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DETERMINATION OF A TERNARY MIXTURE OF PENICILLIN-G SODIUM SALT, PENICILLIN-G PROCAIN SALT AND DIHYDROSTREPTOMYCIN SULPHATE, BY THIRD-DERIVATIVE SPECTROPHOTOMETRY

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Summary—Ternary mixtures of antibiotics, *i.e.* penicillin-G sodium salt, penicillin-G procain salt and dihydrostreptomycin sulphate salt, are assayed by 'zero-crossing' third-derivative spectrophotometry.

Calibration plots follows Beer's law up to 40 μg/ml of penicillin-G sodium at 222.5 nm ($r = 0.9997$), 46 μg/ml of penicillin-G procain at 217 nm ($r = 0.9999$) and 36 μg/ml of dihydrostreptomycin sulphate at 211.5 nm ($r = 0.9999$), in the presence of one another.

Detection limits at $p = 0.05$ level of significance were calculated to be, respectively, 0.66, 0.41 and 0.25 μg/ml.

The procedure is rapid, simple, nondestructive and does not require solution of equations. The method was successfully applied for determining laboratory mixtures and commercial injections.

Derivative spectrophotometry is an analytical technique of great utility for resolving mixtures with overlapping spectra. The fundamental principles and conventions have been announced in the pioneering works of O'Haver and Green[1] and Fell *et al.*[2–4] Although the use of derivative spectra is not new, it has only become practical with the development of microcomputer technology which allows almost instant generation of derivative spectra.[5–16] Recently, derivative spectrophotometry has received increasing attention with regard to the assay of drugs and in systems of clinical and biological interest (an extended bibliography can be found in Ref. 23). In the last few years, we described the application of both first and second derivative spectrophotometry to the simultaneous determination of two-components mixtures of several drugs (penicillins, cephalosporins and amino-acids) either as the pure compounds or in their formulations.[17–24]

The derivative procedures reported in literature concern the assay of single compounds or two components in admixture. We are doubtful that simple methods for the spectrophotometric quantitation of more complex mixtures have been described.

The aim of the present work is to show that the 'zero-crossing' third derivative spectrophotometry allows the simultaneous determination of three antibiotics in admixture, with closely overlapping absorption spectra, *i.e.* penicillin-G sodium salt, penicillin-G procain salt (beta-lactam antibiotics) and dihydrostreptomycin sulphate salt (amino-glucoside antibiotic), A, B and C, respectively, for brevity. In therapeutic fields, A and B are particularly effective against Gram-positive bacteria, C against Gram-negative. The presence of B prolongs the effect of A alone for 12–24 hrs. It has been demonstrated that the mixing of A, B and C develops a synergy, *i.e.* a considerable increase of the single activities. Formulations for these antibiotics are administered by intermuscular injections, particularly in the veterinary field, because they are very advantageous in the initial treatment of serious infections when it is still uncertain the identification of the pathogeneous agent and/or in the presence of mixed infections due to both Gram-positive and Gram-negative bacteria. Less usual is the use in the human therapy because of toxicity of streptomycin and its derivatives. A quick and reliable procedure is therefore needed to quantitate mixtures of A, B and C and for quality control of pharmaceutical dosage forms for these antibiotics. The method proposed is very simple and accurate and

applies favourably to either laboratory samples or commercial injections.

Briefly, the method is based on the preliminary location of the zero-crossing wavelengths of third derivative spectra of mixtures of A + B, A + C and B + C. Then, these wavelengths are employed to obtain the calibration graphs of C, B and A, respectively, be measuring the third derivative value of three-component samples in which the concentration of two antibiotics is kept constant and that of the other one variable.

EXPERIMENTAL

Reagents

Stock solutions (0.2 mg/ml in water) were prepared from pure samples of A, B and C (Farmitalia-Carlo Erba, Italy). Injectable dosage forms of Vettrimicina (Vetem S.p.A., Italy), labelled to contain A 0.727 g, B 1.80 g, C 3.0 g and sodium citrate as excipient 0.032 g, were tested.

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 calculates the derivative of the absorbance spectra using the Savitzky–Golay method;[25] a value of delta = 6 was found optimal in connection with both slit width and wavelength interval (delta represents the width of the boundaries over which the derivative is calculated).[25]

Procedure

Suitable volumes of A, B and C stock solutions, expected to contain up to 40 μg/ml of A, 46 μg/ml of B and 36 μg/ml of C were mixed in a 5-ml calibrated flask and diluted to volume with distilled water. Then, the third derivative spectrum of the mixture against water was recorded and the values of the derivative were measured at 222.5, 217 and 211.5 nm for the determination of A, B and C, respectively.

Procedure for injections

The contents of a vial was dissolved in a 500-ml calibrated flask and diluted to volume with distilled water. The assay was completed as described above for the Procedure. The percentage recovery of the three antibiotics was computed from the corresponding regression equations.

RESULTS AND DISCUSSION

Spectrophotometric measurements

In Fig. 1 are reported the zero-order spectra of A, B and C (all 12 μg/ml) and the corresponding sum spectrum of A + B + C, in the 190–350 nm region. The spectra of the three antibiotics closely overlap, hence the traditional Vierordt's and modified Vierordt's methods[26] for assaying mixtures gave erroneous results. We circumvented this problem by making use of the third derivative spectra of the mixtures. (Preliminary tests with first and second derivative, gave inadequate results, certainly due to the extreme closeness of the absorption spectra of A, B and C, with the subsequent poor resolution of both first and second derivative spectra of the mixtures.) The 'zero-crossing' way of measurement[1,26–27] was preferred. In the present instance, this technique, as adapted by us, requires the measurement of the third derivative spectrum of the ternary mixture at the abscissa values corresponding to the zero-crossing wavelengths of the third derivative spectra of binary mixtures of A + B, B + C and A + C. Measurements made at these wavelengths would be a function only of the concentration of the third component (C, A and B, respectively). In practice, the originality of the present method consists of considering the mixtures of two antibiotics as a single component and the remaining antibiotic as the second component.

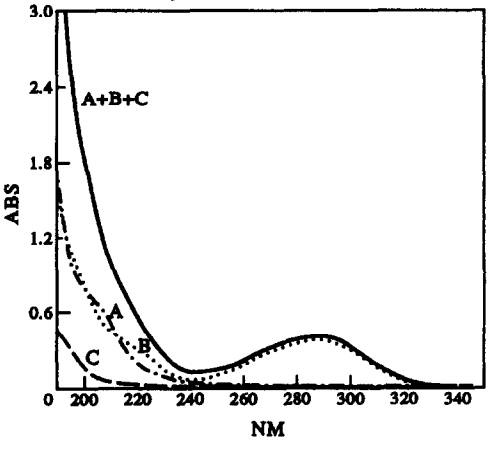

Fig. 1. Absorption spectra of penicillin-G sodium (A), penicillin-G procain (B), dihydrostreptomycin sulphate (C), (all 12 μg/ml) and their sum spectrum (A + B + C). Reference, water.

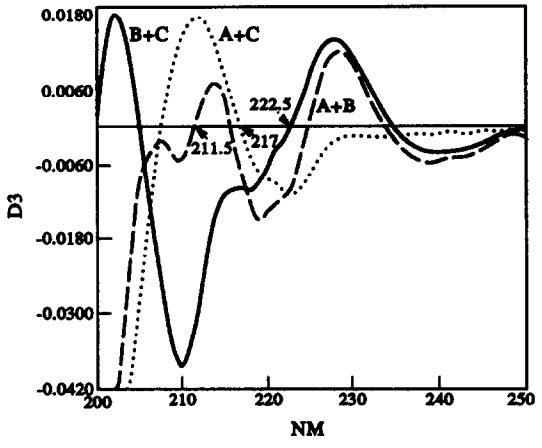

Fig. 2. Third-derivative spectra of the absorbance spectra of binary mixtures of antibiotics: A + B, mixture of penicillin-G sodium and penicillin-G procain; A + C, mixture of penicillin-G sodium and dihydrostreptomycin sulphate; B + C, mixture of penicillin-G procain and dihydrostreptomycin sulphate. All antibiotics 12 μg/ml. Reference, water. The arrows indicate the zero-crossing wavelengths selected for determination of penicillin-G sodium (222.5 nm), penicillin-G procain (217 nm) and dihydrostreptomycin sulphate (211.5 nm).

In this way it is possible to obtain suitable regression equations for each drug.

In Fig. 2 are the third derivative spectra of mixtures of A + B, A + C and B + C, in the 200–250 nm range, with the working zero-crossing wavelengths indicated by arrows. The concentration of each antibiotic in the mixtures is 12 μg/ml. However, a set of preliminary tests have revealed that either shape of spectra or zero-crossings are independent from the concentration of the mixtures. Among the various zero-crossing wavelengths visible in Fig. 2, we selected those which exhibited the best linear response to analyte concentration and/or higher sensitivity, gave a zero (or near to zero) intercept on the lines of regression and were not affected by any other component. All considered, 211.5, 217 and 222.5 nm were found optimal for determination of C, B and A, respectively.

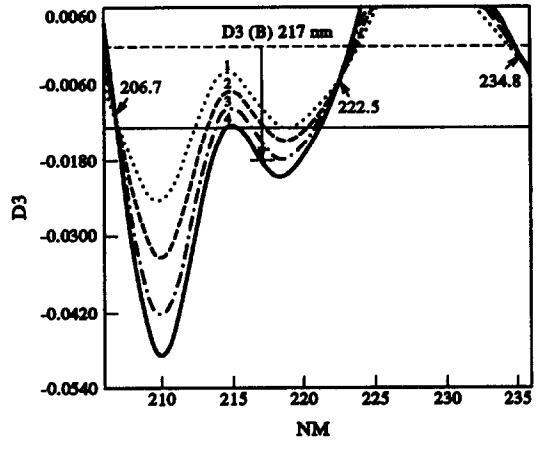

Fig. 3. Third-derivative spectra of ternary mixtures with constant concentrations of penicillin-G sodium (6 μg/ml) and dihydrostreptomycin sulphate (5 μg/ml) and increasing concentration of penicillin-G procain (14, 18, 22 and 26 μg/ml, curves 1 to 4). Reference, water. The wavelengths of isosbestic points are indicated by arrows.

In Fig. 3 are reported examples of third derivative spectra of samples with constant concentrations of A and C plus varying quantities of B. The values of third derivative, D3(B), at 217 nm (zero-crossing of third derivative of two component mixture of A + C) were proportional to the concentration of B and independent from the presence of A and C. Analogous results were obtained with mixtures with constant concentrations of B and C plus increasing concentration of A (at 222.5 nm) and with samples with constant A and B concentrations plus increasing quantities of C (at 211.5 nm).

It is interesting to note in Fig. 3, distinct isosbestic points, *i.e.* the spectra converge, as expected, to the zero-crossing wavelengths of third derivative spectra (not shown) of the single components B, which confirms a mutual independence between the concentration of the analyte B and that of the two other antibiotics in admixture, A and C, over the full range of concentrations investigated. The same behaviour was observed with the two other ternary mixtures.

'Interaction studies'[2,18,21,23,24,29] were performed, with a constant concentration of A, B and C, respectively, and varying B + C, A + C and A + B concentrations, at 222.5, 217 and 211.5 nm, respectively. These measurements confirmed the mutual independence between the derivative amplitudes of A and B + C, B and A + C and C and A + C, in a large enough concentrations ratio.

Calibration graphs and statistical analysis of results

The regression equations calculated with the method described above are assembled in Table 1, together with correlation coefficients, variance[21,30] and detection limits[21,30,31] at $p = 0.05$ level of significance. The detection limits were calculated by means of a statistical treatment of calibration data. Method and equations are extensively reported in Refs 21, 30 and 31.

Table 1. Statistical analysis of the determination of A, B and C in mixture by third-derivative spectrophotometry*

Drug	Wavelength (*nm*)	Regression equations†	Correlation coefficient	Variance (s_0^2)	Detection limit ($\mu g/ml$)
A	222.5	D3(A) = 4.69E − 05 − 8.86E − 04Ca	0.9997	7.32E − 08	0.66
B	217	D3(B) = −3.19E − 04 − 6.77E − 04Cb	0.9999	1.65E − 08	0.41
C	211.5	D3(C) = 2.13E − 04 − 4.94E − 04Cc	0.9999	3.13E − 09	0.25

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

†Ca, Cb, Cc, concentration of antibiotics, $\mu g/ml$; number of standard specimens, $n = 10$. Level of significance, $p = 0.05$.

Beer's law holds up to 40 μg/ml of A, 46 μg/ml of B and 36 μg/ml of C. Both linearity of the lines of regression and negligible scatter of experimental points are clearly evidenced by correlation coefficients and variances.

As in previous papers,[18-20,22-24] we found it interesting to know the absolute error Sc [30] in the determination of a given concentration of A, B or C, calculated by means of statistical analysis of regression equations in Table 1. The error is minimal at about 22 μg/ml of A, 28 μg/ml of B and 22 μg/ml of C.

The bar charts in Fig. 4 (also obtained by statistical analysis of regression equations), configure the confidence limits[30] at the $p = 0.05$ level of significance, in the determination of A, B and C. These were drawn in a particular way,[17-24,32-33] *i.e.* as uncertainty % on concentration (relative error) tp Sc/c (%), against the concentration of A, B and C, respectively. The tolerance limits are not exceeded and the results are satisfactory in each case. Obviously, Sc and, hence, tp Sc/c are not experimental values, but these follow from theoretical calculations based on calibration data used in determining the regression equations assembled in Table 1. Clearly, if the concentrations of A, B or C in the standard specimens were changed, the corresponding bar graphs would have to be re-examined; however, these allow a direct display of relative uncertainty on concentration of the three antibiotics over the full range of concentrations tested, and are a guide to the level of precision that may be expected from the application of the analytical procedure proposed.

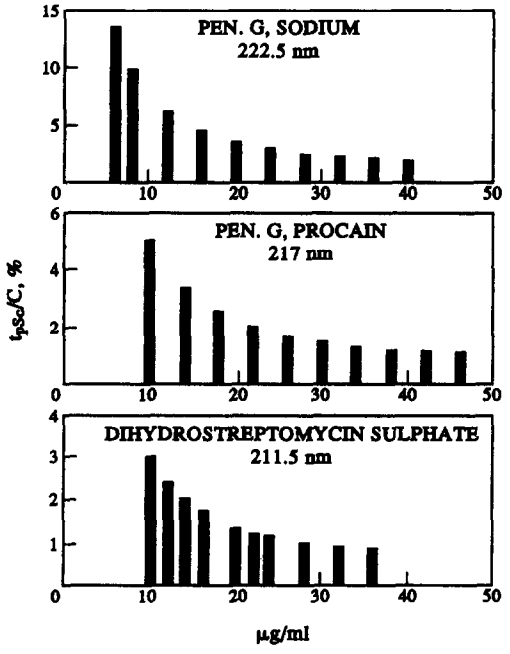

Fig. 4. Bar graphs of the variation of confidence limits at $p = 0.05$ level of significance, under form of uncertainty per cent on the concentration (relative error) of penicillin-G sodium, penicillin-G procain and dihydrostreptomycin sulphate, each one in mixture with the two other antibiotics. Graphs drawn by statistical calculations based on the third-derivative method, at 222.5 nm, 217 nm and 211.5 nm, respectively.

Accuracy and precision. To test accuracy and precision, 10 replicate determinations of laboratory mixtures of A, B and C were carried-out. The results reported in Table 2, appear satisfactory.

Assay of pharmaceuticals. The method was applied to the recovery of A, B and C in injections containing these antibiotics in admixture (see Experimental Section, under Reagents). As previously reported, no separation step was necessary (the excipients did not interfere). The tests were performed as described in Experimental Section. Table 3 shows the results of 10 successive determinations on two different batches of vials. Both accuracy and precision are highly gratifying; the recoveries conform very well to the label claims, also taking in account the level of accuracy with which manufacturers generally prepare their formulations.

Table 2. Replicate determinations on synthetic mixtures of A, B and C*

Mixtures nominal value ($\mu g/ml$) A	B	C	A (222.5 *nm*)†	B (217 *nm*)†	C (211.5 *nm*)†
8	10	20	8.06 ± 0.05 (0.66)	10.05 ± 0.05 (0.50)	20.17 ± 0.08 (0.40)
25	12	15	24.95 ± 0.09 (0.36)	12.10 ± 0.07 (0.58)	15.15 ± 0.08 (0.53)
12	18	24	11.97 ± 0.05 (0.42)	18.18 ± 0.09 (0.50)	24.25 ± 0.09 (0.37)

*A, penicillin-G sodium; B, penicillin-G procain; C, dihydrostreptomycin sulphate.
†Mean ± standard deviation ($\mu g/ml$) for 10 determinations, with RSD (%) in parentheses.

Table 3. Recovery of A, B and C from injections*

Injections	Recovery (%)† A (222.5 *nm*)	B (217 *nm*)	C (211.5 *nm*)
Vettrimicina (Batch no. 1)	101.6 ± 0.9	101.6 ± 0.8	102.9 ± 0.7
Vettrimicina (Batch no. 2)	102.1 ± 0.6	102.5 ± 0.6	103.1 ± 0.7

*A, penicillin-G sodium; B, penicillin-G procain; C, dihydrostreptomycin sulphate.
†Mean and standard deviation for 10 determinations, given as percentage of the nominal content (the labelled content of Vettrimicina and the company are reported under Experimental Section).

In conclusion, the above findings demonstrate that the described procedure, although conceptually and experimentally straightforward, enables accurate and reproducible quantitation of complex mixtures of drugs (or other compounds) with strictly overlapping spectra.

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