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# Evaluation of the stability of gentamicin in different antibiotic carriers using a validated MEKC method

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ABSTRACT

The quality control of gentamicin in different antibiotic carriers, using MEKC as stability-indicating method is described. Baseline separations of gentamicin C1, C1a, C2, C2a and C2b and, furthermore the impurities and degradation products garamin (GARA), 2-deoxy-streptamine (DSA) and sisomicin (SISO) were achieved with a background electrolyte containing 20 mM deoxycholic acid, 15 mM β-cyclodextrin and 100 mM tetraborate (pH 10.0). After derivatization with *o*-phthaldialdehyde reagent (OPA), UV detection at 340 nm was possible. The method was validated with respect to selectivity, limit of detection (LOD) and quantification (LOQ) of the impurities, linearity, accuracy, precision and robustness. Evaluation of four different antibiotic carriers stored under stability conditions according to the International Conference on Harmonization (ICH) guidelines and older pharmaceutical formulations disclosed good stability.
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## 1. Introduction

The aminoglycoside antibiotic gentamicin, produced by *Micromonospora purpurea*, is active against aerob gram-negative bacteria. It is used intravenously for life-threatening bacterial infections, as well as locally as liquid or semi-liquid preparations in ophthalmology, in antibiotic carriers, like polymethylmethacrylate (PMMA) beads and PMMA bone cement [1] or in orthopaedics. These biomaterials were used in modern medicine such as joint prostheses or bone infection. Collagen-gentamicin sponges were an alternative for the beads in the local treatment of bone and soft tissue infections. Bone cements fulfil several functions, especially the fixation of the prosthesis. The risk of attracting infectious microorganisms has been a drawback in biomaterials. To avoid infections, the bone cement itself was used as antibiotic carrier. With this method the surface of the implant and the surrounding area of the fixed prostheses can be treated with high local drug levels, but without systemic side effects. Nevertheless, the potentially ototoxic and nephrotoxic activity of gentamicin has to be taken into account, as well as the quality of the used bulk material and the stability in the final formulations in general.

Gentamicin is a composition of gentamicin C1, C1a, C2, C2a and the minor component C2b [2]. Due to the lack of UV chromophores and the structural similarity of the components, differing only in their degree of methylation on the purpurosamine ring (Fig. 1), the analysis is challenging. In the Ph. Eur. [2] a HPLC method with a styrene-divinylbenzene copolymer column and pulsed amperometric detection is described. The amounts of C1, C1a and the sum of C2, C2a and C2b are limited to 20–40%, 10–30% and 40–60%, respectively; furthermore each impurity is restricted to 3% and the sum of all impurities to 10%.

Several methods for the determination of gentamicin have been described in the literature, focusing on HPLC and CE. In HPLC several detection techniques were used: pre-column derivatization with UV [3] or fluorimetric detection [4], direct detection with pulsed electrochemical detection (PED) [5], where Cai et al. [6] put the focus on the integration, and with the evaporative light scattering detection (ELSD) [7,8]. The latter has the advantage that no reference material is necessary due to nearly equal response factors for molecules with similar molecular masses and structure formulae. However, Manyanga et al. [9] compared two methods for direct detection using ELSD and PED, concluding a modified LC-PED method to be more sensitive and selective.

For CE the situation is similar: CZE with indirect UV detection [10], direct UV detection due to the formation of UV-absorbing borate complexes [11,12] or direct UV detection with a reversed EOF [13]. Potential gradient detection is also possible in CE [14]. After derivatization with *o*-phthaldialdehyde separation was obtained

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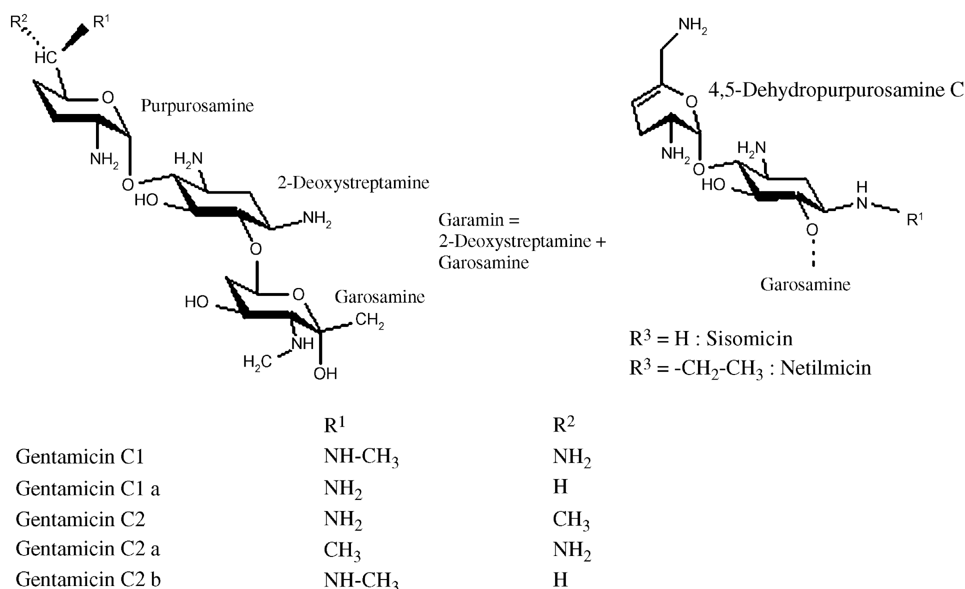


**Fig. 1.** Structural formulae of gentamicin and related substances.

using CZE [15,16] or MEKC conditions [17]. Using the latter method, a huge number of batches of gentamicin bulk material were analysed [18], as well as by NMR spectroscopy as orthogonal techniques [19,20]. Winter et al. [21] used the NMR data for a statistical classification.

The aforementioned methods aim at different purposes, e.g. therapeutic drug monitoring [22] or characterization of impurities [23]. The intention of this study was the evaluation of gentamicin and its impurities and degradation products in different antibiotic carriers within the framework of stability measurements. Stability-indicating measurements revealed the degradation of gentamicin in dextrose solution [24]. In contrast, the assessment of gentamicin in pharmaceutical formulations (injection ampoules, eye drops and eye cream) with chemiluminescent detection after derivatization with *o*-phthaldialdehyde [25] showed no differences between determined concentration and nominal value.

Stability is often verified by means of a method of a monograph in the Ph. Eur. or USP, as described in the test “related substances”. However, investigations by Ghinami et al. [26] and others clearly demonstrated that the Ph. Eur. aminoglycoside methods are not robust. Therefore, we applied the MEKC method with *o*-phthaldialdehyde derivatization described already by Wienen and Holzgrabe [17]. So far this method was not validated according to ICH guidelines, and no stability measurements were reported. The method will be subjected to different gentamicin carriers and bone cements, one of them containing gentamicin and clindamycin, after isolation from the different pharmaceutical formulations.

## 2. Experimental

### 2.1. Instrumentation

CE measurements were performed on a HP$^{3D}$-CE (Agilent Technologies, Waldbronn, Germany) equipped with a DAD detector and the Chemstation 08.03 Software. The detection wavelength was set at 340 nm. Fused-silica capillaries were purchased from Polymicro (BGB Analytik, Schloßböckelheim, Germany) with a total length of 33.0 cm, an effective length to the detector of 24.5 cm, an internal diameter of 50 μm and an external diameter of 375 μm. The capillary cartridge was thermostated at 34 °C.

### 2.2. Chemicals and materials

Gentamicin sulphate, used for the validation, 2-desoxystreptamine (DSA), clindamycin hydrochloride and the stability samples were provided by Heraeus Medical GmbH (Wehrheim, Germany). Sisomicin (SISO) USP reference standard was purchased from Promochem (Wesel, Germany). Garamin hydrochloride (GARA) was a gift from Merck (Darmstadt, Germany). Phthaldialdehyde, deoxycholic acid sodium salt monohydrate, boric acid, and picric acid were purchased from Fluka (Buchs, Switzerland); sodium tetraborate decahydrate, sodium hydroxide and potassium hydroxide from Grüssing (Fillsum, Germany); dichloromethane, thioglycolic acid, and methanol from Merck (VWR-International, Darmstadt, Germany); β-cyclodextrin from Wacker (München, Germany). All samples and buffers were prepared using ultrapure-Milli-Q water (Millipore, Milford, MA, USA) and filtered through a 0.22 μm filter (Carl Roth GmbH, Karlsruhe, Germany) prior to use.

### 2.3. Methods and conditions

The samples were injected at the anodic end of the capillary under pressure of 50 mbar for 5 s. The separations were performed using a 100 mM sodium tetraborate running buffer (pH 10.0) containing 20 mM of deoxycholic acid and 15 mM β-cyclodextrin. The background electrolyte was prepared by dissolving adequate amounts of sodium tetraborate, deoxycholic acid and β-cyclodextrin in water, and adjusting the pH to the required value with a 1.0 M sodium hydroxide solution.

New capillaries were conditioned at 34 °C rinsing with 0.1 M HCl for 20 min, with water for 2 min, with 0.1 M NaOH for 20 min and again with water for 3 min. Before running a series of experiments, the capillaries were conditioned at 34 °C rinsing with water for 2 min, with 0.1 M HCl for 10 min, with water for 2 min, with 0.1 M NaOH for 10 min and finally with water for 5 min. Between each run the capillaries were rinsed at 34 °C with 0.1 M HCl for 1.5 min (6 bar), with water for 1.5 min (6 bar), with 0.1 M NaOH for 2 min (6 bar), with water for 2 min (6 bar) and background electrolyte for 3 min (8 bar) as preconditioning and with water for 3 min (10 bar) as post-conditioning. At the end of a working day, the cap-

illaries were rinsed at 34 °C with water for 10 min. Capillary wash cycles were performed at a pressure of 900 mbar, unless otherwise indicated.

### 2.4. Sample preparation

A stock solution (7 mg/ml) of picric acid, used as an internal standard (IS), was added to all gentamicin samples to reach a final concentration of 0.14 mg/ml. For validation of the method the gentamicin samples spiked with different levels of impurities were prepared by mixing corresponding amounts of gentamicin sulphate and each impurity stock solution in water. The amounts used depended on the validation parameters; details are reported in Section 3.2.

PMMA-placebo beads as pharmaceutical formulations were applied to determine precision and accuracy. The stock solutions of gentamicin sulphate and the impurities were added to PMMA-placebo beads and the mixture was subjected to the preparatory procedure (see Section 2.4.1).

To test the stability of the different gentamicin formulations, PMMA beads (Septopal®, 4.95 mg gentamicin/bead), PMMA mini-beads (Septopal®, 1.87 mg gentamicin/mini-bead) and PMMA bone cement (Copal®, gentamicin 2.34% (m/m) and clindamycin 2.34% (m/m); Osteopal®, gentamicin 1.23% (m/m)) were subjected to a preparatory procedure to gain the stock solutions.

#### 2.4.1. Sample pre-treatment

In order to obtain the nominal gentamicin stock solutions of 0.990 mg/ml for the PMMA beads and 0.898 mg/ml for the PMMA mini-beads, five PMMA beads or twelve PMMA mini-beads, respectively, were dissolved in 50 ml dichloromethane. The organic phase was extracted three times with 50 ml water. The collected aqueous phases were evaporated to dryness (40 °C, 72 mbar). Then the residue, dissolved in 15 ml water, was added to 500 μl IS stock solution and the solution was diluted with water to 25.0 ml.

In case of the bone cements 3.00 g material and 250.00 g water were weighed in a flask and stirred for 30 min. For Copal® 45 ml bone cement the solution was centrifuged for 6 min at 4000 U/min and 40.0 ml of the supernatant were evaporated to dryness (40 °C, 72 mbar). The residue was dissolved in 7 ml water, 200 μl IS stock solution were added and this solution diluted to 10.0 ml with water. The final nominal concentration of the gentamicin stock solution was 1.124 mg/ml. For Osteopal® 55 ml bone cement the solution was centrifuged for 6 min at 4000 U/min and 50.0 ml were evaporated to dryness (40 °C, 72 mbar). The residue was dissolved in 3 ml water, 100 μl IS stock solution added and the solution diluted to 5.0 ml with water. The final nominal concentration of the gentamicin stock solution was 1.476 mg/ml.

#### 2.4.2. Sample derivatization

Derivatization was performed with *o*-phthaldialdehyde reagent (OPA): 260 mg of *o*-phthaldialdehyde was dissolved in 800 μl of methanol and sonicated to aid dissolution. 6 ml of sodium borate solution (pH 10.4, 30 mM) and 520 μl thioglycolic acid were added and the solution was adjusted to pH 10.4, using a solution of 8 M potassium hydroxide. This solution was diluted with sodium borate solution (pH 10.4, 30 mM) to 10.0 ml. The OPA solution was stored at 4 °C and used within 3 days after preparation [27]. It has to be stored for 90 min prior to the first use.

450 μl of sample stock solution were mixed with 160 μl OPA and diluted with methanol to 1.0 ml. This solution was vortexed and heated in a water bath at 60 °C for 4 min. After cooling to room temperature the solution was filtered in a vial appropriate for CE.

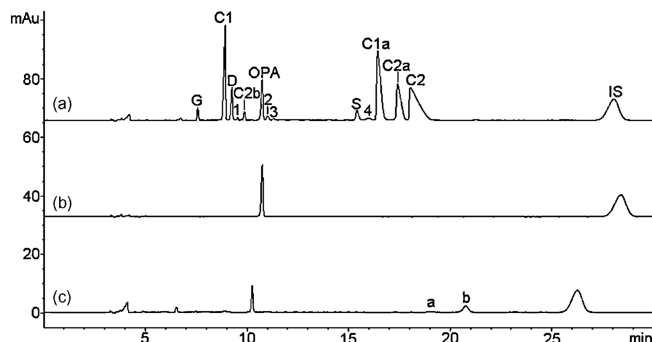


**Fig. 2.** Electropherogram of: (a) gentamicin and possible impurities garamin, 2-deoxystreptamin and sisomicin; (b) blank with the internal standard picric acid; (c) matrix-blank. C1, C2b, C1a, C2a, C2—gentamicin compounds; known impurities: G—garamin, D—2-deoxystreptamin, S—sisomicin; unknown impurities: 1–4; OPA—*o*-phthaldialdehyde reagent; IS—Internal Standard; matrix peaks: a, b.

## 3. Results and discussion

### 3.1. Method optimisation

The MEKC method developed by Wienen and Holzgrabe [17] was slightly modified: the temperature was elevated from 25 to 34 °C in order to decrease the analysis time. This method allows the separation of all major and minor components of gentamicin and the known impurities GARA, DSA, and SISO, as well as unknown impurities (Fig. 2a). SISO serves as a lead impurity for the quality of gentamicin lots [17,20].

### 3.2. Method validation

The modified method, developed for the stability evaluation of gentamicin in different formulations, was validated according to the International Conference on Harmonization (ICH) Guidelines Q2(R1) [28] with respect to selectivity, LOD, LOQ, linearity, accuracy, robustness, and precision. Picric acid at a concentration of 0.14 mg/ml was used as an IS in order to compensate fluctuations of migration times.

#### 3.2.1. Selectivity

In order to assign the signals originating from OPA and IS, a blank containing only the IS picric acid at a concentration of 0.14 mg/ml was carried out. As can be seen in Fig. 2b, no interference with gentamicin-peaks and the known impurities occurred.

Matrix samples were used to determine accuracy and precision. Therefore, a second blank (Fig. 2c) was recorded, using the IS picric acid 0.14 mg/ml and matrix. The blank of the matrix showed two additional signals (a and b) between the main gentamicin peaks and the IS peak, which were not interfering with the peaks of interest.

#### 3.2.2. LOD, LOQ, and linearity

The LOD and LOQ were determined in threefold, using the signal-to-noise ratio of 3:1 and 10:1, respectively. Both values were determined for the known impurities GARA, DSA, and SISO

**Table 1**
LOD and LOQ for the impurities and the minor gentamicin component C2b

| Component | LOD | | LOQ | |
|---|---|---|---|---|
| | μg/ml | %[a] | μg/ml | %[a] |
| Garamin | 1.95 | 0.10 | 6.5 | 0.33 |
| 2-Deoxystreptamine | 1.30 | 0.07 | 4.5 | 0.23 |
| Sisomicin | 3.50 | 0.18 | 12.0 | 0.60 |
| Gentamicin C2b | 2.85 | 0.14 | 9.5 | 0.48 |

[a] Relative to 2 mg/ml gentamicin sulphate stock solution.

**Table 2**
Linearity data gentamicin components/impurities

| Gentamicin/impurity | Slope | Intercept | r |
|---|---|---|---|
| C1 | 0.0019 | −0.037 | 0.9985 |
| C2b | 0.0002 | −0.0044 | 0.9478 |
| C1a | 0.0019 | −0.0548 | 0.9983 |
| C2a | 0.0011 | −0.0595 | 0.9982 |
| C2 | 0.0019 | −0.0843 | 0.9994 |
| Garamin | 0.0075 | −0.0437 | 0.9880 |
| 2-Deoxystreptamine | 0.0202 | −0.0101 | 0.9962 |
| Sisomicin | 0.0073 | −0.0228 | 0.9883 |

*y*: relative peak area = ratio of corrected peak area of gentamicin/impurity to internal standard; *x*: total concentration of gentamicin sulphate/impurity.

**Table 3**
Accuracy of the method tested at 2.4%, 3%, and 3.6% impurity levels relative to 1.6, 2.0 and 2.4 mg/ml gentamicin sulphate

| Impurity | Accuracy (%) | R.S.D. (%) |
|---|---|---|
| Garamin | 97.14 | 9.71 |
| 2-Deoxystreptamine | 103.21 | 6.96 |
| Sisomicin | 117.33 | 9.27 |

(Table 1). Gentamicin C2b belongs to the major components being present in small portions of 0.5–3% only. In the working standard used, the portion was found to be 1.55%. The LOD and LOQ of gentamicin C2b were evaluated as a representative for unknown impurities (Table 1). The linearity was determined twice by using six concentrations of a spiked stock solution. For gentamicin sulphate the 100% value was set to 2 mg/ml. For the impurities the 100% value was set to 3%, which is in line with the limit for each impurity reported in the Ph. Eur. [2]. Therefore, the sample concentration for each impurity was 60 μg/ml and a spiked stock solution was correspondingly prepared. The linearity ranged from 30% to 120% relative to 2 mg/ml gentamicin sulphate for the gentamicin components (Table 2) and relative to 60 μg/ml for the impurities (Table 2). As gentamicin sulphate working standard contains a minor portion of GARA, this was taken into consideration in the determination of the linearity. Calibration curves were obtained by plotting the concentration levels of the compounds against the peak-area ratios. All data are summarized in Tables 1 and 2. In all cases, regression lines with correlation coefficients ($r$) were above 0.998 for the gentamicin components, except the minor compound C2b (0.9478), and above 0.988 for the impurities.

### 3.2.3. Accuracy

The accuracy for the known impurities GARA, DSA and SISO was evaluated using recovery measurements at 80%, 100% and 120% levels [29]. The degradation product GARA is of great importance for stability measurements, because it is a real intermediate, containing DSA and garosamine (Fig. 1); thus two cleavage steps are necessary to liberate DSA by degradation.

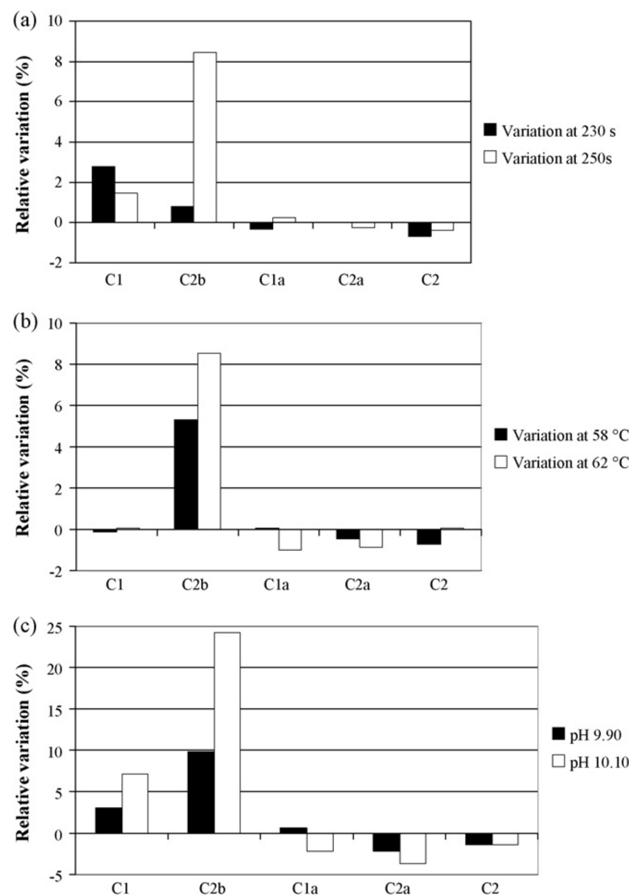


**Fig. 3.** Relative variation of the peak area in percentage by normalisation procedure for the gentamicin compounds at small variation of (a) the time of derivatization; (b) the temperature of derivatization; (c) the pH of the buffer.

SISO does not normally occur in gentamicin; however, it was previously found in many samples and defined as a major impurity for the appearance of numerous unknown impurities of high amount [17]. Therefore, SISO was taken into account due to its marker function.

The recovery $W$ was calculated as follows: $W = (S1 - S2)/S3 \times 100$ with $S1$: sample solution plus impurities with matrix (B), $S2$: sample solution (C), and $S3$: solution of impurities (A).

The three solutions $S1$, $S2$ and $S3$ were taken through all steps of the method. Thus, a sample of 2 mg/ml gentamicin sulphate spiked with GARA, DSA and SISO of 3% (w/w) each was used as 100% level. A threefold determination was performed for all components on each level. PMMA placebo beads dissolved in dichloromethane were used as a matrix. Due to the fact that the amount of available GARA was limited, it was only possible to determine the 80% level

**Table 4**
Intra-day and inter-day precision of a spiked gentamicin stock solution with matrix

| | Intra-day precision (n = 6) | | | | | | Inter-day precision (n = 12) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RMT (s) | R.S.D. (%) | Area ratio | R.S.D. (%) | Peak area (%) | R.S.D. (%) | RMT (s) | R.S.D. (%) | Area ratio | R.S.D. (%) | Peak area (%) | R.S.D. (%) |
| Garamin | 0.290 | 3.33 | 0.117 | 7.46 | 1.105 | 4.89 | 0.286 | 3.75 | 0.116 | 6.72 | 1.089 | 4.60 |
| C1 | 0.335 | 3.18 | 1.374 | 5.80 | 14.991 | 2.75 | 0.331 | 3.66 | 1.387 | 5.08 | 15.021 | 2.10 |
| 2-Deoxystreptamin | 0.352 | 3.12 | 0.280 | 4.59 | 3.220 | 1.31 | 0.348 | 3.52 | 0.279 | 3.59 | 3.192 | 2.98 |
| C2b | 0.370 | 3.04 | 0.116 | 14.74 | 1.403 | 13.20 | 0.366 | 3.49 | 0.112 | 13.93 | 1.345 | 13.07 |
| Sisomicin | 0.518 | 2.01 | 0.174 | 3.41 | 3.075 | 2.67 | 0.519 | 1.38 | 0.172 | 4.46 | 3.056 | 4.37 |
| C1a | 0.609 | 2.23 | 1.388 | 2.92 | 27.599 | 1.25 | 0.605 | 2.38 | 1.391 | 2.81 | 27.665 | 1.19 |
| C2a | 0.645 | 2.10 | 0.774 | 3.22 | 16.285 | 1.01 | 0.641 | 2.21 | 0.774 | 3.34 | 16.300 | 1.30 |
| C2 | 0.671 | 2.01 | 1.406 | 2.47 | 30.764 | 1.45 | 0.667 | 2.10 | 1.402 | 3.04 | 30.734 | 1.12 |

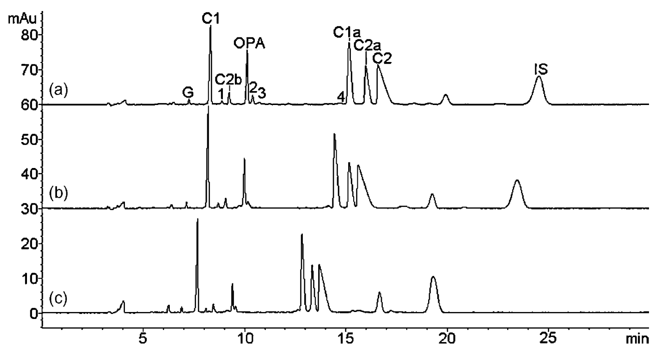


**Fig. 4.** Electropherogram of PMMA beads at: (a) 0 month; (b) 6 months, accelerated conditions; (c) 24 months, long term conditions; C1, C2b, C1a, C2a, C2—gentamicin compounds; known impurities: G—garamin; unknown impurities: 1–4; OPA—*o*-phthaldialdehyde reagent; IS—Internal Standard.

in presence of the matrix. The data are summarized in Table 3. All accuracy values for the impurities are in a range between 80% and 120% (R.S.D. ≤ 10%).

### 3.2.4. Precision

The determination of the precision was performed using a gentamicin sulphate solution (2 mg/ml) spiked with the impurities GARA, DSA and SISO, and the matrix. The stock solution obtained from extraction and evaporation was injected six times on 1 day (intra-day precision) and the following day (inter-day precision). Analysis was performed for the relative migration time and the relative peak area using the IS picric acid, and for the peak area in percentage using the normalisation procedure (Table 4). Acceptable relative standard deviations (R.S.D. ≤ 10%, except C2b ≤ 15%) were found. Using the normalisation procedure even R.S.D. ≤ 5% was found, except C2b (R.S.D. ≤ 15%). Compared with the other gentamicin components, C2b is a low level compound, therefore higher variations are acceptable.

### 3.2.5. Robustness

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations of the method parameters [28]. Here, variations of the time ±10 s and temperature ±2 °C of derivatization, and the pH of the running buffer ±0.1 were considered. The solution, containing 2.0 mg/ml gentamicin sulphate and 0.14 mg/ml IS, was analysed twice using either condition. The data of robustness for the gentamicin compounds with respect to the peak area in percentage by normalisation procedure are summarized in Fig. 3a–c. Except for gentamicin C2b, small variations resulted in still acceptable, but not negligible relative variations of the peak area. Similar results were obtained when using the IS (data not shown). Due to the fact, that the normalisation procedure is more common for multi-component drugs, it was further used.

## 3.3. Stability

The MEKC method, which was proved to be accurate, precise and robust, was used for stability tests of gentamicin containing formulations for local antibiotic treatment in orthopaedic surgery [30]: PMMA beads and PMMA mini beads (Septopal®), and PMMA bone cement (Copal® and Osteopal®). Twelve batches of PMMA beads and PMMA mini-beads were tested for stability. In addition, four

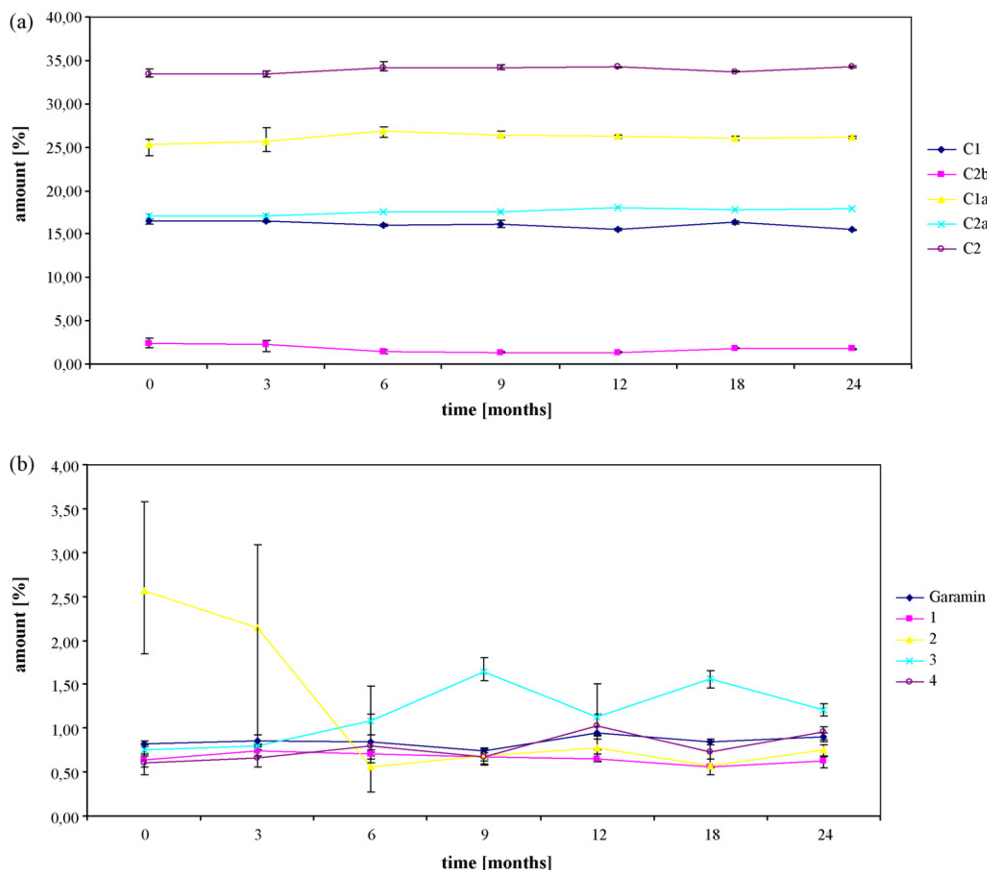


**Fig. 5.** Amount of PMMA beads by normalisation procedure under long term conditions (a) gentamicin compounds; (b) impurities; reported is the average out of three independent determinations, the minimal and maximal value is reported additionally.

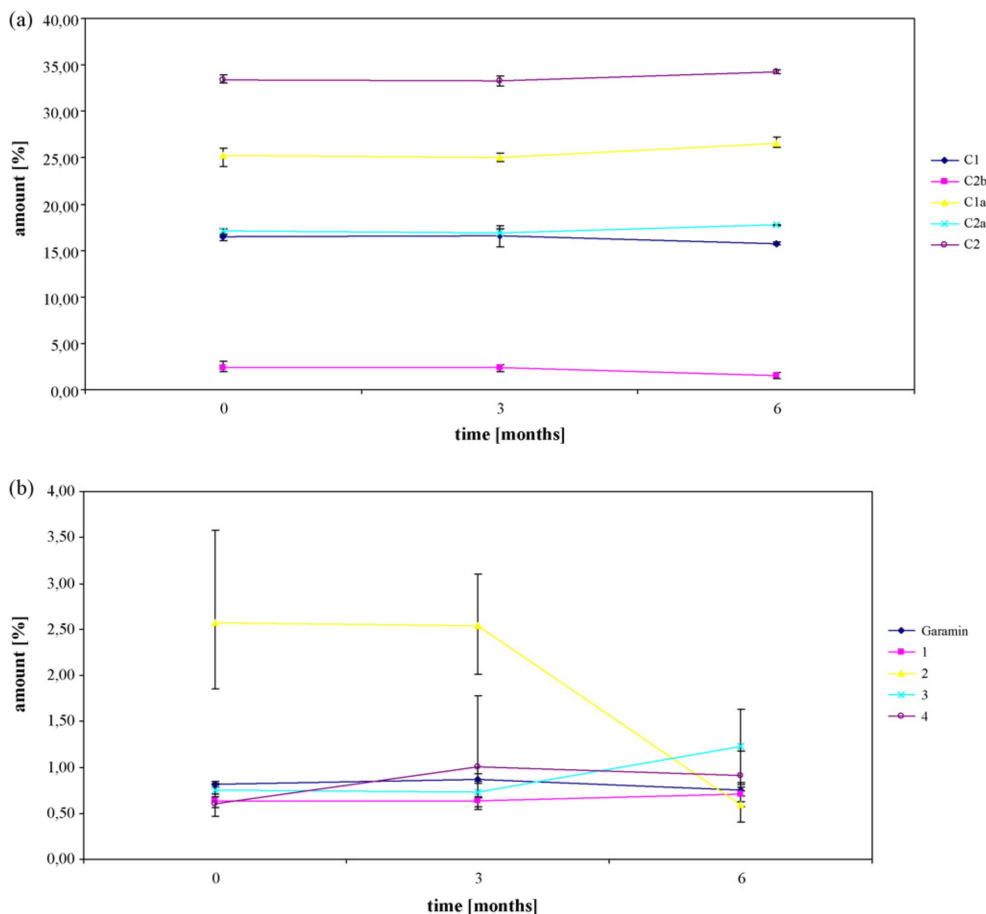


**Fig. 6.** Amount of PMMA beads by normalisation procedure under accelerated conditions (a) gentamicin compounds; (b) impurities; reported is the average out of three independent determinations, the minimal and maximal value is reported additionally.

batches of two different bone cements were studied. One of these bone cements additionally contained clindamycin, which had no influence on the determination of gentamicin due to the lack of primary amines, which could react with the OPA, as well as no absorption in the region of the detection wavelength. The antibiotic carriers were stored under accelerated (40 °C, 75% relative humidity) and long term (25 °C, 60% relative humidity) conditions. The stability test was performed according to the ICH guideline Q1A(R2) [31]. For the long term conditions the frequency of testing was every 3 months over the first year, every 6 months over the second year, and should be annually thereafter through the proposed shelf life. At the accelerated storage condition a minimum of three points from a 6-month study is recommended.

For gentamicin bulk material a stability of at least 5 years has already been reported [27]. Prior to the stability, real time samples (14 PMMA beads and PMMA mini-beads, stored between 1995 and 2006) were analysed (data not shown). Even in the oldest samples DSA as a known degradation product could not be detected. The variations, marked, e.g. by the pattern of the triad of gentamicin C1a, C2a and C2, were supposed to originate from the used gentamicin bulk material. Gentamicin seems to be stable in the formulations, as no additional peaks appeared at 6 months accelerated and 24 months long term conditions (Fig. 4). Representative results using the normalisation procedure of one batch of PMMA beads are shown in Figs. 5 and 6. The amount of GARA varies in the different formulations, but no increase was seen in the formulations during the storage. The unknown impurities differ in their amounts, but their number is stable. Since their amounts vary even between independent measurements of the same sample, the fluctuations are considered as a variation of the method and not as degradation.

For the sum of mass, calculated using the IS with the linearity curves as calibration curves, only variations and no decrease of gentamicin were recognized, while the amount of GARA was constant over the time (data not shown).

## 4. Conclusion

The MEKC method developed earlier [17] was slightly modified and validated without any problems for the purpose of routine analytics of gentamicin containing antibiotic carriers in the frame of stability evaluation. Therefore the focus was set on the degradation impurities GARA, DSA, and the marker impurity SISO [18,20]. All samples were without SISO and the related numerous unknown peaks, so it can be concluded that the quality of the bulk gentamicin is high. An increase in the number or amount of unknown peaks was not noticed. The stability of gentamicin in different antibiotic carriers was demonstrated.

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