Original Article

Mahidol University Journal of Pharmaceutical Sciences 2014; 41 (1), 1-10

Analysis of Biocidal Oligoguanidines and Oligobiguanides by High Performance Liquid Chromatogaphy and Mass Spectrometry

*T. Buchberger, M. Himmelsbach and W. Buchberger**
Institute of Analytical Chemistry, Johannes-Kepler-University Linz, Altenbergerstrasse 69, A-4040 Linz (Austria)

Abstract

Oligoguanidines and oligobiguanides are in general use as antiseptics and disinfectants. They consist of complex mixtures of oligomers with different structures and varying chain length, so that a thorough chemical characterization of these products remains challenging. In the present work, high performance liquid chromatography was investigated as a tool for separation of oligoguanidines obtained from polymerization of guanidine and a diamine, as well as of oligobiguanides obtained from hexamethylenebiscyanoguanidine and hexamethylenediamine. Efficient separations could be achieved under reversed-phase conditions using gradient elution with water (acidified by 0.1% trifluoroacetic acid) and acetonitrile. Structure elucidation of separated peaks was done by mass spectrometric detection. This approach was not only suited for characterization of products obtained under different synthesis conditions, but could also be successfully applied to residue analysis in environmental water samples after extraction and preconcentration by solid-phase extraction. Preconcentration factors up to 10000 were possible by mixed-mode sorbents exhibiting both reversed-phase and ion-exchange properties.

Keyword: Oligoguanidines; Oligobiguanides; Biocides; Liquid chromatography; Mass spectrometry

INTRODUCTION

Polycationic compounds like oligoguanidines or oligobiguanides (see Figure 1 and Figure 2) have become important biocides that may be widely used for infection control in medicine and in other areas where efficient disinfection is required[1]. The action of oligoguanidines and oligobiguanides consists in binding to the phospholipids of bacterial membranes. This binding destroys the homogenous distribution of phospholipids in membranes and leads to cellular leakage and loss of essential cellular components, followed by the death of the cell. Contrary to these phenomena, there is only little interaction with human and animal cells which provides significant safety during application. A major advantage of these polycationic biocides is the fact that the potential to induce the development of resistant bacteria strains is very low or even nonexistent.

Oligoguanidines are easily synthesized by polycondensation of guanidine hydrochloride and an aliphatic diamine like 1,6-hexamethylenediamine[2]. More recently, efforts have been made to synthesize analogous compounds based on different amines and to compare the antibacterial activities[3, 4]. Besides aliphatic diamines, polyetheramines (also called jeffamines) like 2,2´-(ethylenedioxy) bis(ethylamine) have been used as the amine component of oligoguanidines [5]. A main problem with the products of this synthesis reaction is the fact that a range of oligomers is generated which presents a challenge for comprehensive analytical characterization.

Oligobiguanides are typically synthesized from hexamethylenediamine and hexamethylenebiscyanoguanidine[6]. Similar to oligoguanidines, they consist of mixtures of different oligomers.

For investigations of the chemical structures obtained during the synthesis of

***Corresponding author:** Institute of Analytical Chemistry, Johannes-Kepler-University Linz, Altenbergerstrasse 69, A-4040 Linz, Austria
Phone: +43 732 2468 8724 Fax: +43 732 2468 8679 Email: wolfgang.buchberger@jku.at

oligoguanidines, matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS)[5-8] as well as electrospray ionization (ESI) MS4-9 have been used. These studies revealed several types of oligomers, including linear, cyclic and branched structures. The most important ones are shown in Figure 1. In case of polyhexamethylene biguanide, oligomers of six different linear structures could be identified which possess combinations of amine, cyanamine, guanidine or cyanoguanidine end groups (Figure 2) [10].

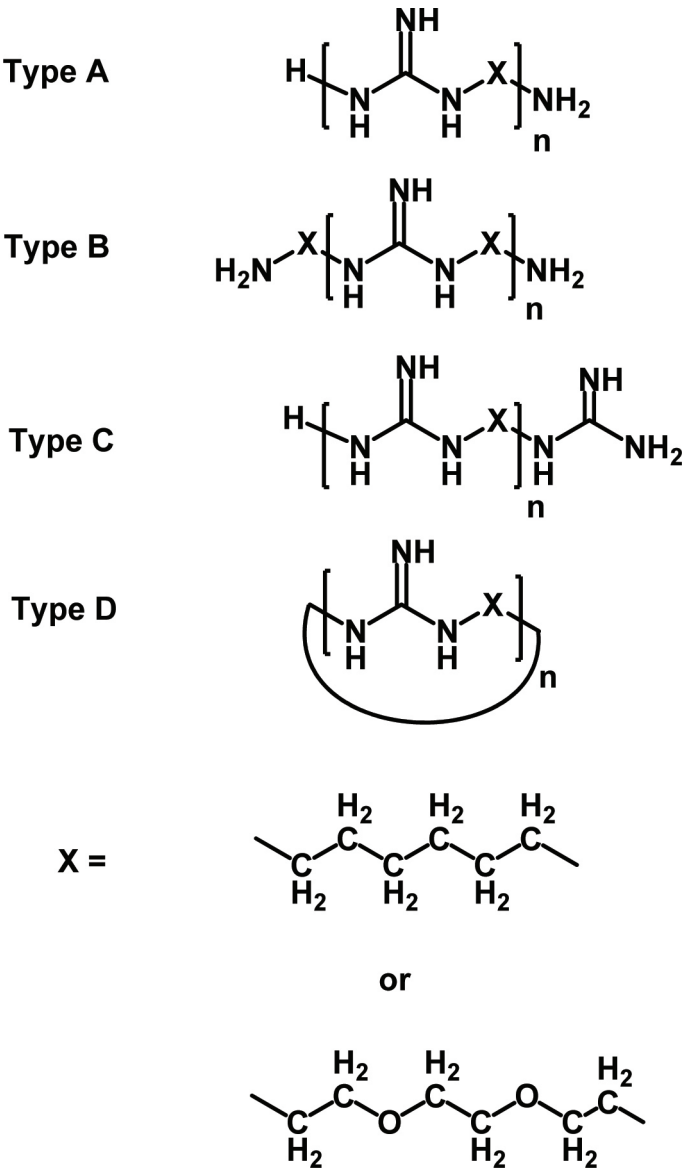

Figure 1. Typical structures of oligoguanidines obtained by polymerization of guanidine with hexamethylenediamine or 2,2´-(ethylenedioxy)bis(ethylamine).

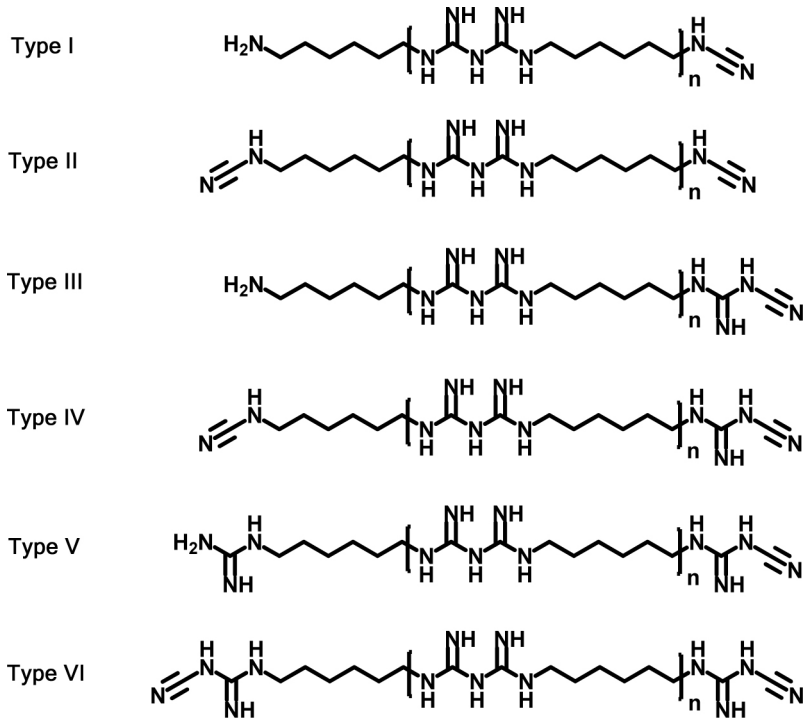

Figure 2. Typical structures reported in the literature [10] for polyhexamethylene biguanide.

Although the MS techniques mentioned above had turned out as most helpful in structure elucidation, a proper separation of the mixture of oligomers by chromatographic or electrophoretic techniques may be even more helpful for quantitative analysis of the components of a product obtained under certain synthesis conditions, especially when such separation methods are directly hyphenated with MS detection. Rudnev and Dzherayan[11] have been the first to demonstrate the potential of capillary electrophoresis for oligoguanidines. Such a technique had later been refined by Buchberger *et al*.[12], who also developed methods based on high-performance liquid chromatography (HPLC) in combination with MS. This technique allowed the identification of additional types of oligomers like species terminated by a hydroxy group, or linear strucures generated by formation of bonds between two amine molecules of the reaction mixture.

The present work intends to show the potential of HPLC with either UV or MS detection as a generic method for characterization of polymerization mixtures obtained under different reaction conditions or from different monomers. In addition, the application of this method for residue analysis of oligoguanidines in environmental samples after proper preconcentration is shown.

MATERIALS AND METHODS

Samples

Oligoguanidines were synthesized according to procedures reported in the literature[5] with slight modifications. 15 mmoles of guanidine hydrochloride (Sigma-Aldrich, Steinheim, Germany) together with 15 mmoles of an amine, namely 2,2´-(ethylenedioxy)bis(ethylamine) (Fluka, Buchs, Switzerland) or hexamethylenediamine (Sigma-Aldrich), were weighed into a 5 ml glass vial and heated to 160°C for periods up to 15 h. After cooling, the product was dissolved in water to give a stock solution of 250 g/l.

The same procedure was also used for synthesis of oligoguanidines containing three monomers, namely guanidine and both hexamethylenediamine and 2,2´-(ethylene-

dioxy)bis(ethylamine). In this case, a molar ratio of 2:1:1 of the three monomers was selected for the synthesis.

As an example for oligobiguanides, a commercially available antiseptic solution containing 0.02% of polyhexamethylene biguanide hydrochloride (PHMB) was used.

In all these cases, the samples were directly injected onto the HPLC column after appropriate dilution.

For residue analysis in environmental water samples, surface water from the river Danube was collected, filtered through 1.5 µm glass microfiber filters (VWR International, Radnor, PA, USA), and buffered by addition of 10 ml 4 M phosphoric acid to 1 l sample and adjusting the pH to 4 by adding aqueous sodium hydroxide. Subsequently, a solid-phase extraction (SPE) step was performed. A polymeric mixed-mode sorbent containing weak cation exchange groups (Strata-X-CW, 500 mg cartridge, from Phenomenex, Aschaffenburg, Germany) was used. The sorbent was pretreated with 10 ml methanol followed by 10 ml of high-purity water. Then 1 l of the pH-adjusted surface water sample was applied to the cartridge. Afterwards, a washing step with 5 ml methanol / water (50 / 50, v/v) was done. For elution of the preconcentrated analytes, 5 ml of 2 M hydrochloric acid in methanol were used. The eluate was brought to dryness and reconstituted in 100 µl of high purity water for injection onto the HPLC column.

Instrumentation for HPLC and MS

Separations were performed on an 1100 HPLC system (Agilent, Palo Alto, CA, USA) equipped with vacuum degasser, quaternary pump, autosampler (50 µl injection volume), and UV-visible diode array detector. MS detection was performed with a 6510 quadrupole / time-of-flight (QTOF) instrument (Agilent) or a 6420 triple quadrupole (QqQ) instrument (Agilent), both with electrospray ionization in the positive mode.

The chromatographic column was a ODS-AQ 3 µm from YMC (Schermbeck, Germany) with a length of 250 mm and an inner diameter of 3 mm. Gradient elution was done with a binary gradient consisting of aqueous 0.1 % (v/v) trifluoroacetic acid (Merck, Darmstadt, Germany) and acetonitrile (HPLC gradient grade from J.T. Baker, Deventer, The Netherlands) at a flow rate of 0.4 ml min^{-1} and a temperature of 25°C.

RESULTS AND DISCUSSION

HPLC with UV or MS detection for characterization of oligoguanidines

Reversed phase stationary phases such as C18-modified silica were used for the separation of oligoguanidines and oligobiguanides. These analytes show good water solubility and therefore seem less suited for reversed phase separation due to low retention times. Nevertheless, the addition of a small amount of trifluoroacetic acid to the mobile phase consisting of mixtures of water and acetonitrile increased the retention sufficiently by the formation of ion pairs with the positively charged analytes. Generally, the performance of C18-modified silica may depend on the surface chemistry used for the preparation of the material. Unfortunately, sufficient information about the surface chemistry is often not available from manufacturers so that selection of stationary phases must be done by a trial-and-error approach. In the present case, materials with a polar endcapping yielded the best performance.

The number of peaks fully separated in the chromatogram was strongly dependent on the gradient composition of the mobile phase. Generally, the separation required a start of the gradient at 100 % water (containing 0.1% trifluoroacetic acid) and 0% acetontrile. The slope of the gradient needed to be optimized depending on the analytical requirements (such as separation of all oligomers as comprehensively as possible, or separation of a limited set of compounds to obtain a pattern typical for a certain product, or even separation of only three or four main components used as marker compounds). A typical gradient suited for separation and structure elucidation of a large number of oligomers included an isocratic period of 5 min with 100 % of aqueous 0.1 % trifluoroacetic acid, followed by the increase of the acetonitrile content at

a rate of 0.2 % per minute. Together with a flow rate of 0.4 ml/min (selected for reasons of compatibility with an MS detector) a run time of more than 2 h may be required for the separation, depending on the specific type of oligoguanidines. On the other hand, a separation performed for residue analysis of oligoguanidines in environmental samples may be based on a much steeper gradient (resulting in significant lower analysis time) because it is sufficient to quantitate a few representative main compounds of the product. In case of routine product control, a less expensive UV detector operated at 200 nm may be sufficient instead of an MS detector once the identity of the different peaks has been established.

A typical HPLC separation obtained for a polymerization product of the polyetheramine 2,2´-(ethylenedioxy)bis(ethylamine) and guanidine is shown as chromatogram 1 in Figure 3. Structure elucidation of the peaks was done by QTOF MS detection. The labelling of the peaks in chromatogram 1 in Figure 3 is done in the way that for example A2 means an oligomer of type A according to Figure 1 with n=2. Oligomers with n up to approximately 10 could be identified. This is the typical range of polymerization degrees mentioned in the literature for oligoguandidines[7]. It is fair to say that despite the very flat gradient of the mobile phase the resolution of minor components with n higher than 10 was no longer sufficient and did not allow a structure elucidation by the mass spectra. Here the method clearly reaches the limits of its capabilities. Analysis of samples obtained after different polmerization times (from 2 to 15 h) indicated that increased reaction times lead to an increase of larger linear oligomers and a decrease of shorter oligomers (as can be expected), whereas the amount of cyclic products remained constant after a reaction time of approximately 2 h.

The MS spectra used for identification of the oligomers typically showed singly charged ions for compounds with up to two or three subunits, and doubly charged ions for compounds with three to five subunits. With higher n, the charge number was also increased.

Chromatogram 2 in Figure 3 demonstrates the applicability of the same chromatographic method for a product obtained from a different amine component, namely hexamethylenediamine, and guandine. The labelling is done in the same way as for chromatogram 1. A comparison of the two chromatograms leads to the conclusion that similar distributions of the various analogous oligomers were obtained in both cases, although some minor differences do exist, such as the occurrence of the cyclic structure D which is mainly found in the product from the polymerization with the polyetheramine.

Finally, chromatogram 3 in Figure 3 shows an even more complex mixture, namely the product from the polymerization of guanidine and a mixture of hexamethylenediamine and 2,2´-(ethylenedioxy)bis (ethylamine). Some of the peaks identified by QTOF MS detection are labelled in a way that A, B, C, and D again refer to the type of structures given in Figure 1. The first number in the bracket shows the number of polyetheramine units in the structure, and the second number shows the number of hexamethylenediamine units in the structure.

HPLC with UV or MS detection for characterization of oligobiguanides

As mentioned in the introduction, in practice biocidal oligobiguanides are used as an alternative to oligoguanidines. Structures so far suggested for polyhexamethylene biguanide based on MALDI-MS measurements without chromatographic separation[10] are shown in Figure 2. It appeared interesting to apply the same analytical approach used for oligoguanidines also to oligobiguanides. As it can be seen in Figure 4, a range of oligomers could be separated. Various peaks could be identified and correlated with the structures suggested so far in the literature and given in Figure 2. These peaks are labeled in a way that for example III1 means type III with n=1. In addition, a few other peaks (labeled as a, b, c, d, and e) could be found corresponding to new structures that are listed in Figure 5.

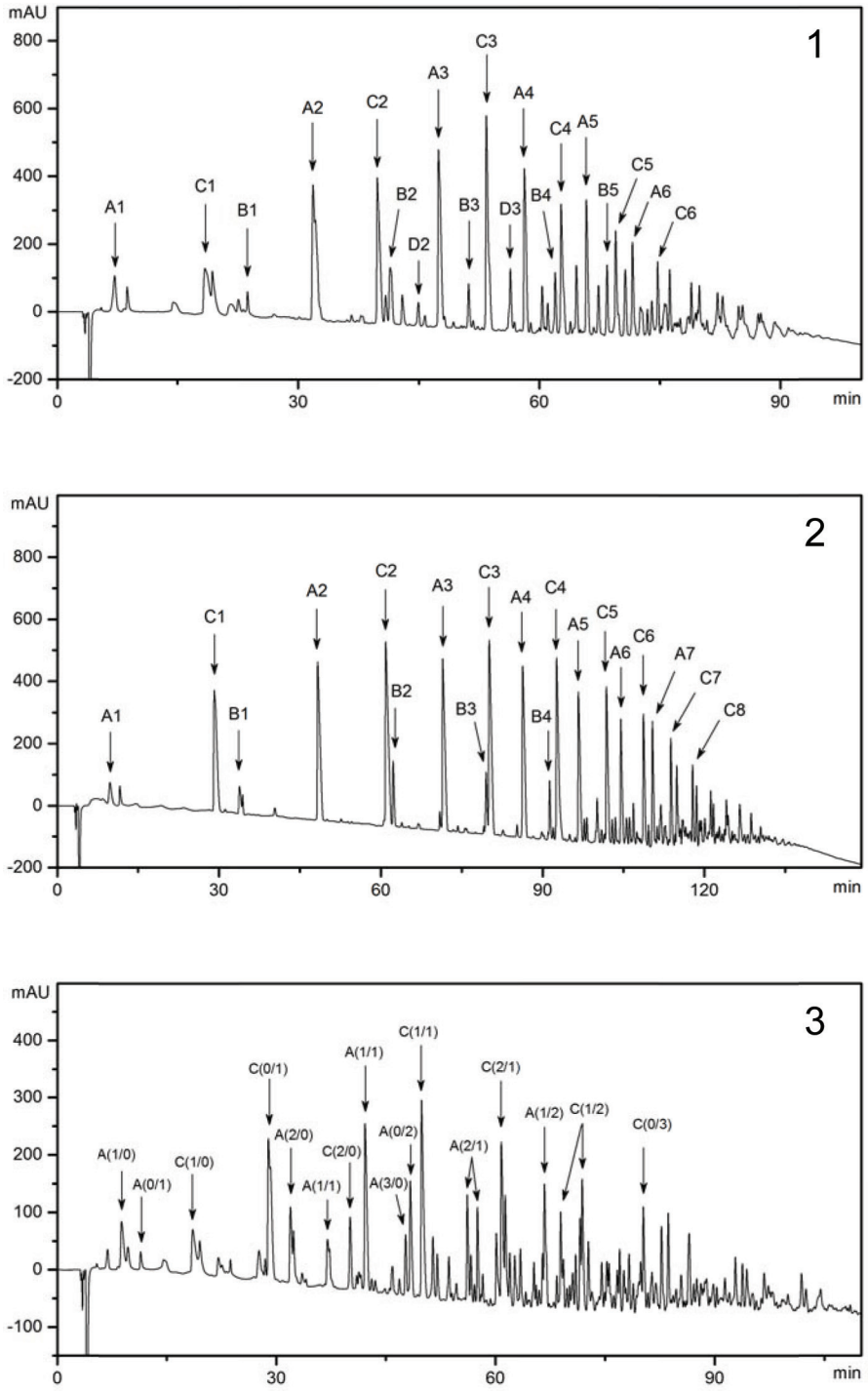

Figure 3. HPLC separations of oligoguanidines obtained by polymerization of guanidine and 2,2′-(ethylenedioxy)bis(ethylamine) (chromatogram 1), guanidine and hexamethylenediamine (chromatogram 2), and guanidine and both 2,2′-(ethylenedioxy)bis(ethylamine) and hexamethylenediamine (chromatogram 3). For peak assignment see discussion in the text.

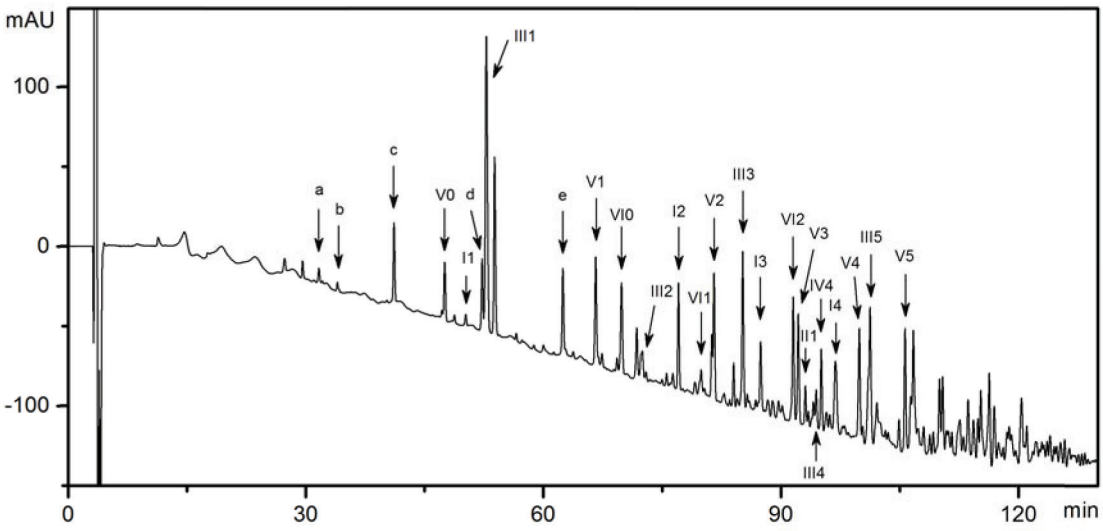

Figure 4. HPLC separation of polyhexamethylene biguanide. For peak assignment see discussion in the text.

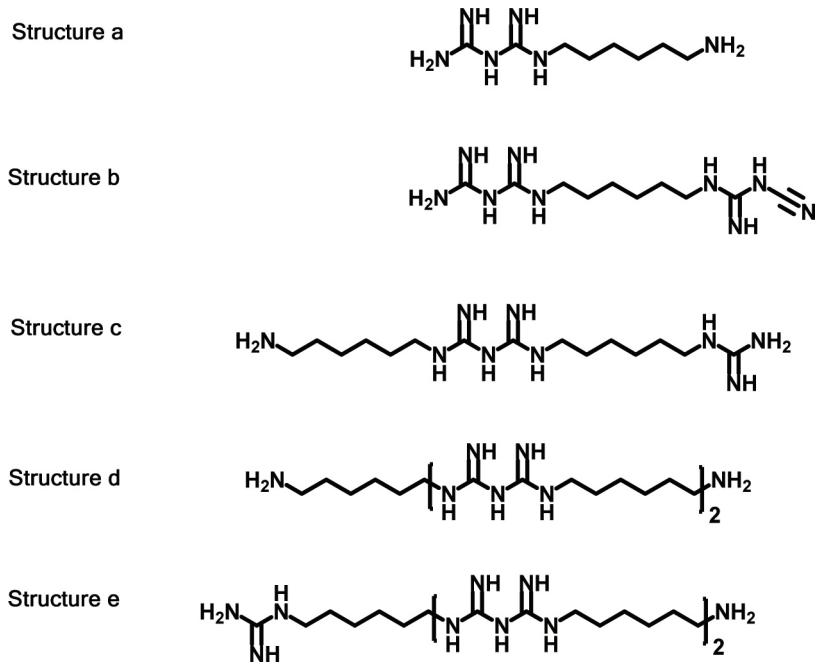

Figure 5. Structures of components of polyhexamethylene biguanide found besides those reported in the literature.

Residue analysis in environmental water samples

Nowadays it is well-known that residues of pharmaceutials and personal care products may turn up in the environment. The use of oligoguanidines and oligobiguanides as disinfectants and antiseptics may lead to the release of these compounds into natural aquatic systems. Recent studies indicated that polyhexamethylene biguanide is quite stable in surface water samples[13]. Up to now, adequate analytical methods have not yet been established for quantitative analysis at sub-µg level. In this context, it seemed necessary to investigate appropriate preconcentration strategies for water samples. Solid-phase extraction is nowadays one of the most common techniques to achieve efficient preconcentration of xenobiotics from aqueous sample.

Preliminary experiments with reversed-phase SPE sorbents showed insufficient performance for oligoguanidines and oligobiguanides, especially for those with lower molecular mass. Therefore, mixed-mode phases were employed that exhibited both reversed-phase and cation-exchange properties. It turned out that weak cation-exchange functionalities (carboxylate groups) were well suited with respect to the elution step after the sample loading. An acidic eluent will protonate the carboxylate group and decrease the exchange capacity, thereby allowing easy elution by 2 M HCl in methanol. Such conditions allowed the preconcentration of 1 l of water samples (larger volumes have not yet been tried) with recovery better than 90 %. A preconcentration factor of 10000 is possible if the eluate from the SPE procedure for 1 l water sample is brought to dryness and reconstituted in 0.1 ml solvent.

For environmental water samples it is less interesting to quantitate every oligomer that may be present in a biocidal product. Instead, a more straightforward approach is the selection of a few components of the oligomeric mixture as marker compounds representative for the whole mixture. In case of the product of 2,2´-(ethylenedioxy)bis (ethylamine) and guanidine, the components A2 and A3 as well as C2 and C3 (see chromatogram 1 in Figure 3) were selected. For separation of this limited number of analytes, a much steeper gradient with an increase of acetonitrile of 2 % per min was used. The chromatographic system was hyphenated with a QqQ mass spectrometer which is nowadays state-of-the-art for quantitative trace analysis. The transitions used for QqQ measurements were the following m/z values: 182.6 / 174.2 for A2, 269.2 / 174.2 for A3, 203.6 /174.1 for C2, and 290.3 / 157.1 for C3. A chromatogram of a surface water sample spiked at a total concentration of 2.5 µg/l is shown in Figure 6.

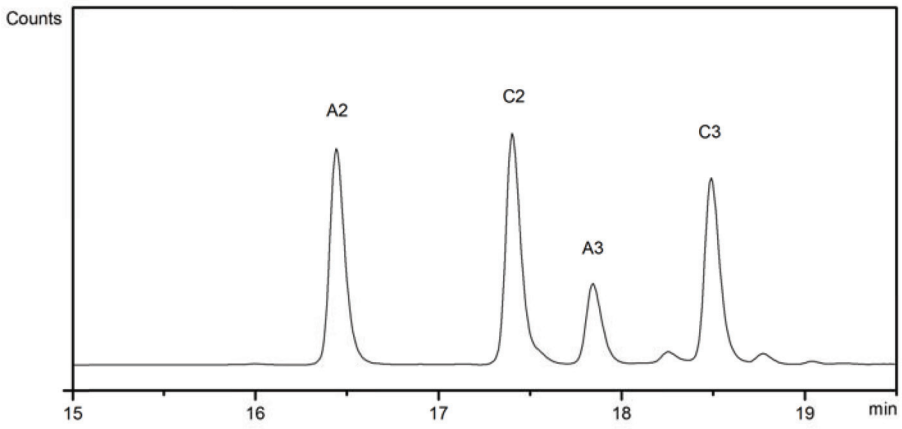

Figure 6. HPLC separation with QqQ MS detection of a surface water sample spiked with 2.5 µg/l of a mixture of oligoguanidines (synthesized from guanidine and 2,2´-(ethylenedioxy) bis(ethylamine)) and preconcentrated by solid-phase extraction. MS transitions belonging to the components A2, C2, A3, and C3 were recorded.

It should be kept in mind that this spiking concentration refers to the total amount of the mixture of oligomers. The exact concentrations of the components A2, A3, C2, and C3 in the mixture are unknown and cannot be readily determined because pure standards of the different oligomers are not available. The true spiking concentrations of the four analytes may be one to two orders of magnitude lower than the total concentration given above. Detection limits in the analysis solution after the preconcentration step are estimated to be around 0.025 mg/l of total concentration. It is fair to say that the high preconcentration factor resulted in suppression effects in ESI (that are generally well known) caused by matrix components, which decreased peak intensities depending on the type of water samples. Such effects may deteriorate the detection limits by a factor of 2.

The same preconcentration strategy may be applied to other oligoguanidines and oligobiguanides, although the full range of possible applications has not yet been completely exploited so far.

CONCLUSIONS

Within this work, it was possible to establish generic reversed phase HPLC conditions for the chemical characterization of biocidal oligoguanidines and oligobiguanides. Hyphenation with ESI MS allows a reliable elucidation of the structures of different oligomers present in products obtained from the polymerization reaction. For routine quality control of commerical products, UV detection at 200 nm will be sufficient. In the future, it may be interesting to put the focus on the quantitation of single oligomers in a product, which has not yet been achieved due to lack of pure standard compounds.

REFERENCES

1. Gilbert P, Moore LE. Cationic antiseptics: diversity of action under a common epithet, *J Appl Microbiol* 2005; 99: 703-715.
2. Zhang Y, Jiang J, Chen Y. Synthesis and antimicrobial activity of poylmeric guandidine and biguanidine salts, *Polymer* 1999; 40: 6189-6198.
3. Zhou Z, Wei D, Guan Y, *et al*. Extensive *in vitro* activity of guanidine hydrochloride polymer analogs against antibiotic-resistant clinically isolated strains, *Mat Sci Eng C* 2011; 31: 1836-1843.
4. Wei D, Guan Y, Ma Q, *et al*. Condensation between guanidine hydrochloride and diamine/multi-amine and its influence on the structures and antibacterial activity of oligoguanidines, *e-Polymers* 2012; 12: 848-857.
5. Feiertag P, Albert M, Ecker-Eckhofen E, *et al*. Structural characterization of biocidal oligoguanidines, *Macromol Rapid Commun* 2003; 24: 567-570.
6. Kaehn K. Polihexanide: A safe and highly effective biocide *Skin Pharmacol. Physiol* 2010; 23:(suppl) 7-16.
7. Albert M, Feiertag P, Hayn G, *et al*. Structure-activity relationships of oligoguanidines - influence of counterion, diamine, and average molecular weight on biocidal activities. *Biomacromol* 2003; 4: 1811-1817.
8. Hwang HJ, Nam J, Yang SI, *et al*. MALDI-TOF analysis of polyhexamethylene guanidine (PHMG) oligomers used as a commercial anti-bacterial humidifier disinfectant, *Bull. Korean Chem Soc* 2013; 34: 1708-1714.
9. Wei D, Ma Q, Guan Y, *et al*. Structural characterization and antibacterial activity of oligoguanidine (polyhexamethylene guanidine hydrochloride), *Mat Sci Eng C* 2009; 29: 1776-1780.
10. O´Malley LP, Hassan KZ, Brittan H, *et al*. Characterization of the biocide polyhexamethylene biguanide by matrix-assisisted laser desorption ionization time-of-flight mass spectrometry, *J Appl Polym Sci* 2006; 102: 4928-4936.
11. Rudnev AV, Dzherayan TG, Determination of polyhexamethyleneguanidine by capillary electrophoresis, *J Anal Chem* 2006; 61: 1002-1005.

12. Buchberger WW, Hattinger I, Himmelsbach M, Characterization of mixtures of biocidal oligoguanidines by capillary electrophoresis and high-performance liquid chromatography coupled to mass spectrometry, *J Chromatogr A* 2009; 1216: 113-118.
13. Lucas AD, Environmental fate of polyhexamethylene biguanide, *Bull Environ Contam Toxicol* 2012; 88: 322-325.