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Validation and in vitro characterization of antibiotic-loaded bone cement release

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Abstract

Antibiotic-loaded polymeric bone cement is used in orthopedic surgery to deliver local high concentrations of antibiotics to the tissues. The precise mechanism by which antibiotic is released from the polymeric matrix is still not very well known. This research was conducted to investigate the in vitro drug release behavior of antibiotic from acrylic bone cement. A spectrophotometric method for the quantitative analysis of gentamicin sulfate using *o*-phtaldialdehyde as a derivatizing reagent was thoroughly validated, in order to assure a minimum quality level of the measures. The method proved to be quick, reliable, less expensive than methods such as polarization fluorescence immunoassay and others, and therefore more convenient for the routine analysis of the numerous samples generated during in vitro liberation assays. The release of gentamicin from commercial CMW1® acrylic bone cement samples was investigated following proposed in vitro release experiments.

Keywords: Gentamicin; o-Phtaldialdehyde; Polymeric bone cements; Validation PMMA; Release assays

1. Introduction

Few complications in reconstructive orthopedics pose a more challenging obstacle to clinical success than infection, and bacterial osteomyelitis remains an important and daunting orthopedic and clinical problem. A variety of prophylactic and therapeutic techniques have been designed to reduce the incidence and impact of infection. Conventional treatment using systemic antibiotics is expensive, prone to complications and often unsuccessful. One management method utilizes surgical implantation of antibiotic polymethylmethacrylate (PMMA) bone cements for local delivery of antibiotics (Adams et al., 1992; Gerhart et al., 1993). This method should make it possible to reach high local drug levels while maintaining low systemic levels.

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Due to the importance that these systems have for orthopedic surgery, the liberation of antibiotics from PMMA polymeric matrix has been the object of different in vitro and in vivo studies (Elson et al., 1977; Hill et al., 1977; Stephen and Trippel, 1986; Schmidt et al., 1995; Mader et al., 1997; Masri et al., 1998; Klekamp et al., 1999; Penner et al., 1999). Because of the differences found in the results produced by many of these studies and the difficulties in comparing non-uniform designed studies, the data generated is not enough to reach firm conclusions and the mechanism of this release is still not very well known, thus the exact role of antibiotic-loaded bone cement in the prophylaxis of infection is yet to be determined. A drug delivery system should modulate the released drug amount and the rate of the release. For PMMA bone cement, additional investigation will be required to accomplish this.

Gentamicin is one of the most common antibiotics for incorporation in the acrylic bone cement due to its wide antibacterial spectrum and because, unlike many antibiotics, it is stable at the high temperatures reached during the curing of PMMA (Hill et al., 1977). in vitro release assays require a large amount of measures of gentamicin concentration in different test solutions, so quick, reliable and inexpensive methods for its quantification are needed.

A wide variety of methods can be used to quantify aminoglycoside antibiotics, including fluorescence polarization immunoassay (FPIA; Gurtler et al., 1995), enzyme-linked immunosorbent assay (ELISA; Ara et al., 1995; Kolosova et al., 1998), enzyme-immunoassay (EMIT; Miglioli et al., 1993), microbiologic (Reamer et al., 1998), and also chromatographic and spectrophotometric methods. Chromatographic methods are the ones used in most pharmacopoeias (British Pharmacopoeia, 1998; Real Farmacopea Española, 1997) for the quantitative analysis of gentamicin. In spite of its reliability the main drawback of chromatographic methods is that it is time-consuming. Radioenzymatic methods (ELISA, EMIT) and radioimmunoassays (PFIA, TDX), also quite reliable, are very expensive.

Spectrophotometric methods are usually rapid, sensitive and economical (Ayad et al., 1999). Since gentamicin does not absorb ultraviolet nor visible light, an indirect method is required for its spectrophotometric assay. Ninhydrin colorimetric reaction is commonly used for the qualitative identification of several drugs containing amino groups (Clark's, 1989). Ninhidrin reacts with primary and secondary amines present in the gentamicin molecule to yield chromophoric products. A validated quantitative colorimetric assay for gentamicin (Frutos et al., 2000) was described employing ninhydrin as derivatizing agent; the new method was compared with the official chromatographic method (British Pharmacopoeia, 1998) obtaining excellent results, in the calibration range 30–120 μg/ml, as well as a considerable saving of time and resources.

Different derivatizing agents that react with amino groups to yield chromophoric products have been used for the detection of aminoglycosides using high performance liquid chromatography (HPLC) with fluorescence detection (Real Farmacopea Española, 1997). New spectrophotometric procedures were developed for the analysis of tobramycin and other aminoglycosides (Sampath and Robinson, 1990), using the derivatizing agents *o*-phtaldialdehyde, fluorescamine and dansyl chloride. These derivatizing agents react with amino groups to yield chromophoric and fluorogenic products. The authors compared different spectrophotometric methods that permitted the quantitative analysis and the best results were obtained with *o*-phtaldialdehyde as a derivatizing agent.

The purpose of this investigation was the in vitro release characterization of gentamicin sulphate from loaded CMW1® bone cement. The measurements were made using a modified Sampath method previously validated.

2. Materials and methods.

2.1. Materials

Gentamicin sulfate (lot CH-10520) was kindly donated by Laboratories Normon S.A. (Spain). CMW1® bone cement, with gentamicin (ref. 3015–040) was purchased from De Puy Ibérica

S.A. (Spain). 2-propanol was obtained from Scharlab, S.L. (Spain). Deionized Milli Q water was used for all the reagents, when needed.

Buffered saline solution pH 7.4 was prepared as described in the British Pharmacopoeia: disodium hydrogen phosphate anhydrous 2.38 g, mono-potassium hydrogen phosphate anhydrous 0.19 g and sodium chloride 8 g (purchased from Panreac Quimica S.A., Spain).

o-Phtalaldialdehyde reagent was formulated using *o*-phtaldialdehyde and 2-mercaptoethanol obtained from Sigma-Aldrich, methanol obtained from Scharlau and 0.04 M sodium borate obtained from Panreac Química S.A. in distilled water solution.

2.2. Methods

2.2.1. Quantitative analysis of gentamicin sulfate

The procedure proposed by Sampath (Sampath and Robinson, 1990), with a slight modification by Zang et al. (1994) was used for the analysis of gentamicin sulfate. The *o*-phtaldialdehyde reagent was formulated by adding 2.5 g *o*-phtaldialdehyde, 62.5 ml methanol and 3 ml 2-mercaptoethanol to 560 ml sodium borate in distilled water solution. The reagent was stored in a brown bottle in a dark chamber for at least 24 h before use. This reagent can be used only 3 days, then it degrades.

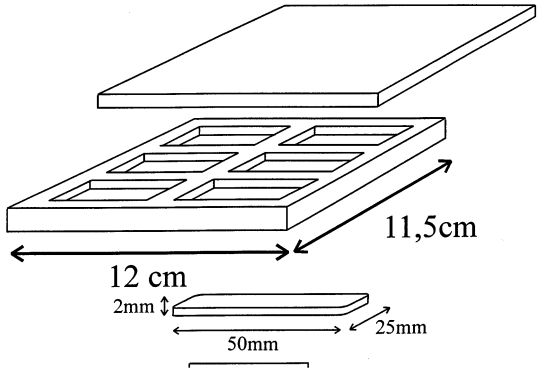

Fig. 1. Mould used for the preparation of bone cement specimens showing approximate shape and dimensions of each replicate.

Gentamicin solution, *o*-phtaldialdehyde, and isopropanol (to avoid precipitation of the products formed) were mixed in similar proportions and stored for 30 min at room temperature. The *o*-phtaldialdehyde reacted with gentamicin amino groups and chromophoric products were obtained, whose absorbances were measured at 332 nm. A Beckman DU-70 Spectrophotometer was used for all the measures.

2.2.2. Manufacturing release specimens.

Gentamicin bone cement specimens were made using CMW1® packs. Each pack of bone cement consists of 40 g of powder component and 18.37 g of liquid component. Appropriate proportions of both components were mixed thoroughly, but carefully to minimize air entrapment, in a suitable recipient. The dough thus formed was then taken in gloved hands and kneaded thoroughly.

In order to characterize the release behavior of gentamicin from bone cement, specimens with a well-defined shape were made (Fig. 1). Before the cement had completely hardened, the dough was introduced in a six-hollowed teflon mould with shape and dimensions as shown in Fig. 1. The mould was then compressed on a hydraulic press until the cement had completely hardened. After ejection, samples were carefully wiped clean and dried in a vacuum chamber for 8 h. Six specimens were obtained from each lot of cement. Each specimen, with round-cornered rectangular slab shape, was carefully weighed and measured. Three different lots of samples with two replicates for each were made, two of them with the proportions of powder and liquid component used in commercial cement and different hydraulic press to prove the possible influence of the hydraulic press. The thirst lot was made with made with less quantity of liquid component and the same hydraulic press of the second lot, to prove the possible influence of power–liquid propotions. Table 1 shows the composition and dimensions of each specimen performed.

2.2.3. Surface characterization

The surface of the specimens was characterized using a JEOL JSM-840 Scanning Electron Microscope (SEM). Samples were covered with a

Table 1
Characteristics of the different bone cement specimens performed

Specimen	Powder/liquid ratio	Replicate	Weight (g)	Gentamicin amount (mg)	Surface (cm^2)	Volume (cm^3)
1	2.05:1	1	2.85	83.233	27.92	2.496
		2	3.14	91.703	28.05	2.62
2	2.05:1	1	2.92	85.277	27.944	2.554
		2	3.29	96.083	28.384	2.75
3	5.71:1	1	3.46	124.207	29.28	3.065
		2	3.27	117.468	29.15	3.158

golden-platinum layer that makes it possible to analyze the surface.

2.2.4. In vitro release of gentamicin sulfate from bone cement

Release amounts of gentamicin were measured using a special system (Fig. 2). Each sample was introduced into 250 ml of saline buffered pH 7.4 solution in a thermostatized glass reactor. The test solution was maintained at 37°C and stirred at 150 rpm. Aliquots (3 ml) of the solution were withdrawn at appropriate time intervals, passed through a 0.45 μm membrane filter, and diluted with the dissolution medium, when necessary, for the spectrophotometer.

3. Results and discussion

3.1. Optimum reaction time

Fig. 3 shows a scan of gentamicin sample measured in order to determine the maximum. A well-defined peak can be seen at 332 nm. The disturbance in the curve at 260–290 nm region is due to derivatizent agent.

In order to determine the optimum reaction time for the formation of the *o*-phtalaldialdehyde–gentamicin complex, eight samples were prepared by adding 3 ml of a gentamicin standard solution (103.25 μg/ml), 3ml of isopropanol and 3 ml of *o*-phtalaldialdehyde reagent. Each samples was read in the spectrophotometer at different reaction times. Results are shown in Fig. 4, in which it can be observed that the absorbance of the samples shows a maximum (1.0997) at 30 min.

3.2. Validation of analytical procedure

Validation of the analytical procedure was performed according to the International Conference on Harmonization (ICH Topic Q 2B, 1996).

For evaluation of the specificity of the procedure, different solutions using pH 7.4 phosphate buffer as solvent, were prepared: (i) gentamicin reference solution, (ii) polymerized bone cement, (iii) non-polymerized bone cement, (iv) polymerized bone cement and gentamicin and (v) non-

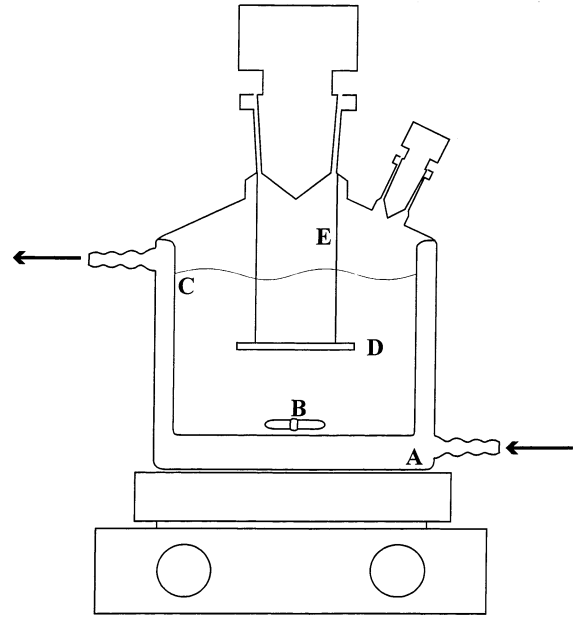

Fig. 2. Glass reactor used for the in vitro release assays. (A) Distilled water 37 ± 0.1°C, (B) magnetic stirrer, (C) buffered saline solution (pH 7.4), (D) bone cement sample, (E) iron-steel cable.

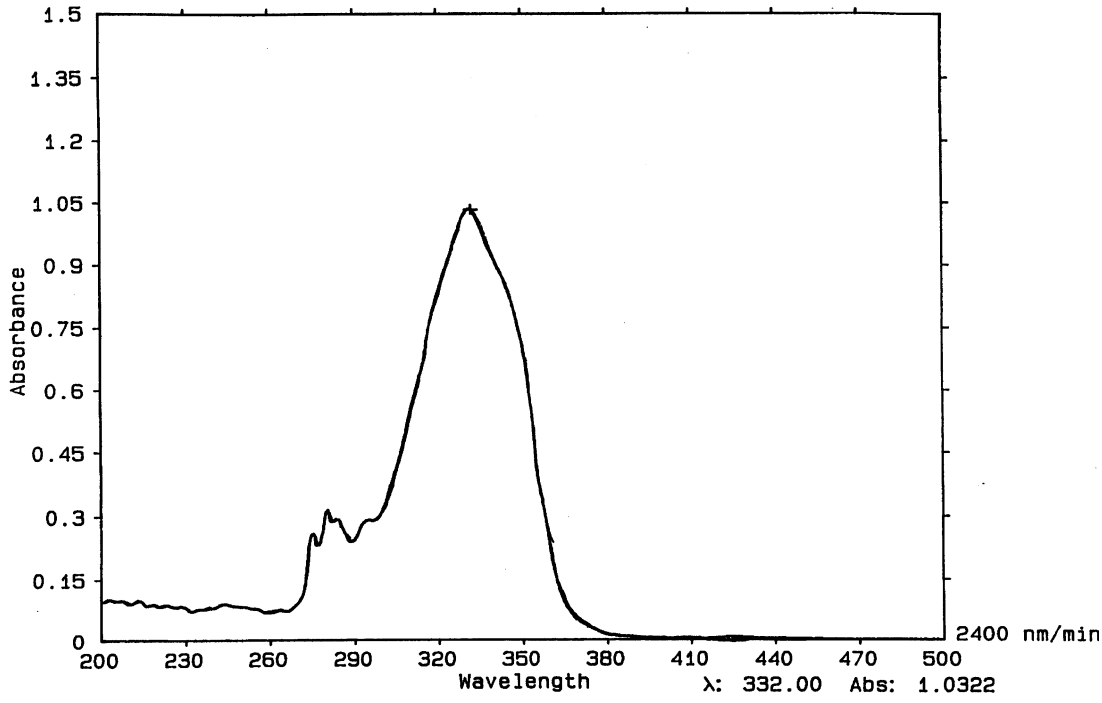

Fig. 3. Absorption spectra scan of gentamicin sulfate sample.

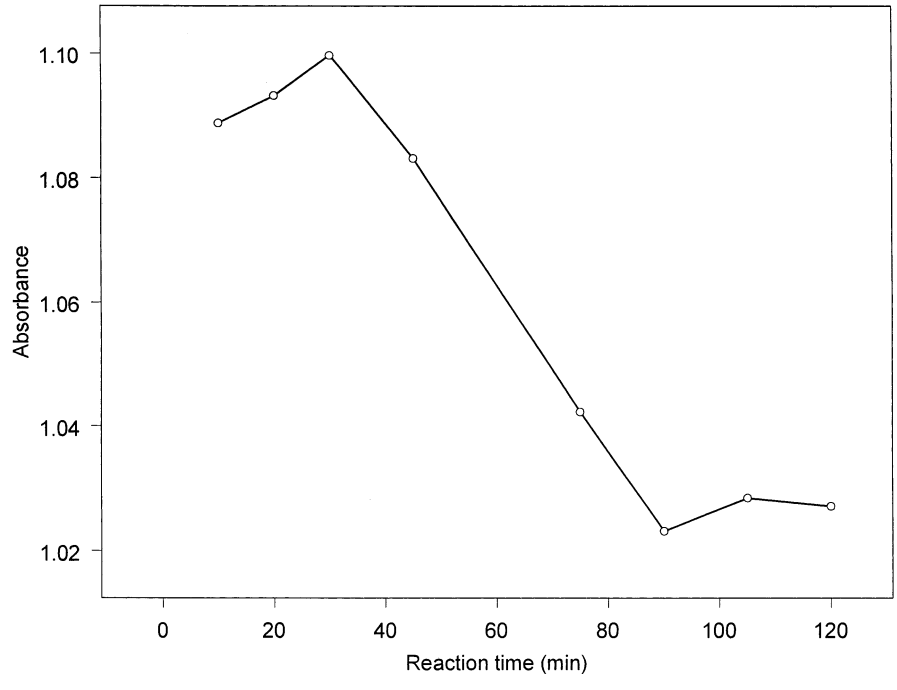

Fig. 4. Determination of the optimum reaction time for the formation of the gentamicin–*o*-phtalaldialdehyde complex.

Table 2
Regression analysis to prove the lineanity[a]

Parameter	Estimate	S.E.	t-Statistic	P-value
Intercept	0.0015	0.00540	0.279	0.7826
Slope	0.0098	0.00005	176.7	0.0000

[a] Linear model: absorbance $= a + b \times$ conc.

polymerized bone cement and gentamicin solution.

Each solution's absorbance was measured inthe spectrophotometer. Neither non-polymerized nor polymerized cements presented a quantifiable absorbance value, so there was no reaction between the *o*-phtaldialdehyde reagent and the components of the cement. On the other hand, the absorbance value of gentamicin reference solution agreed with the samples containing gentamicin with polymerized and non-polymerized bone cement, solutions (iv) and (v). The results prove that there was no interference from components of the cement matrix and there was no gentamicin adsorption on the cement surface. Therefore, it can be concluded that the method is specific for the gentamicin release from CMW1® bone cement.

In order to determine the linearity of the method, twenty-four standard aqueous gentamicin solutions at six different concentration levels were prepared. The replicates at each level were analyzed randomly. The output in Table 2 shows the results of fitting a linear model to describe the relationship between concentration and absorbance. The equation of the fitted model was: absorbance $= -0.0011 + 0.0098 \times$ concentration.

The correlation coefficient was 0.9996, indicating a relatively strong relationship between the variables. The r^2 statistic indicates that the model as fitted explains 99.93% of the variability in absorbance. The residual standard error (S.E.) was 0.012 on 22 degrees of freedom. Fig. 5 displays the linearity plot of absorbance versus concentration showing the best fit line and 95% confidence bands. Fig. 6 displays a plot of residuals versus the fitted values, which indicates no abnormality. A variance test was performed to

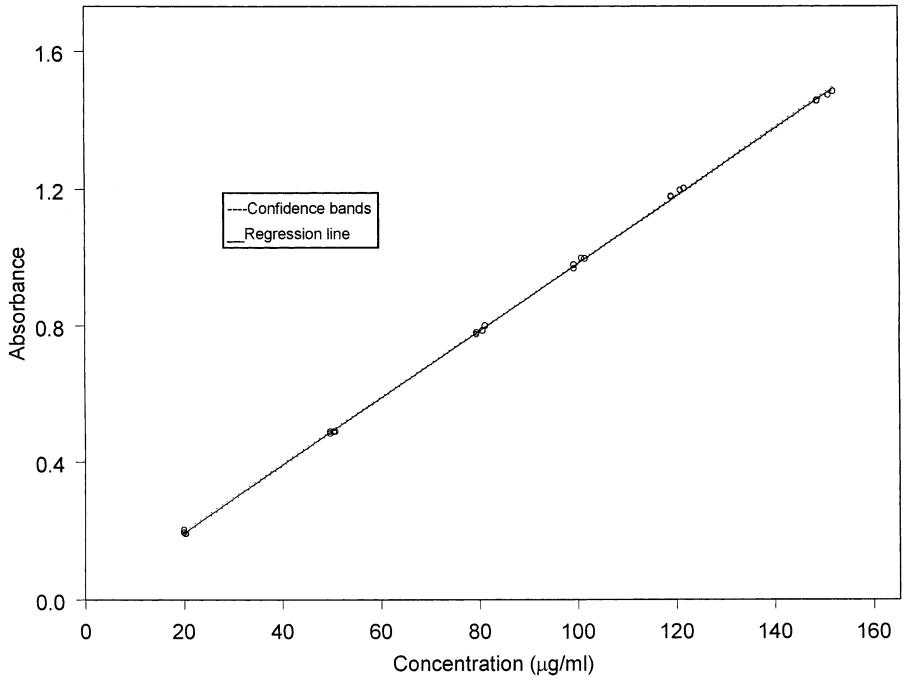

Fig. 5. Calibration curve for absorbance versus concentration of gentamicin, showing the best fit line (solid), the 95% confidence bands (dotted) and the individual data points (open circles).

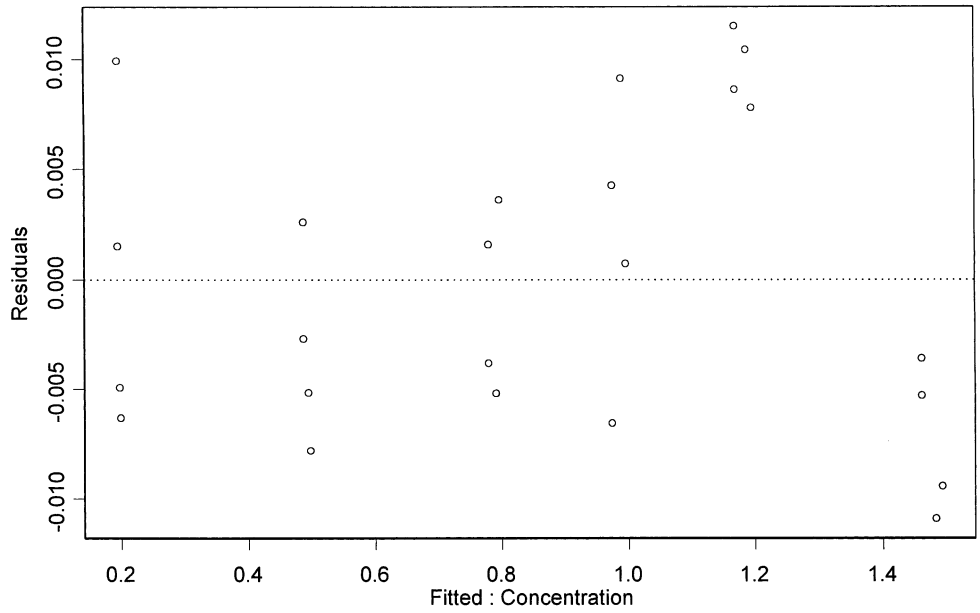

Fig. 6. Plot of residuals versus fitted values showing that no trend could be observed in the data.

Table 3
Analysis of variance with lack-of-fit

Source	Sum of squares	Degrees of freedom	Mean square	*F*-ratio		*P*-value
Model	4.1133604	1	4.33604	31230.87		0.0000
Residual	0.00305	22	0.00014			
Lack-of-fit	0.00185	16	0.00012		0.58	0.8231
Pure error	0.00120	6	0.00020			
Total (corr.)	4.3391	23				

compare the variances of the absorbance at each of the six levels of concentration. Since the *P*-value, 0.18, of Bartlett's statistics is greater than 0.05 there was not a statistical difference amongst the variances at 95% confidence level. That result showed that the homoscedasticity of the data is fulfilled. Table 3 shows an analysis of variance with lack of fit. Since the *P*-value in the ANOVA table is less than 0.01, there is a statistically significant relationship between absorbance and concentration at the 99% confidence level. The lack of fit test, also included in Table 3, is designed to determine whether the selected model is adequate to describe the observed data, or whether a more complicated model should be used. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate values of the independent variable (concentration). Since the *P*-value for lack-of-fit in the ANOVA table is greater than 0.10, the linear model appears to be adequate for the observed data. These results prove that the linearity of the method proposed is suitable for our requirements.

For evaluation of the precision estimates repeatability and intermediate precision were performed. According to ICH repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure. Standard gentamicin solution samples were ana-

lyzed at three different concentration levels. Six independent replicates were measured at each level. Results are shown in Table 4. A Bartlett's test for absorbance at the different levels was performed to evaluate the repeatability of the method. The value of Bartlett's statistic was 1.394 with an associated P-value of 0.101; therefore, there was no statistically significant difference between the variance of the levels. The method thus shows an adequate repeatability.

Intermediate precision of the procedure was evaluated by means of a 8 day $\times$ 2 replicates experimental design, at three different concentration levels: 75, 100 and 125 μg/ml. For each concentration level, a Bartlett's test for absorbance on the different days was performed to evaluate the intermediate precision of the analytical procedure. The value of Bartlett's statistic was 1.00 with an associated P-value of 0.999; therefore, there was no statistically significant difference between the variance of days. The method thus shows an adequate intermediate precision.

The calibration range was established through consideration of the practical range necessary. It was derived from linearity studies: 20–150 μg/ml. Detection and quantification limits, LD and LQ respectively, were determined according to ICH recommendation (ICH Topics Q 2B, 1996). Using the approach based on the S.E. (standard error) of the response and the slope. The detection and quantification limits may be expressed as:

$$LD = \frac{3.3\sigma}{S};\ LQ = \frac{10\sigma}{S}.$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve. The slope S may be calculated from the calibration curve of analyte, 9.8×10^{-3}. The estimate of σ, 3.4×10^{-3}, was carried out based on the standard deviation of the blanks. In order to determine these limits, measurement of the magnitude of analytical background response was performed by analyzing 10 samples (3 ml phosphate buffer + 3 ml isopropanol + 3 ml o-phtaldialdehyde reagent), including the cement matrix, and calculating the standard deviation of these responses. The calculated detection limit was: $LD = 1.1$ μg/ml, and the quantification limit was: $LQ = 3.4$ μg/ml

Table 4
Repeatability test results

Concentration (μg/ml)	Absorbance	Coefficient of variation (%)
49.25	0.4874	1.66
	0.4924	
	0.4961	
	0.5101	
	0.4902	
	0.4901	
98.5	0.9892	1.58
	0.9836	
	1.0045	
	0.9907	
	1.0273	
	0.9954	
147.75	1.5248	1.59
	1.4885	
	1.5332	
	1.5252	
	1.4881	
	1.4779	

3.3. In vitro liberation assays

The use of combinations of cement and antibiotic is based on the principle that the antibiotic will be gradually released from the cement over time. It is a complex process and one with a critical bearing on the development of any clinical application.

Fig. 7 shows cumulative drug released profiles of gentamicin sulfate from specimens of CMW1® antibiotic bone cements. Each point in the plot represents the mean of four independent experiments corresponding to lots 1 and 2. There were no significant differences between both lots. Lot 3 measures could not be used to the release model fitting due to its low and erratic drug release. The plot of the cumulative amount of drug dissolved against time can be described by the semi empirical equation $y = a + bt^n$, where y is the cumulative released amount, t is the release time, and a, b and n are constants. The first term in the second hand of equation represents the burst effect and the second term, known as power low of time model

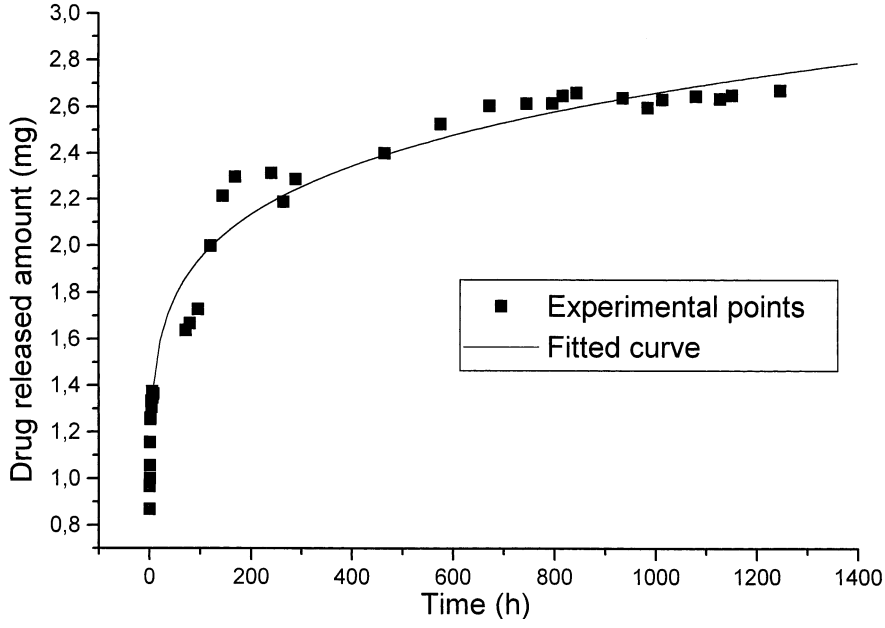

Fig. 7. Mean amount of cumulative gentamicin sulfate release from specimens of CMW1 bone cement over each sampling interval for the 8-week study period.

(Korsmeyer et al., 1983), predicts that the amount of drug released is exponentially related to the release time. Parameters in the model were estimated by minimizing the sum of square residuals. All computations were made using a statistical software package, S-PLUS (S-PLUS, 2000). For release in the conditions described in materials and methods fitted parameters were $a = 0.332$, $b = 0.7769$ and $n = 0.1591$ and residual standard error was 0.0699 on 33 degrees of freedom ($r^2 = 0.9787$). In Fig. 7 it can be observed that most of the gentamicin sulfate was released within the first hours of the experiment. The amount of gentamicin in the test solution subsequently remains almost constant so there seems to be no further release. In addition most of the gentamicin incorporated to the cement is not released and more than 90% of the total amount remains in the cement matrix. The mean release rate profile is shown in Fig. 8; the inset on the panel shows the in vitro rate of gentamicin released from polymeric matrix in the first 3 h. The release rate throughout the first day was high, particularly on the first hour and rapidly decreased until the third hour, and a constant release rate was attained by the first week, which was maintained throughout the experimental period.

Large initial antibiotic bursts were observed for the polymer–gentamicin specimen. The initial burst was due to the dissolution of drug particles adsorbed to the slab surface or due to the diffusion of gentamicin particles close to the surface. PMMA is a highly hydrophobic polymer, and thus impervious to gentamicin diffusion; therefore gentamicin will be released from the matrix through voids, cracks and imperfections of the system. SEM was used to clarify the surface of the specimen, before and after the release assay was performed (Figs. 9 and 10, respectively). Electron microscopy observations showed a irregular surface before the assay (see Fig. 9), and voids and cracks can be noticed in the SEM microphotograph accomplished after the assay (see Fig. 10). The additives of the acrylic cement such as barium sulfate or gentamicin sulfate may be the cause of these imperfections of the matrix. The circular voids in may be due to gentamicin sulfate particles dissolved.

Only gentamicin molecules located in the su-

perficial layers of the system, where the test solution can penetrate to dissolve them through the pores and cracks of the matrix, will be released. When these molecules are dissolved, the antibiotic liberation from the system will last no longer because the polymeric matrix will cover those molecules located inside the matrix and isolate them from the test solution, so they cannot be released to the medium.

When the liquid proportion added to the pow-

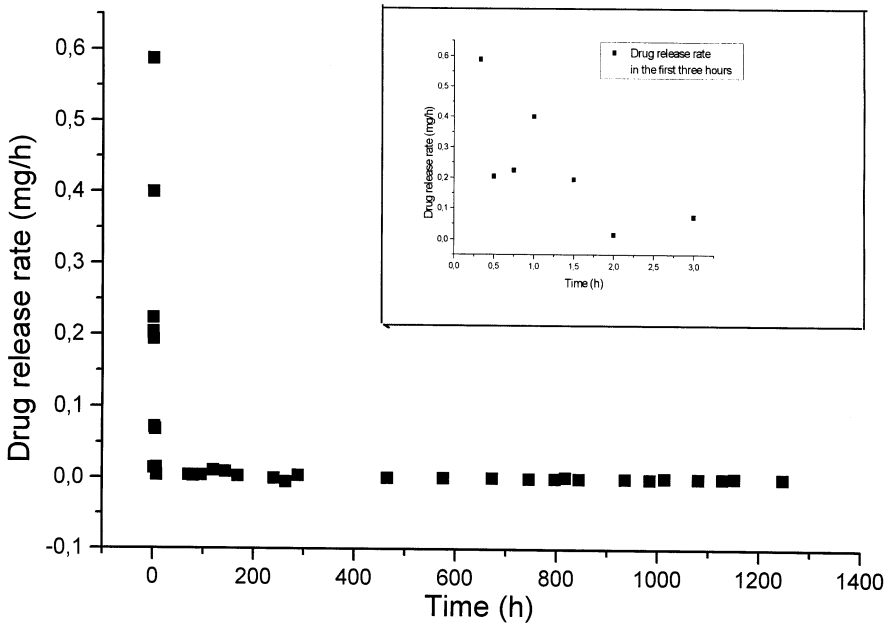

Fig. 8. Mean rate gentamicin sulfate release from specimens of CMW1 bone cement over each sampling interval for the 8-week study period. The inset shows the rate of gentamicin released from polymeric matrix in the first 3 h.

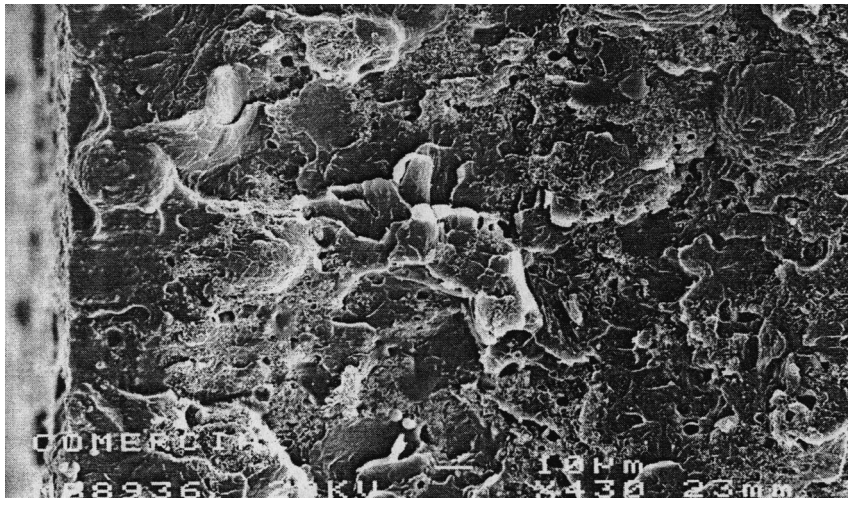

Fig. 9. Scanning electron microphotograph showing an irregular surface specimen before the release assay was performed. Photograph taken at a magnification of $\times 430$.

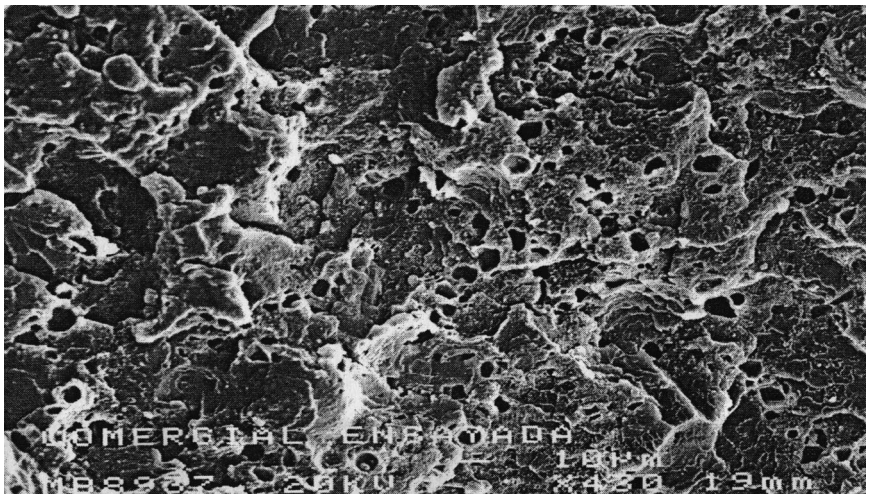

Fig. 10. Scanning electron microphotograph showing that voids and cracks can be noticed on the surface specimen after the release assay was performed. Photograph taken at a magnification of $\times 430$.

der is decreased, the polymerization reaction is defective. The monomer may not be sufficient for the reaction to take place in full, so more cracks and imperfections will appear in the matrix, allowing a further penetration of the test solution. This can lead to a more sustained release of the antibiotic, and to a higher amount of gentamicin being released.

4. Conclusions

The validation test performed has shown that the method used for the quantitative analysis of gentamicin sulfate has an adequate quality level, and that measures of gentamicin released from bone cements can therefore be performed by means of this method. The greatest advantage of the proposed procedure versus the published method (Frutos et al., 2000) was a considerable increase in the sensitivity (68.3%) and calibration range (20–160 μg/ml); moreover, the *o*-phtaldialdehyde-gentamicin complex is more stable than the ninhidrin–gentamicin complex.

in vitro assays for gentamicin released from bone cements showed that acrylic bone cements do not allow a complete release of gentamicin incorporated to them. The release of gentamicin may be due to defects and imperfections (pores, cracks and voids) of the polymeric matrix that facilitate the penetration of the test solution to leach gentamicin particles. The effect of the amount of liquid component added to the cement matrix has been studied. Decreasing the amount of monomer incorporated leads to an increase in the amount of gentamicin released and to a more sustained release, probably due to a defective polymerization reaction and thus to the appearance of more defects in the cement matrix.

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