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A validated quantitative colorimetric assay for gentamicin

P. Frutos [a,*], Susana Torrado [a], M.E. Perez-Lorenzo [a], G. Frutos [b]

[a] *Department of Pharmaceutical Technology, Faculty of Pharmacy, Complutense University, Av Complutense s/n, Madrid 28040, Spain*

[b] *Department of Statistics and Operational Research, Faculty of Pharmacy, Complutense University, Av. Complutense s/n, Madrid 28040, Spain*

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Abstract

A colorimetric procedure was developed for the quantification of gentamicin. The method was based on the ninhydrin reaction with primary and secondary amines present in the gentamicin. This reaction produces a purple colour. The effects of several factors including pH, ninhydrin concentration and reaction time were investigated to optimize the assay method. Using the assay protocol, the absorption of the gentamicin–ninhydrin mixtures at 400 nm had a linear relationship with the gentamicin concentration ranging from 30 to 120 μg/ml. The colorimetric gentamicin assay reported herein is of great practical value because it is reproducible, sensitive, simple and extremely inexpensive.

Keywords: Gentamicin; Ninhydrin; Colorimetric assay; Validation

1. Introduction

Conventional treatment of osteomyelitis involves repeated surgical removal of dead bone tissue, coupled with repeated irrigation of the wound and prolonged systemic administration of antibiotics. Different improved methods of treatment have been developed including the spherical implants containing different antibiotics [1] or more recently, antibiotic bone cement mixtures [2]. The use of antibiotic-containing poly(methyl methacrylate) was introduced by Buchholz and colleagues in 1970 [3]. The main advantage of these treatment methods as compared with conventional therapy is that they provide high concentrations of antibiotics [4] in a limited blood circulation area and also in the infected bone. This delivery method may reduce the side effects that results from systemic administration of antibiotics.

The selection of antibiotics for use in the poly(-methyl methacrylate) (PMMA) cements should be made based on the antibiotic stability characteristics at both body temperature and the highest temperature reached during the setting of PMMA. In addition the antibiotic should present a high germicide activity in order to allow a low dosage so as to avoid possible modifications in the mechanical properties of the cement.

* Corresponding author. Fax: +34-1-3941723.
E-mail address: pafrutos@eucmax.sim.ucm.es (P. Frutos)

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The antibiotic selected for the present work was gentamicin. This drug is widely used because of its broad spectrum activity and thermal stability. Gentamicin is a member of the aminoglycoside class of antibiotic substances. It may be quantified using polarization fluorescence immunoassay (PFIA) [5], enzyme-linked immunosorbent assay (ELISA) [6], enzyme-immunoassay (EMIT) [7], fluorescence-immunoassay (TDX) [7], microbiologic [8], and chromatographic methods [9]. However most of these methods lack sensitivity and reproducibility (microbiologic), are uneconomical for multiple samples over protracted time periods (PFIA, ELISA, EMIT and TDX), or are time consuming (chromatographic) [10].

For drugs that obey the Beer–Lambert Law, spectrophotometric methods of analysis of a single component in solution are usually rapid, sensitive and economical [11]. Since gentamicin poorly absorbs ultraviolet and visible light, an indirect spectrophotometric method is necessary for its assay. Ninhydrin colorimetric reaction is commonly used as a general method for the qualitative identification of several drugs containing amino groups [12]. In the present work a new spectrophotometric procedure for the quantitative analysis of gentamicin using ninhydrin as derivatizing agent was developed, validated and compared with an official liquid chromatographic method [13].

2. Experimental

2.1. Materials

2.1.1. Ninhydrin reagent

1.25% (w/v) aqueous ninhydrin solution.

2.1.2. pH 7.4 phosphate buffer [14]

A 50-ml aliquot of a 0.2 M monobasic potassium phosphate solution and 39.1 ml of a 0.2 M sodium hydroxide solution were mixed and diluted to 200 ml with water.

2.1.3. pH 9.0 alkaline borate buffer [14]

A 50-ml aliquot of a 0.2 M boric acid solution and 20.8 ml of a 0.2 M sodium hydroxide solution were mixed and diluted to 200 ml with water.

2.1.4. Phthalaldehyde reagent

Boric acid (2.47 g) was dissolved in 75 ml of water, then the pH was adjusted to 10.4 with a 45% w/v solution of potassium hydroxide and sufficient water was added to produce 100 ml. Phthalaldehyde (1 g) was dissolved in 5 ml of methanol, then 95 ml of the boric acid solution and 2 ml of mercaptoacetic acid were added and the pH was adjusted to 10.4 with the potassium hydroxide solution. This reagent was protected from light and used within 3 days of preparation.

The PMMA cement used was CMW 1 Radiopaque (De Puy International).

2.2. Equipment

A Beckman DU-6 spectrophotometer and a Gilson liquid chromatograph, with an autosampler and UV/Vis detector set at 330 nm and a Spectra Physics-4270 integrator.

2.3. Spectrophotometric method

2.3.1. Effect of the pH buffer solution on the ninhydrin–gentamicin reaction

Gentamicin samples were prepared in the buffer solutions (pH 7.4 and 9) at concentrations of 100, 200 and 1000 μg/ml. Assay samples (10 ml) were mixed with freshly prepared ninhydrin reagent (0.1 ml) and heated in a water bath at 95°C for 5 min. The tubes were then cooled in an ice-water bath. The UV–visible spectra over the wavelength range of 200–700 nm were measured using the mixture of ninhydrin and the respective buffer solution at the appropriate concentrations as the blanks.

2.3.2. Effect of the ninhydrin concentration on the ninhydrin–gentamicin reaction

The optimum ninhydrin–gentamicin ratio for the gentamicin–ninhydrin reaction was determined by adding varying volumes of a ninhydrin solution to a known constant concentration of gentamicin.

A gentamicin solution of 200 μg/ml was prepared in the pH 7.4 phosphate buffer. Aliquots (5 ml) of this gentamicin solution were mixed with different volumes of the ninhydrin reagent and

different volumes of pH 7.4 phosphate buffer to obtain a sample final volume of 10 ml (Table 1). The blanks were obtained in a similar manner except that pH 7.4 phosphate buffer was added in the place of the aliquots of gentamicin solution. Samples were heated and cooled as described above and the UV–visible absorption spectra were measured over the wavelength range of 200–700 nm.

2.3.3. Effects of heating time on the ninhydrin–gentamicin reaction

A gentamicin solution of 100 μg/ml was prepared in the pH 7.4 phosphate buffer. Aliquots (5 ml) of this gentamicin solution were mixed with 1 ml of the ninhydrin reagent and heated in a water bath at 95°C for different times (5, 10, 15, 20, 30 and 45 min). The assay samples were then cooled in an ice-water bath and the visible absorbances were measured at 400 nm. Mixtures of pH 7.4 phosphate buffer and ninhydrin reagent (5:1) were immersed in the water bath for the same time as the samples and used as blanks.

2.4. Chromatographic method

The HPLC chromatographic method selected for the present work is described in the gentamicin sulphate monograph of the British Pharmacopoeia [13]. The HPLC system comprised a Gilson (Middleton, WI) 305 pump and a Gilson 231 XL automatic sampler attached to a Rheodyne injection valve (100-μl sample loop). Detection of the analytes was accomplished using a Gilson 116 variable-wavelength UV detector. Data were recorded on a Spectra-Physics SP 4270 Integrator (San José, CA).

The mobile phase was a solution containing 5.5 g of sodium heptane sulphonate in a mixture of 700 vol. methanol, 250 vol. water and 50 vol. of glacial acetic acid. The mobile phase was filtered through a 0.45-μm Millipore filter prior to use. A 12.5×4.6 cm column packed with 5 μm Nucleosil C_{18} was used. The flow rate was 1.5 ml/min. The injection volume was 100 μl and ultraviolet detection was at 330 nm (0.05 aufs).

For sample solution 5 ml of methanol and 4 ml of phthalaldehyde reagent were added to 10 ml of different gentamicin sulphate solutions in water, this mixture was mixed and sufficient methanol was added to produce 25 ml, heated in a water bath at 60°C for 15 min and cooled.

2.5. Validation of spectrophotometric method

2.5.1. Linearity

The linearity of the method was evaluated by analysing a series of gentamicin sulphate standards. According to the International Conference on Harmonisation [15], at least five concentrations must be used. In this study seven concentrations were chosen, ranging between 30 and 120 μg/ml. Fifteen samples were prepared, the repeated runs were genuine repeats and not just repetitions at the same reading; this approach will provide information on the variation in ab-

Table 1
Samples assayed to evaluate the effect of the ninhydrin concentration on the ninhydrin gentamicin reaction

Sample	Gentamicin solution (ml)	pH 7.4 buffer (ml)	Ninhydrin reagent (ml)	Gentamicin conc. (μg ml^{-1})	Ninhydrin conc. (mg ml^{-1})
1	5	4.5	0.5	100	0.625
2	5	4	1	100	1.250
3	5	3.5	1.5	100	1.875
4	5	3	2	100	2.500
5	5	2.5	2.5	100	3.125
6	5	2	3	100	3.750
7	5	1.5	3.5	100	4.375
8	5	1	4	100	5.000
9	5	–	5	100	6.250

sorbance between samples of same concentration. The assay was performed according to experimental conditions previously established.

2.5.2. Precision

Repeatability and intermediate precision experiments were performed at three concentration levels 30, 100 and 120 μg/ml. The data for each concentration levels were evaluated by one-way ANOVA. A 8 days × 2 replicates design was performed.

2.5.3. Range

The calibration range was established through consideration of the practical range necessary according to the use of the gentamicin concentration present in samples obtained in the in vitro dissolution test of the gentamicin bone cements.

2.5.4. Detection and quantification limits

According to ICH recommendations [15] the approach based on the S.D. of the response and the slope, was used for determining the detection limit. The quantification limit was also calculated.

2.5.5. Selectivity

In order to check the selectivity of the spectrophotometric method, the results of the gentamicin spectrophotometric assay of different samples containing all the cement components were compared with that obtained from an original gentamicin sample. The concordance grade of the results is a selectivity measure.

For the selectivity study the following samples were prepared and immersed in a water bath maintained at 37°C and agitated continuously for 24 h:

Gentamicin reference solution (100 μg/ml). The solvent for this reference solution was pH 7.4 phosphate buffer.

Polymerized bone cement in pH 7.4 buffer solution.

Non-polymerized bone cement in pH 7.4 buffer solution.

Polymerized bone cement in gentamicin reference solution (100 μg/ml).

Non-polymerized bone cement in gentamicin reference solution (100 μg/ml).

3. Results and discussion

Only the samples prepared with the pH 7.4 phosphate buffer presented a purple colour so that this phosphate buffer was selected as the solution medium for the gentamicin–ninhydrin reaction.

Fig. 1 shows a scan of gentamicin sample of 1000 μg/ml prepared with the pH 7.4 phosphate buffer. The spectrophotometric scan showed three maxima near 315, 400 and 550 nm.

The effects of several factors on the gentamicin–ninhydrin reaction were investigated. When the ninhydrin concentration was gradually raised from 0.625 to 6.250 mg/ml different spectra were obtained and all showed the three maxima previously described. As an example, the scans obtained with ninhydrin concentrations of 1.875 and 4.375 mg/ml are shown in Fig. 2a and Fig. 2b, respectively. As the ninhydrin concentration was increased, the maximum nearest to 550 nm presented a small displacement while the maximum nearest to 400 nm remained fixed. Furthermore, the absorbance values at 400 nm of the samples with a ninhydrin concentration from 1.875 to 5 mg/ml were very similar and close to 0.720. Accordingly, in the experiments designed to check the effect of the heating time on the ninhydrin–gentamicin reaction, only absorption at 400 nm was measured and a ninhydrin concentration of 2.08 mg/ml was employed.

As shown in Table 2, as the heating time was increased the absorbance values were raised. As a function of time, the absorbance for the experimental data included in Table 2 follows the multiplicative model below:

$$\text{Absorbance} = 0.192 \times \text{time}^{0.405}$$

with a determination coefficient $r^2 = 0.97$.

A reaction time of 15 min was selected as the optimum one because the absorbance values obtained at this time (0.565 and 0.583) would allow the assay of slightly concentrated samples without dilution. The absorbance values at 400 nm of the ninhydrin–gentamicin mixtures that were heated for 15 min at 95°C remained virtually unchanged for at least 4 h when kept on ice and protected from light. However the spectrophotometric ex-

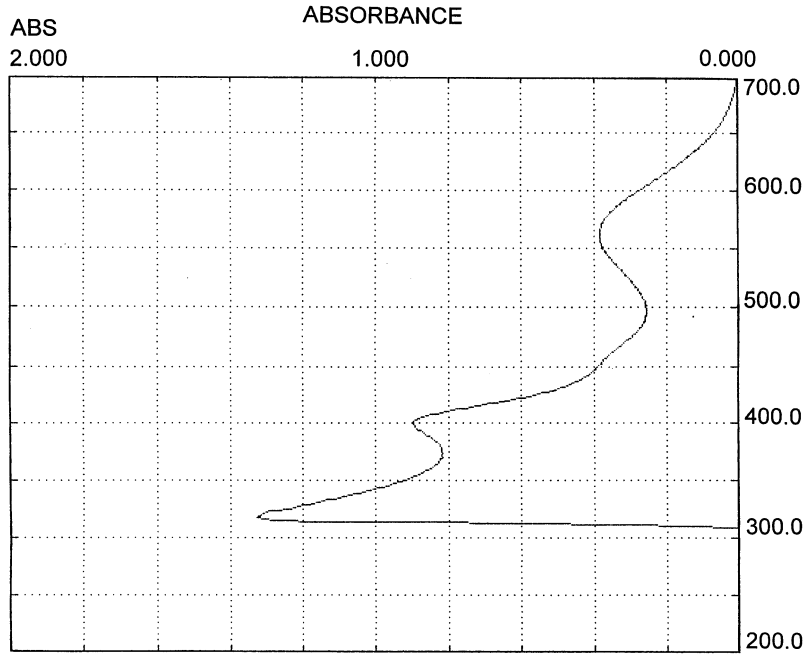

Fig. 1. Absorption spectrum of a gentamicin sample of 1000 μg/ml in pH 7.4 phosphate buffer.

amination of all the samples was performed within 1 h.

According to the previously obtained results, the optimum experimental conditions for the gentamicin–ninhydrin spectrophotometric method were as follows: 5 ml gentamicin solution, 1.5 ml ninhydrin reagent, absorbance at 400 nm, a heating temperature of 95°C and a reaction time of 15 min. The validation assays were performed following these experimental conditions.

Chromatographic separation of the gentamicin complex showed it to consist of four major components designated C_1, C_2, C_{1a} and C_{2a}. According to the British Pharmacopoeia [13], when the chromatograms are recorded under the conditions described in the gentamicin monograph, the retention time of the component C_2 is 10–20 min and the peaks are well separated with relative retention times of about 0.13 (reagent), 0.27 (component C_1), 0.65 (component C_{1a}), 0.85 (component C_{2a}) and 1.00 (component C_2). In our chromatographic conditions the next relative retention times with respect to component C_2 were obtained: 0.16 (reagent), 0.61 (component C_1), 0.75 (component C_{1a}), 0.84 (component C_{2a}) and 1.00 (component C_2).

The pharmacopoeia specify that the test is not valid unless the resolution factor between the peaks due to components C_{2a} and $C_{2,}$ is at least 1.3; under our chromatographic conditions, this resolution factor was 2.2.

Fig. 3 shows a chromatogram of a gentamicin solution of 100 μg/ml.

Table 3 shows the results of fitting a linear model to describe the relationship between absorbance and concentration of gentamicin sulphate. According to these results, absorbance as a function of concentration follows a linear model, given by the equation:

$$\text{Absorbance} = -0.106 + 6.73 \times 10^{-3} \times \text{Concentration}$$

The correlation coefficient was 0.9996. The R^2 statistic indicates that model as fitted explains 99.915% of the variability in absorbance. The residual S.E. was 0.00647.

Plots represented in Figs. 4–7 were constructed using the graphics function of S-PLUS 4 statisti-

cal package [16]. Fig. 4 is the linearity plot relating to absorbance and concentration showing the best fit line and 95% confidence bands for the true mean value of absorbance; visual observation of this plot shows that there are no outliers with respect to the model. Fig. 5 displays a plot of residuals versus the fitted values, which indicates no abnormality. A one-sided F-test ($\alpha = 0.05$) was performed to compare the variances at the highest and lowest levels of concentration. The observed F-value (0.8155), and the coefficient interval for the ratio of the variance, which extends from 0.02 to 31.8, showed that the assumption of the homoscedasticity of the data is fulfilled. In Fig. 6, a plot of absorbance versus the fitted values is displayed, the line Absorbance = Fitted absorbance, also drawn, shows the linearity of the assay. Fig. 7 shows a plot of Cook's distance

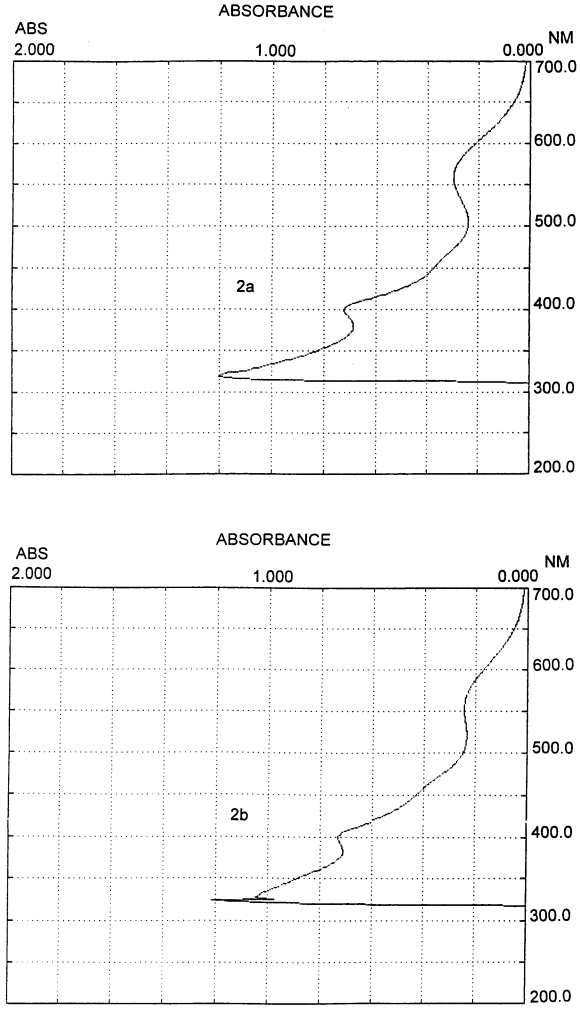

Fig. 2. Absorption spectra of a gentamicin sample (200 μg/ml) in pH 7.4 phosphate buffer with ninhydrin concentrations of 1.875 mg/ml (a) and 4.375 mg/ml (b).

Table 2
Absorbance values of a gentamicin sample of 100 μg/ml (5 ml) and ninhydrin reagent (1 ml) heated in a water bath at 95°C for different times

Time (min)	Absorbance (A.U.)
5	0.357
	0.367
10	0.473
	0.481
15	0.565
	0.583
20	0.708
	0.715
30	0.772
	0.789
45	0.841
	0.839

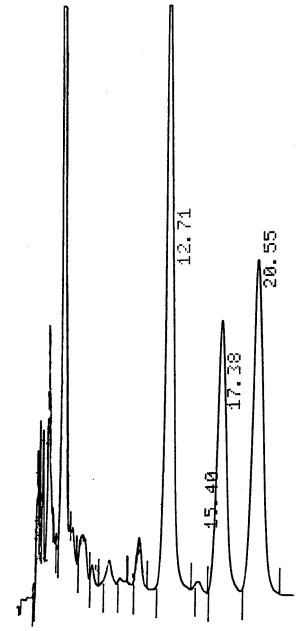

Fig. 3. High performance liquid chromatogram of gentamicin solution (100 μg/ml) using the conditions described in the British Pharmacopoeia [13].

Table 3
Regression analysis[a]

Parameter	Estimate	S.E.	t-statistic	P-value
Intercept	−0.10646	0.004499	−23.66	0.0000
Slope	0.00673	0.000054	123.61	0.0000

[a] Linear model: Absorbance $= a + b \times$ Concentration; correlation coefficient, 0.99957; R^2, 99.91%; S.E. of estimate, 0.00647.

values versus the observation number displaying three heavily influential observations in the regression coefficients. The three most extreme values are identified in the residual plot and the Cook's distance plot.

When there are repeat runs in the data [17], pooling the internal sums of squares from all the repeat runs, the pure error mean square is the total of the 'within repeats' sum of squares divided by the total of the corresponding degrees of freedom.

The sum of squares corresponding to lack of fit can by obtained by subtracting the pure error sum of squares from residual sum of squares. Mean squares due to pure error estimates σ^2, variance of variable response, Y_i, according to de basic assumptions made in the model. Mean square due to lack of fit estimates σ^2 plus a bias term, if the model is inadequate. The lack of fit test is performed by comparing the mean square due to lack of fit to the variability between observations at replicate values of the concentration of gentamicin sulphate, mean square due to pure error.

Analysis of variance with lack of fit are shown in Table 4. Since the P-value in the ANOVA table, corresponding to F-test for significance of regression, is 0.0000 there is a statistically significant relationship between absorbance and concentration of gentamicin sulphate. The P-value for the lack of fit test is greater than 0.10, therefore the lack of fit is not significant.

The same calibration data were fitted to a weighted regression model, with different weighting factors, but the visual evaluation of the residual plots did not prove that the goodness of the fit would have improved. That agrees with the behaviour of the variance and with the visual observation of the residual plot (Fig. 5).

Data were also fit to several curvilinear models which allow the calibration curve to display a

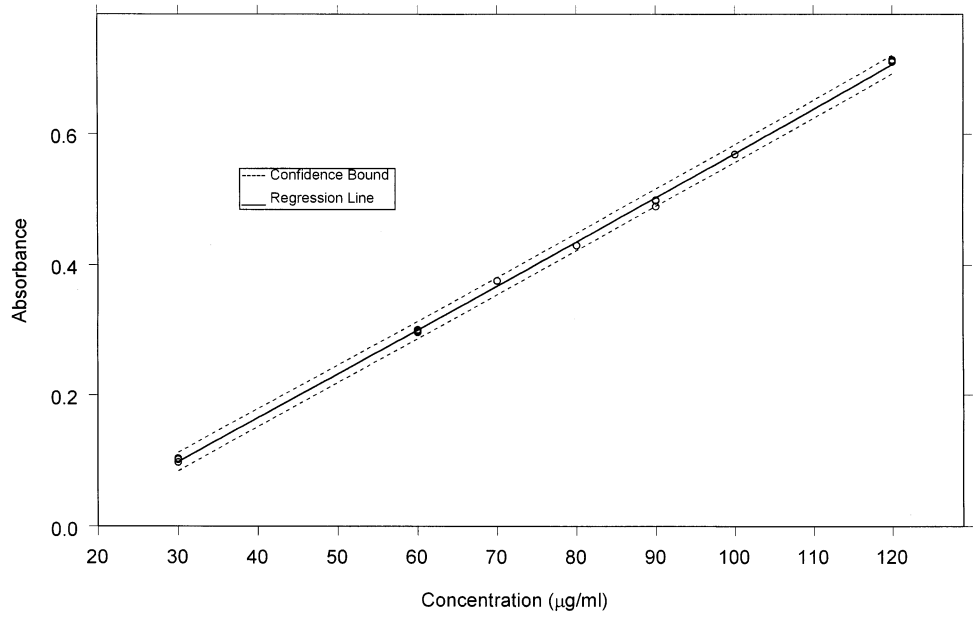

Fig. 4. A typical calibration curve for absorbance versus concentration of gentamicin, showing the best fit line (solid), the 95% confidence bands (dotted) and the individual data points (open circles).

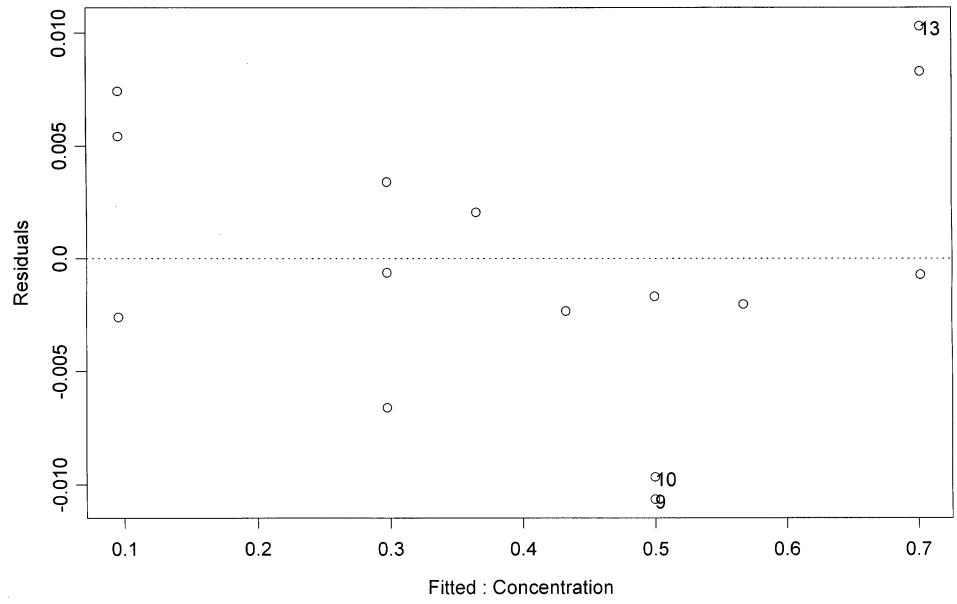

Fig. 5. Plot of residuals versus fitted values showing that no trend could be observed in the data.

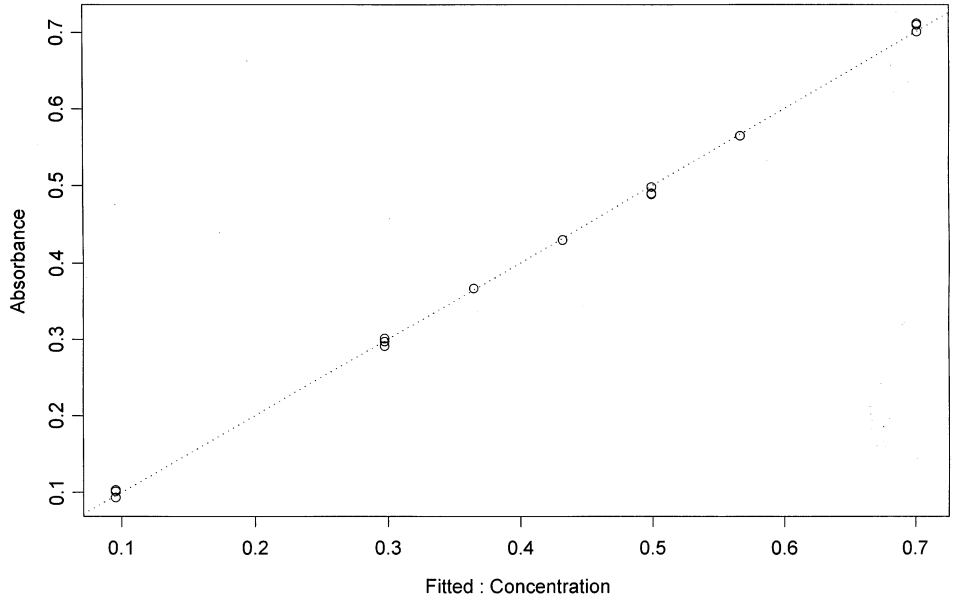

Fig. 6. A plot of absorbance versus fitted values for gentamicin analysis illustrating the linearity of assay over the calibration range.

degree of curvature over the concentration range. In Table 5 the comparison of alternative models is listed. As can be observed, the linear model yields the highest R^2 value with 99.91%, of the models fitted, so that the linear model can be recommended for the observed data.

For evaluation of the precision estimates, repeatability and intermediate precision were per-

formed at three concentration levels. Table 6 shows three univariate analyses of variance for each concentration level. It can be seen from Table 6 that since the *P*-value of the *F*-test is always greater than 0.05, there is not a statistically significant difference between the mean absorbance from one level of day to another at the 95% confidence level.

According to the approach established in the methods section, the calibration range was 30–120 μg/ml. The corresponding detection and quantification limits expressed in concentration units were DL = 0.392 μg/ml and DQ = 1.188 μg/ml respectively.

With regard to the selectivity of the spectrophotometric method, the samples of the cements, both polymerized and non-polymerized in pH 7.4 buffer solution, did not present a quantifiable absorbance value so there was not a reaction between ninhydrin and the components of the cement. On the other hand, the samples containing polymerized cement in reference gentamicin solution were assayed and the gentamicin value obtained agreed with the gentamicin concentration of the sample, therefore, there was no gentamicin adsorption on the cement. So, we can conclude that the proposed method is selective for the gentamicin release assay of the PMMA implant cements. The spectrophotometric method

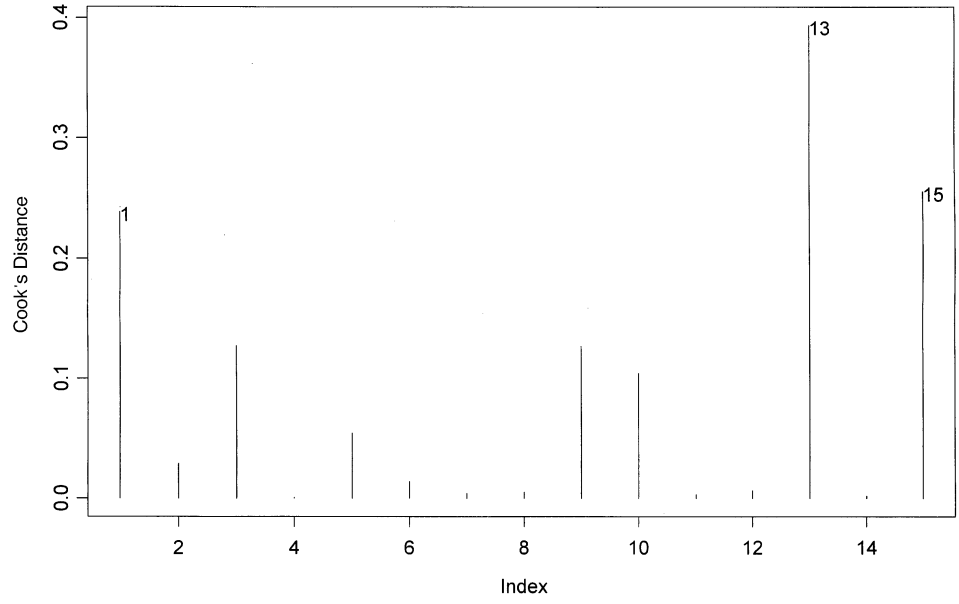

Fig. 7. Plot of Cook's distance values versus observation numbers showing three influential observations in the regression coefficients.

Table 4
Analysis of variance with lack of fit

Source	Sum of squares	Df	Mean squares	*F*-ratio	*P*-value
Model	0.641028	1	0.641028	15 279.04	0.0000
Residual	0.000545	13	0.000041		
Lack of fit	0.000321	5	0.000064	2.30	0.1418
Pure error	0.000224	8	0.000028		
Total (corr.)	0.641574	14			

Table 5
Comparison of alternative models

Model	Correlation	R^2 (%)
Linear	0.9996	99.91
Multiplicative	0.9973	99.45
Logistic	0.9926	98.52
Square root-X	0.9922	98.44
S-curve	−0.9918	98.38
Double reciprocal	0.9917	98.35
Square root-Y	0.9913	98.26
Log Probit	0.9908	98.16
Logarithmic-X	0.9737	94.82
Exponential	0.9615	92.44
Reciprocal-X	−0.9146	83.65

was compared with an official chromatographic procedure [13]. The release of antibiotics from the cements is erratic and much of the drug may also be retained within the PMMA, sometimes as much as 90% of the load [18] so, it does not make any sense to perform the usual recovery experiments. The linearity was the parameter used to compare the proposed method with the official chromatographic one. The calibration of both methods gave a r^2-value of 0.999 for the spectrophotometric method and a 0.992 for the chromatographic method. This low value of the chromatographic method may be due to the concentration of gentamicin (100 μg/ml) for which this method is optimised. However, the lower expected gentamicin concentrations of the 'in vitro' release from the loaded cements made us choose a concentration range from 20 to 80 μg/ml. The low determination coefficient value of the chromatographic method was attributed to the low sensibility (0.05 aufs) of the lamp selected to analyse this low concentration range. Therefore the proposed spectrophotometric method presented a better linearity at low concentrations than the official chromatographic one.

The proposed spectrophotometric method proved to be reproducible, sensitive and more convenient for routine analysis of the numerous samples generated during in vitro dissolution procedures compared with the official chromatographic method and also for assay of gentamicin pure form and pharmaceutical formulations without any amine containing excipient.

In comparison with conventional HPLC analysis, this colorimetric assay is much faster especially for large numbers of assays and is extremely economical, since only dilute water ninhydrin solution is needed. The ninhydrin reactive also presents the advantage of easy preparation and it may be used within 3 days of preparation. In addition, no radioactive labels are used, so this assay is safe and does not cause environmental contamination.

It should be pointed out that the gentamicin assay conditions (buffer solution pH 7.4) presented in this paper were designed mainly based on in vitro studies to determine the release of the antibiotic from the hardened PMMA bone cements, as these do not interfere with detection.

In summary, it seems likely that the absorption of the ninhydrin–gentamicin mixture at 400 nm

Table 6
Analysis of variance for repeatability and intermediate precision

Abs. level	Source	Sum of squares	Df	Mean squares	F-ratio	P-value
Lower	Between	3.4×10^{-5}	7	4.8×10^{-6}	0.12	0.9938
	Within	3.14×10^{-4}	8	3.9×10^{-5}		
	Total (corr.)	3.48×10^{-4}	15			
Intermediate	Between	2.5×10^{-4}	7	3.6×10^{-5}	0.56	0.7733
	Within	5.2×10^{-3}	8	6.6×10^{-5}		
	Total (corr.)	7.8×10^{-4}	15			
Upper	Between	3.5×10^{-4}	7	4.9×10^{-5}	0.42	0.8651
	Within	9.5×10^{-4}	8	1.2×10^{-4}		
	Total (corr.)	1.3×10^{-3}	15			

observed in this study can be attributed to a ninhydrin gentamicin derivative complex. A simple colorimetric gentamicin assay was developed based on this feature. These protocols may serve as a general guide to develop similar spectrophotometric assay methods for the analysis of other aminoglycosides.

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