.7	1.3	14.8	98.9	1.1
	Flucloxacillin	45	45.8	101.7	1.7	44.3	98.5	1.5
<b>Mix. IV</b>								
1	Amoxicillin	100	100.5	100.5	0.5	99.4	99.4	0.6
	Dicloxacillin	300	301.3	100.4	0.4	298.7	99.6	0.4
2	Amoxicillin	100	101.1	101.1	1.1	99.2	99.2	0.8
	Dicloxacillin	100	100.8	100.8	0.8	99.7	99.7	0.3
3	Amoxicillin	40	40.1	100.3	0.3	39.9	99.7	0.3
	Dicloxacillin	200	200.3	100.2	0.2	198.2	99.1	0.9
4	Amoxicillin	120	121.3	101.1	1.1	118.9	99.1	0.9
	Dicloxacillin	120	120.9	100.8	0.8	119.2	99.3	0.7

\*RRMSE = Relative Root Mean Squared Error

**Table 7.** Results obtained by applying CLS and PCR analysis to the commercial dosage forms of the studied  $\beta$ -lactam antibiotics.

Dosage form	Component	Claimed (mg)	CLS			PCR			Reported method[15] (% $\pm$ S)
			Found (mg)	Found (% $\pm$ S)	RRMSE (%)	Found (mg)	Found (% $\pm$ S)	RRMSE (%)	
Ampiflux <sup>®</sup> capsules	Ampicillin	250	249.8	99.92 $\pm$ 0.30 t = 1.882 F = 6.250	0.08	248.0	99.20 $\pm$ 0.35 t = 0.108 F = 4.592	0.80	99.24 $\pm$ 0.75
	Flucloxacillin	250	251.8	100.72 $\pm$ 0.32 t = 1.646 F = 3.776	0.72	248.5	99.40 $\pm$ 0.46 t = 2.288 F = 1.898	0.60	100.19 $\pm$ 0.62
Dipenacid <sup>®</sup> vial	Ampicillin	250	250.7	100.28 $\pm$ 0.10 t = 0.110 F = 9.000	0.28	248.6	99.44 $\pm$ 0.50 t = 2.555 F = 2.778	0.56	100.30 $\pm$ 0.30
	Dicloxacillin	250	251.6	100.60 $\pm$ 0.20 t = 2.671 F = 1.322	0.64	250.8	100.32 $\pm$ 0.30 t = 0.871 F = 1.701	0.32	100.13 $\pm$ 0.23
Flumox <sup>®</sup> capsules	Amoxicillin	250	252.0	100.80 $\pm$ 0.34 t = 1.840 F = 5.263	0.80	247.9	99.16 $\pm$ 0.63 t = 2.096 F = 1.533	0.84	100.10 $\pm$ 0.78
	Flucloxacillin	250	253.7	101.00 $\pm$ 0.50 t = 2.274 F = 1.538	1.48	248.3	99.32 $\pm$ 0.60 t = 2.255 F = 1.068	0.68	100.19 $\pm$ 0.62
Amoclox <sup>®</sup> capsules	Amoxicillin	250	251.4	100.56 $\pm$ 0.60 t = 1.046 F = 1.690	0.56	248.1	99.24 $\pm$ 0.60 t = 1.954 F = 1.690	0.76	100.10 $\pm$ 0.78
	Dicloxacillin	250	250.3	100.12 $\pm$ 0.40 t = 1.131 F = 1.822	0.12	249.0	99.60 $\pm$ 0.70 t = 2.175 F = 1.680	0.40	100.46 $\pm$ 0.54

### Multivariate Calibration Analysis

The absorption spectra of the studied drugs are shown in Figures 1-4. As can be seen, a considerable degree of spectral overlapping occurs in the region from 250 to 320 nm, for the components of mix I, II and IV, and from 266 to 320 nm for the components of mix III. The degree of spectral overlapping was given by  $(D_i)^{0.5}$ . In case of the presently studied compounds, the spectra lead to  $D_i = 0.879, 0.812, 0.555$  and  $0.496$  implying a 93.80, 90.13, 74.50 and 70.73% of spectral overlap for mix I, II, III and IV, respectively.

All the spectral characters for the studied mixtures were found to agree well with the requirements (overlay spectra of different concentrations of the drugs). Table 5 shows the actual and predicted amounts  $\pm$  errors (%) of the studied drugs. The results confirm the high degree of agreement and indicate that both methods are suitable for analysis in the given domain for each drug.

Several laboratory prepared mixtures were subjected to the CLS and PCR analysis in order to confirm the suitability of the calibration model for determination of the studied drugs in the pharmaceutical sample solutions. Table 6 summarizes the results obtained for the suggested laboratory prepared binary mixtures. As could be seen, the concentrations predicted by the model are very close to the real concentrations, the results in all cases were satisfactory. It can be observed from this set of results that the drug mixture determination is perfectly feasible and the

multivariate calibration model allows a significant reduction of errors in relation to the determination by univariate calibration techniques.

On the other hand, the results for commercial dosage forms and laboratory prepared mixtures with comparable concentrations were found closely matched. This indicated that the present or added excipients and additives did not interfere with the determinations. Moreover, the results for dosage form were compared with those obtained by applying official methods. As shown in Table 7, the results are in good agreement with those of the reported procedure as indicated by the calculated  $t$  and  $F$  values.

### Conclusion

The proposed derivative, derivative ratio, CLS and PCR methods can be used for the simultaneous determination of four  $\beta$ -lactam antibiotic binary mixtures; ampicillin with flucloxacillin, ampicillin with dicloxacillin, amoxicillin with flucloxacillin and amoxicillin with dicloxacillin as binary mixtures either in their pure powder forms or in their pharmaceutical preparations. The proposed methods are precise, accurate and simple. Also, no separation step is required. They are rapid and do not require any expensive or sophisticated apparatus if compared with the chromatographic methods. So, the proposed methods were completely validated and suitable for quality control laboratories, where economy and time are essential.

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