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Micellar electrokinetic capillary chromatography, high performance liquid chromatography and nuclear magnetic resonance—three orthogonal methods for characterization of critical drugs

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Abstract

Purity assessment of multicomponent drugs such as aminoglycoside antibiotics is still a challenge. For example, the high performance liquid chromatography (HPLC) method reported in the European Pharmacopoeia for gentamicin suffers from various disadvantages, i.e. missing robustness, long retention times and broad peaks. Previously an effective and robust micellar electrokinetic chromatography (MEKC) method has been described which is capable of separating all components and impurities. Since a couple of gentamicin lots collected from international markets showed a high number and high quantities of impurities NMR spectroscopy as a primary analytical method was applied in order to validate the quantification results obtained from MEKC. In this study NMR spectroscopy was found to be a very good orthogonal and complementary method.
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Keywords: Gentamicin impurities; Capillary electrophoresis; NMR spectroscopy

1. Introduction

Purity assessment of small-molecule pharmaceuticals can normally be done by means of high performance liquid chromatography (HPLC). Hence, the three major pharmacopoeiae USP, EP and JP make intensive use of HPLC for evaluation of related substances. Moreover, the EP is going to replace almost all thin layer chromatography methods with HPLC. However, for drugs biotechnologically produced and/or consisting of more than one component the quality control by HPLC is still a challenge. Beside biopharmaceuticals, such as heparins, especially the aminoglycoside antibiotics, makrolide antibiotics, e.g. erythromycin, and peptide antibiotics, e.g. bacitracin, belong to this group of critical drugs. They are typically composed of a couple of main components accompanied by minor components and impurities of a level often higher than 0.1% being the limit allowed for small-molecule drugs. In all cases the composition of the antibiotics depends sensitively on the fer-

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mentation conditions and the subsequent purification applied.

Aminoglycosides are always composed of a variety of structurally related amino sugars having neither a chromophor nor a fluorophor. Whereas kanamycin, neomycin, and paromomycin are characterized by one main component accompanied by some minor components of less than 5% content, gentamicin consists of four major components, i.e. GM C1, C1a, C2 and C2a, and some minor components such as GM C2b, 2-deoxystreptamine (DSA), garamine (GA), sisomicin and netilmicin, the latter two being antibiotics on their own (see Fig. 1).

Due to the close structural relationship of the aminoglycosides and the missing chromophor the evaluation of the composition and related substances is still a challenge for both HPLC [1–3] and capillary electrophoresis (CE) [4,5]. Since edition 4.5 the EP [6] describes a HPLC method utilizing a styrene-divinylbenzene copolymer column and a pulsed amperometric detector for the aforementioned purposes. However, this method suffers from several problems, e.g. the pulsed amperometric detection is not very robust because the electrode has to be frequently cleaned, the special column applied cannot be used as often as, e.g. a RP-18 column, the run time of a chromatogram is longer than 70 min and the main components of gentamicin elutes more than 10 min. The latter two problems are demonstrated in Fig. 2 showing a typical chromatogram obtained with the EP method.

In order to improve the selectivity a micellar electrokinetic chromatography (MEKC) method was recently developed [7] which is capable to separate and quantify both the components of gentamicin and the impurities. However, it suffers

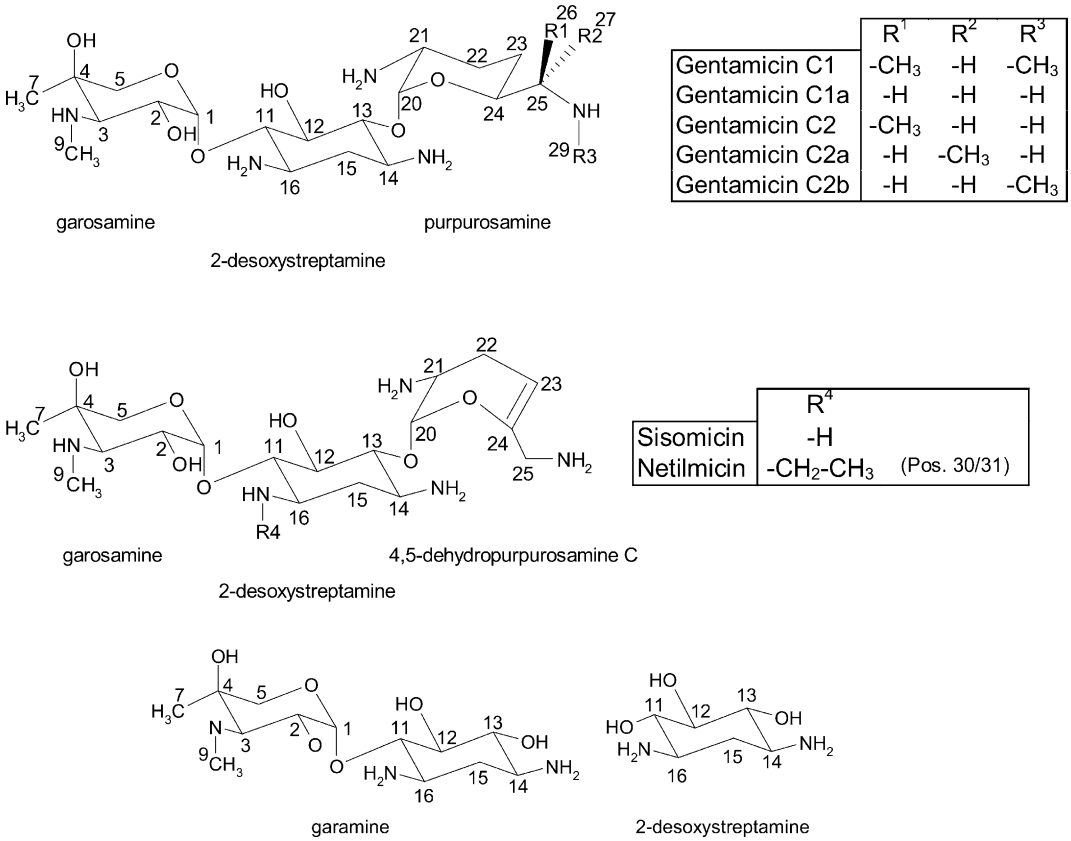

Fig. 1. Structural formulae of the gentamicin components and respective impurities.

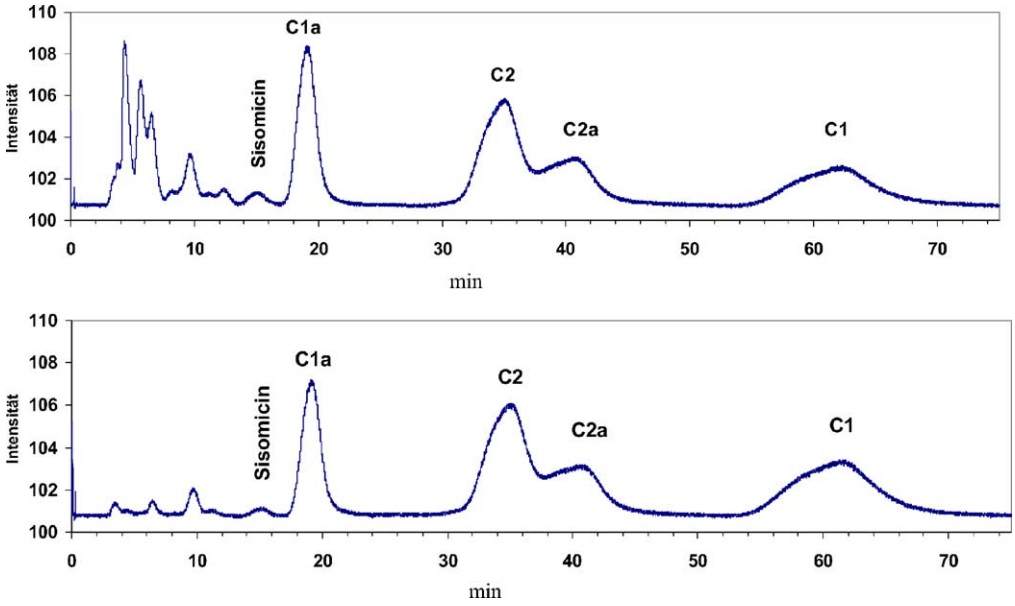

Fig. 2. HPLC chromatogram of a "dirty" (above) and a "pure" sample (below) obtained by the EP method.

from the drawback of a derivatization utilizing the *o*-phthaldialdehyde/thioglycolic acid system which was formerly used in the EP and which can be easily validated. This disadvantage was overruled by the high selectivity and efficiency of the method.

Due to the complexity of gentamicin the purpose of this paper was to check out whether NMR spectroscopy can serve as an orthogonal and complementary method which is able to verify the results obtained from MEKC. A special advantage of NMR spectroscopy is its suitability as a primary method of measurement. A primary method is defined as a method with the highest metrological qualities whose operation can be completely described and understood. Thus, the results can be accepted without referencing to a standard [8]. With other words, when using a primary method the amount of a substance can be determined directly from the physical context without referencing to another substance whose amount has to be determined independently. In NMR spectroscopy the interesting quantity is the signal-to-noise ratio, defined as the pure absorption mode signal divided by twice the noise. Both the absorption mode signal (as a lorentzian line) and the noise can be derived from fundamental constants and parameters specific to the sample and the spectrometer [9]. Due to this fact, the NMR spectroscopy can be used as a primary method of measurement if several aspects, such as ideal pulse angle and relaxation delay are considered [10].

The spectra of all components of gentamicin were assigned in a previous study [11]. Having this information in hands NMR spectra can be used to quantify the components in the mixture. The results will be compared to the findings of the MEKC method.

2. Experimental

2.1. Chemicals

Gentamicin sulfate (chemical reference substance) and sisomicin (chemical reference substance) were purchased from Promochem (Wesel, Germany), desoxystreptamine dihydrochloride and garamine hydrochloride were gifts from Merck (Darmstadt, Germany), netilmicin was a gift from Essex Pharma (Munich, Germany), and gentamicin C2b sulfate (sagamicin, micronomicin) was purchased from Pharm Chemical (Shanghai Lansheng Corporation, China). The gentamicin components C1, C1a, C2 and C2a were separated from a commercial sample of gentamicin sulfate as previously described [11]. The

commercial sample of gentamicin sulphate was a gift from Meiji Seika Kaisha Ltd. (Tokio, Japan).

OPA (for fluorescence, ≥99%), DChol (MicroSelect, ≥99%), picric acid, sodium tetraborate decahydrate (TB, 99.5%), and boric acid were purchased from Fluka/Riedel de Haen (Seelze, Germany), TGA (Reag. Ph. Eur., ≥99.0%), methanol (HPLC grade) and isopropanol from Merck (VWR-International, Darmstadt, Germany), acetonitrile (HPLC grade) from Carl Roth (Karlsruhe, Germany). β-CD was a gift from the Consortium für Elektrochemische Industrie (München, Germany).

2.2. NMR experiments

The first set of experiments was performed on a Bruker Avance 400 MHz NMR spectrometer, operating at 400.13 MHz (^{1}H), equipped with XWIN-NMR software (Version 3.0, Bruker Analytik GmbH, Rheinstetten, Germany) running on Microsoft Windows PC. For ^{1}H NMR spectra, about 15 mg of the gentamicin as mixture of components and about 10 mg of the single components, respectively, were dissolved in 650 μl deuterium oxide. For spiking experiments about 5 mg of each single compound was added to a solution of about 15 mg of gentamicin dissolved in 650 μl D_2O. 64 scans were collected into 64 k data points giving a digital resolution of 0.25 Hz per point. The spectral width was 8278 Hz, the transmitter offset at 6.15 ppm, the flip angle was 30°. Using an acquisition time of 3.96 s and an additional delay of 1 s a pulse repetition period of about 4.96 s results. Prior to Fourier transformation no zero filling was performed but an appropriate window function (exponential multiplication with a line broadening factor of 0.30 Hz) and manual phasing was applied.

The second set of experiments was performed on a Bruker DMX 600 MHz NMR spectrometer using the XWIN-NMR program package Version 2.0. For the spectra 256 scans with a frequency range of 6009.615 Hz were collected into 65536 data points, giving a digital resolution of 0.18 Hz per point. An appropriate window function was applied prior to Fourier transformation in order to enhance the spectral resolution. The samples were measured in D_2O at 300 K. The used flip angle was 90°. The pulse repetition period of 6.45 s consisted of 5.45 s acquisition time and 1 s additional delay.

2.3. Micellar electrokinetic chromatography

Apparatus: All CE experiments were carried out on a HP^{3D}-CE (Agilent Technologies, Waldbronn, Germany) equipped with a DAD detector. The capillaries were purchased from Polymicro (BGB Analytik, Schloßböckelheim, Germany). The fused-silica capillaries were of 50 μm internal diameter and effective length of 24.5 cm.

Buffers: The BGE was composed of 100 mM TB (pH10.0), 20 mM Dchol and 15 mM β-CD in ultrapure Milli-Q water. The samples were loaded by pressure injection applying 50 mbar for 5 s on the anode side and detection at 340 nm was performed at the cathode side. Electrophoresis was carried out at 25 °C and a voltage of 12 kV.

Derivatization: The samples were dissolved in high purity water solution (2.0 mg/ml) containing picric acid (IS, 7 mg/ml). OPA reagent: 650 mg of OPA were dissolved in 2.0 ml of methanol and approx. 15 ml of boric acid solution (pH 10.4, 30 mM). After ultrasonification the solution was adjusted to pH 10.4 using potassium hydroxide solution (8 M). Thioglycolic acid (1.300 ml) was added and pH was adjusted again to 10.4 with potassium hydroxide solution (8 M). This solution was diluted to 25.0 ml with boric acid solution (pH 10.4, 30 mM). Further details can be found in reference [7].

2.4. High performance liquid chromatography

The HPLC was performed according to the EP method [6] using a Merck Hitachi 2000 chromatograph (Merck, Darmstadt, Germany) equipped with a ED40 electrochemical detector (Dionex, Idstein, Germany) and a poly(styrene-divinyl-benzene) PLRP-S (Polymer, Shropshire, UK).

3. Results and discussion

NMR spectra have already been used for checking the identity and the composition of gentamicin by the DAB 9 [12]. For evaluation purposes Calam et al. [13] and Reuter et al. [14] inspected the integrals of the signals of the N–CH_3 and C–CH_3 groups at $\delta = 1.25$ and 1.35 ppm, and 2.75 and 2.95 ppm, respectively, using a 60 MHz spectrometer. Due to impurities both

regions turned out to be crowed in a 400 MHz spectrum. Additionally, the signals are not properly separated for precise quantification. In contrast, the signals of the anomeric hydrogens of the garosamine part and the purposamine part of the molecule are well separated, the latter perfectly mirroring the differences in the structure of all gentamicin components, i.e. the purposamine part. Moreover, between the signals of the anomeric hydrogens of the garosamine part and the purposamine part some additional signals appear in the samples showing a high number and high quantities of impurities in the electropherogram obtained from the MEKC method [7]. Thus, this part of the spectrum seems to be reasonable to be used for evaluation of components and impurities.

In the first step, the four signals of the anomeric purposamine hydrogens have to be assigned to the four main components (Fig. 3). Since the chemical shift can slightly change when changing from a single pure substance to a mixture, the assignment was performed by spiking the mixture with the each component individually. The order with decreasing chemical shift was found to be C2a, C1, C2 and C1a. However, the signal resulting from the anomeric hydrogen of minor component C2b was hidden. Even when using 600 MHz ^{1}H NMR spectra for better signal separation the signal of C2b kept hidden. In order to find the signal, the ratios between the integrals of the anomeric hydrogens were compared to the ratios of MEKC data of the corresponding gentamicin components in two ways. First, the proportions of the four major components C1, C1a, C2 and C2a were considered. From the MEKC data the relative proportions of these four components were calculated using the

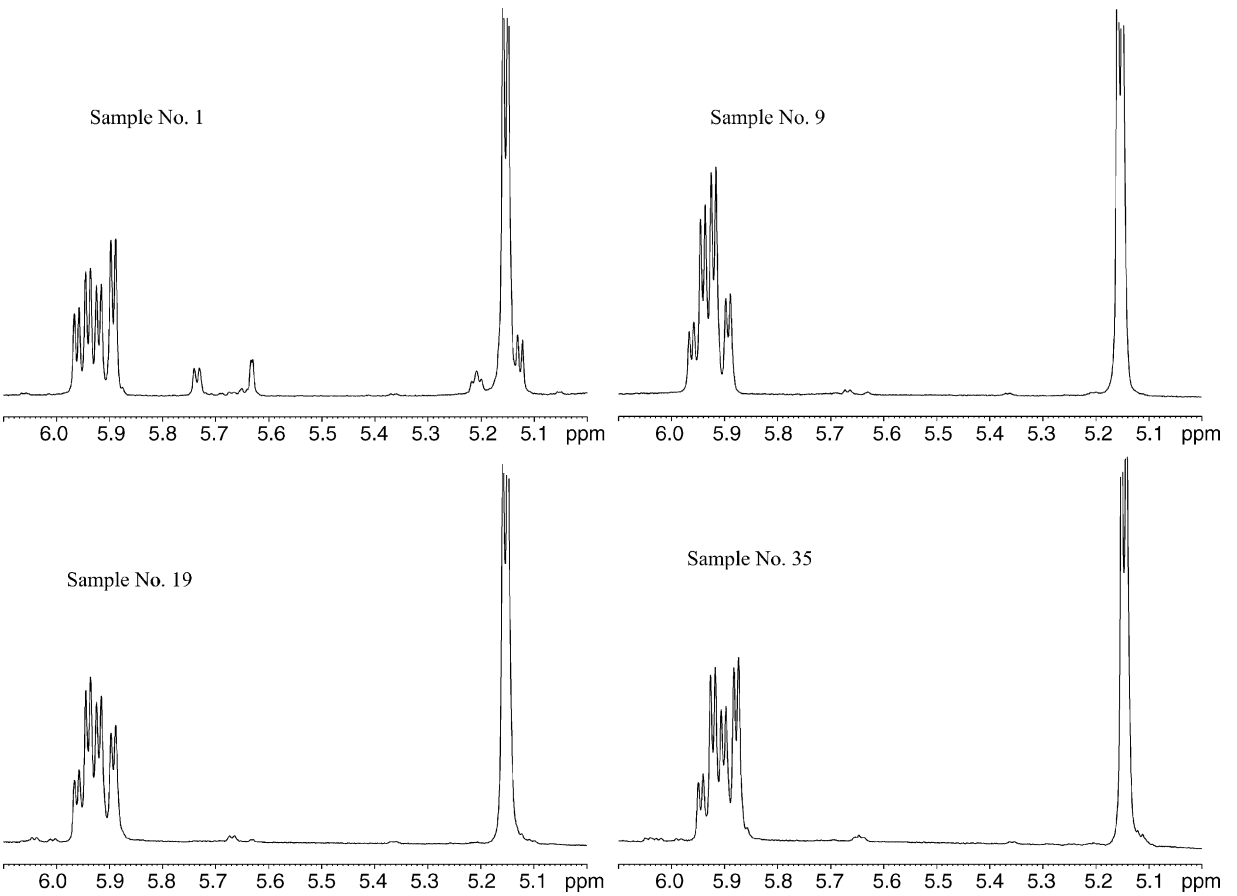

Fig. 3. Anomeric region of the ^{1}H NMR spectra of four representative lots of gentamicin.

normalization procedure and the chemical reference substance. These were compared to the relative proportions as calculated from the NMR spectra taking the MEKC-derived values as 100%. The mean values of the various lots show, that for components C1, C1a and C2 the NMR-derived values are lower then the MEKC-derived values. In contrast, the NMR-value for C2a is clearly higher than the MEKC-derived value. In the next step, gentamicin C2b was additionally considered. From the MEKC data the relative proportions of these five components were calculated. The relative proportions of gentamicin C2a and C2b were added and the sum of these was taken for comparison with the NMR value which was assigned to C2a (see Fig. 4). By doing this, the mean values of the relative proportions from NMR data of all signals show excellent

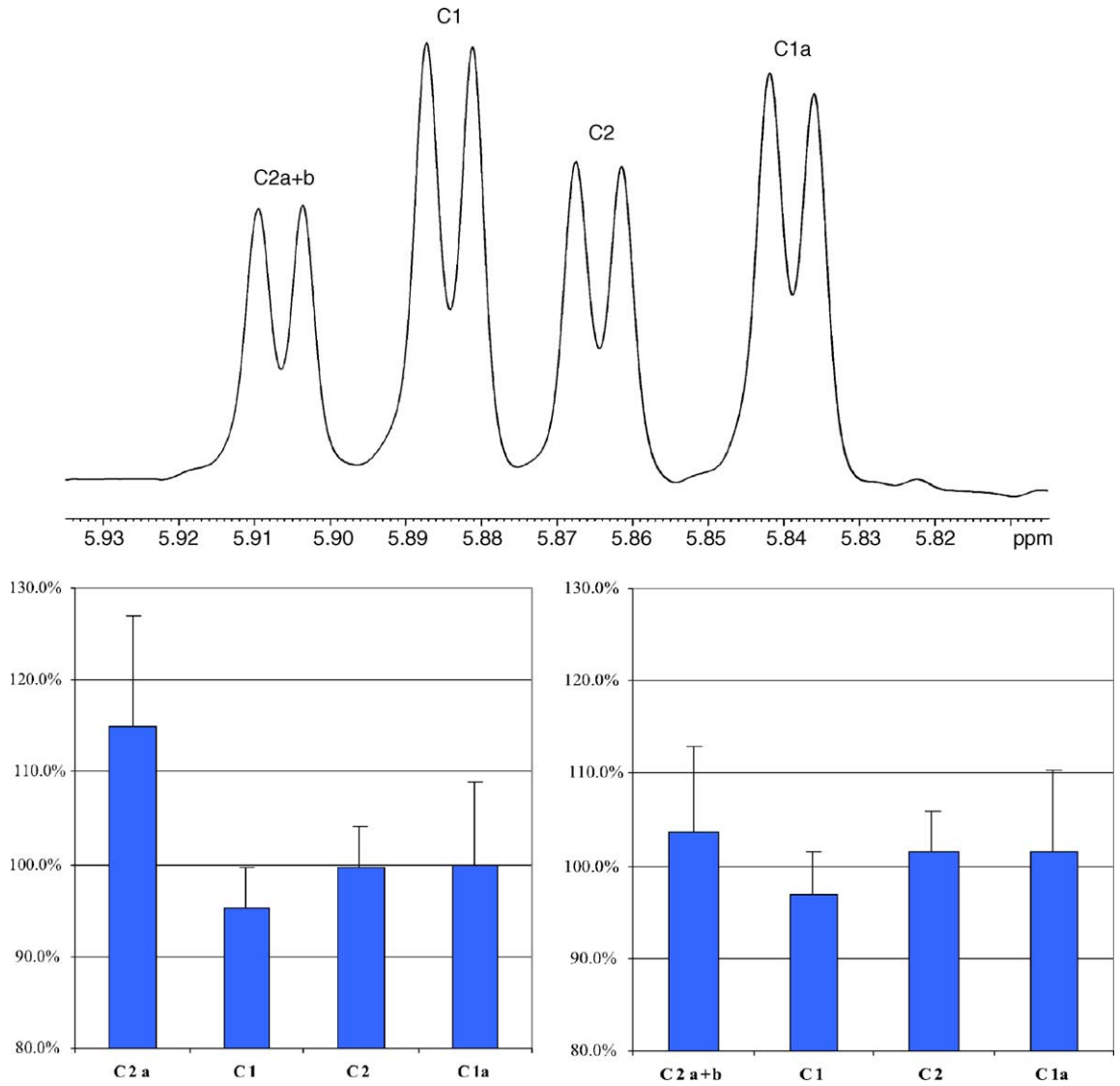

Fig. 4. Assignment of the signals of the gentamicin components C1, C1a, C2, C2a and C2b by comparison of the relative proportion found in NMR and MEKC (top, assignment of the signals of the anomeric hydrogens, left bottom without considering 2b and right bottom considering 2b hidden under signal of hydrogen 2a).

accordance with the MEKC data indicating two facts, first, the NMR signal of C2b is hidden under the signal of C2a which was additionally confirmed by spiking experiments and second, both methods are likely to be complementary.

Due to the fact that NMR spectroscopy is suitable as a primary method of measurement, the results derived from the NMR spectra directly represent the molar relations between the different components. No referencing to a standard and no derivatization procedure has to be applied. So these results should in fact represent the true molar ratios. The accordance with the MEKC evaluation by normalized peak heights suggests that this evaluation of the electropherograms should be the one of choice.

Fig. 3 displays the anomeric region of the NMR spectra of four different lots of gentamicin. The varying height and integrals of the four doublets nicely mirror the varying content of the four main components of the gentamicin lots. Having assigned the signals of the anomeric hydrogens of the main components the additional signals found in sample number 1 were checked out by spiking with netilmicin, sisomicin, desoxystreptamin and garamine. The signals at $\delta = 5.63$ and 5.20 ppm belong to the hydrogens of sisomicin or netilmicin, i.e. H20 and H23, respectively. Due to the structural similarity of sisomicin and netilmicin NMR spectroscopy is not capable of differentiating both. The signal close to the garosamine signal can be assigned to garamine. The signal at

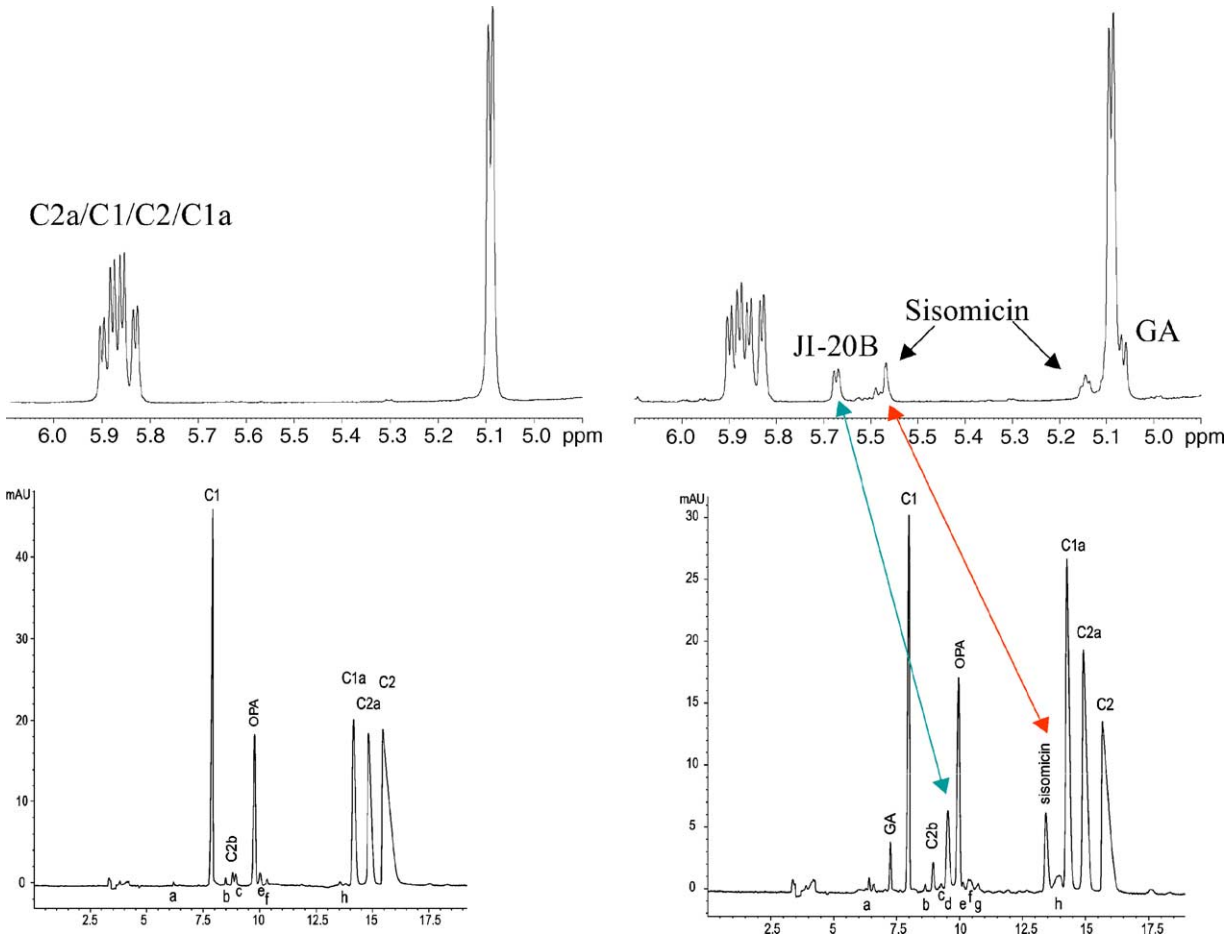

Fig. 5. Comparison of the NMR spectra and electropherograms obtained by MEKC of a pure and a dirty sample.

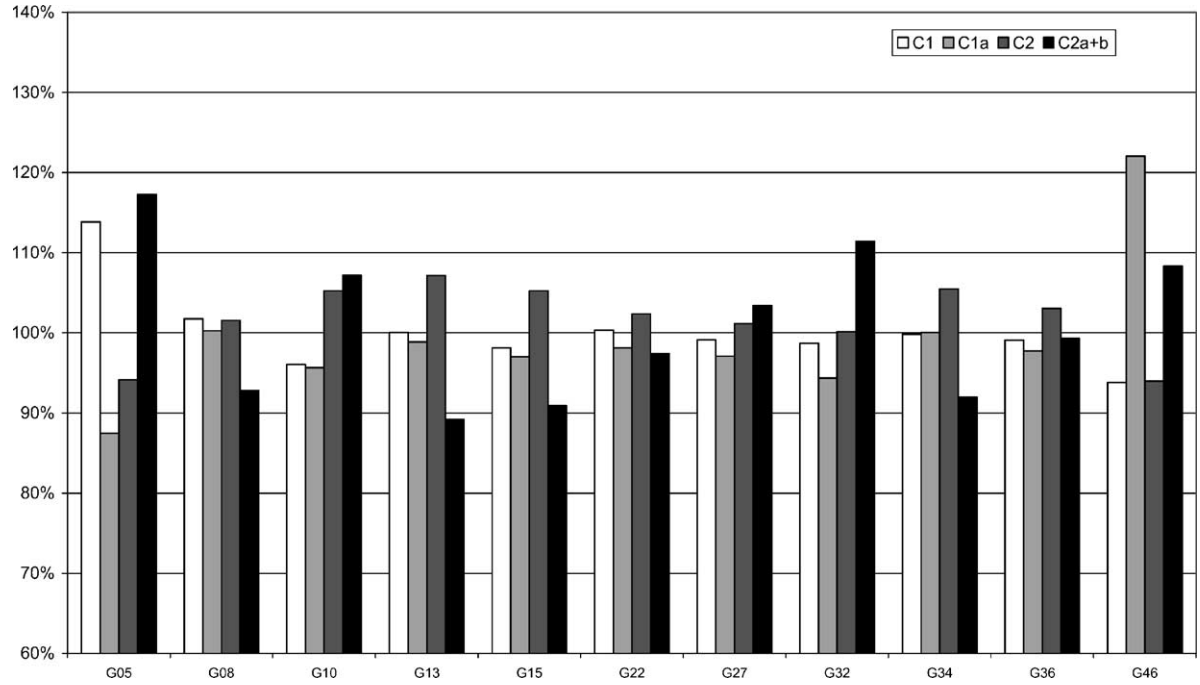

Fig. 6. Relative amount the main components of 11 representative lots of gentamicin evaluated by MEKC and NMR spectroscopy. The MEKC results are taken as true values and each column shows the proportion which could be found using the NMR spectra.

5.73 ppm characterized by almost the same integral as the sisomicin signal at $\delta = 5.63$ ppm may belong to JI-208 (=dihydroxy-gentamicin C2a). The impurities JI-20B and sisomicin often appear in similar amounts [3]. The comparison of peak "d" in the electropherograms with the peak of sisomicin, the comparison of the two signals in the NMR spectra and the comparison of the electropherograms to the NMR-spectra support this assignment (see Fig. 5). Since no reference substance is available it is impossible to confirm the suggestion.

Having assigned all signals in the region of the anomeric hydrogens in the NMR spectrum and the peaks found in the electropherogram of the MEKC the correspondence of both methods can be seen at a first glance by comparing the signal heights of the gentamicin components in the NMR spectra with the corresponding peaks obtained from MEKC (see Fig. 5).

In the next step, the results of the evaluation of the main components of some 40 gentamicin lots collected from the European and American market achieved with both methods were compared. As can be seen from Fig. 6 the findings are in good accordance indicating that the NMR and MEKC method are orthogonal. Organization of the lots of gentamicin in groups results in two main groups, one containing sisomicin and in connection with sisomicin a high number and high amounts of impurities and a sisomicin-free group characterized by a low number and amount of impurities. Examples of both groups are displayed in Fig. 5, the electropherogram and NMR spectrum of the sisomicin-free group on the left hand side and the electropherogram and NMR spectrum of the sisomicin-containing group on the right hand side. Thus, sisomicin seems to be a lead impurity. Both groups can be subdivided in number of subgroups of higher similarity in composition.

4. Conclusion

The MEKC method was found to be more robust than the HPLC method reported in PhEur 4.5 and the NMR spectroscopy was found to be a useful primary method orthogonal and complementary method to MEKC. Comparing the results of the MEKC and

NMR spectroscopy method the following conclusions could be drawn for the samples collected from the market. Two main groups were identified, one containing sisomicin and a huge number of impurities and a sisomicin-free group which turned out to be rather pure. The groups were divided in seven subgroups of similar composition pattern. Thus, the organization in groups previously found by means of capillary zone electrophoresis [15] and MEKC [7] was supported by the NMR results.

In addition, some patterns occurred in samples of different origin, and samples from same sources exhibited different patterns indicating different producers than may known to the MAA holder and known to the authorities. Thus, some samples are supposed to be counterfeit drugs.

Acknowledgements

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