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HPLC-MS/MS of Highly Polar Compounds

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1. Introduction

Complicate high-performance liquid chromatographic (HPLC) interfaces like the moving belt, direct liquid interface, thermospray (Arpino, 1985) fast atom bombardment (FAB) (Caprioli et al., 1986; Garcia & Barceló, 1993; Dell et al., 1988; Larry et al., 1989) characterized the early days of HPLC used in tandem with mass-spectrometry (MS); all of them, apart FAB, were more effective with moderately polar compounds (Strege et al., 1999). After years of difficulties and struggles the introduction of atmospheric pressure ionization by electrospray (ESI) or chemical ionization marked a clear breakthrough: an effective, robust and user friendly HPLC interface was finally available.

As a matter of fact, considering the physicochemical principles of the source, the polar compounds were the first compounds successfully ionized (Griffits, 2008).

Despite the huge potential of ESI for polar and highly polar compounds the applications for the latter proved to be critical due to the fact that effective HPLC separations often require non volatile buffers and/or high water content that are not suitable in MS detection.

Along the years, the development of HPLC columns, specific derivatization methods, new chromatographic techniques and sample preparation approaches have increased the number of applications with highly polar compounds. Three classes of not so widely studied molecules have been selected in this study in order to illustrate the application of HPLC-MS/MS to highly polar compounds; updates as well a review of past data will be presented.

The groups of compounds taken in consideration are: glycosaminoglycans, bisphosphonates and aminoglycoside antibiotics; the first-ones are both endogenous and exogenous while the last 2 classes are of typical pharmaceutical interest.

Obviously other more well-known molecules like peptides and polynucleotides are also included in this class of highly polar compounds but HPLC-MS/MS methods in these particular fields are widely described and probably represent the most common areas of application. It is also important to keep in mind that this chapter doesn't intend to be an exhaustive presentation of the published analytical methods for heparins, bisphosphonates or aminoglycosides, but these compounds are used as models to emphasize the potential of HPLC-MS/MS in the analysis of highly polar molecules.

2. Glycosaminoglycans

2.1 Chemical structure and main features of glycosaminoglycans

Glycosaminoglycans (GAGs) are a family of highly sulphated, complex, polydispersed linear polysaccharides that display a variety of important biological roles. They contain amino-sugars with alternating disaccharide units, which comprise an acidic monosaccharide such as glucuronic or iduronic acid and a basic monosaccharide such as N-acetylglucosamine or N-acetyl-galactosamine; both monosaccharides can be sulphated. In natural biological systems GAGs are mainly linked to proteins to form proteoglycans, with great interest for medical science being structural proteins or receptor as well adhesion molecules (just to mention a few examples) and can be categorized, based on the GAGs structure, into three main structural groups: heparin/heparan sulphate group, dermatan/chondroitin sulphate group and hyaluronan (Mao et al., 2002; Tiayu Peng, 2002). *Heparin and heparan sulphate* occur as proteoglycans in which the glycosaminoglycan chains have different lengths and are composed of disaccharide units consisting of L-iduronic or D-glucuronic acids $\alpha(1-4)$ linked to glucosamine units that can be N-acetylated or N-sulphated (Figure 1).

Heparin and heparan sulphate have closely related structures and consist of similar disaccharide composition except that heparin has a greater content of iduronic acid and is more highly sulphated per polysaccharide chain than is heparan sulphate. The differences between the chemical structure of the sequences of heparin and heparan sulphate explain the biological function related to them. As an example, the specific interaction of heparin with the protein antithrombin III, determining its anticoagulant activity, is mediated by a characteristic pentasaccharide that is not normally present in heparan sulphate and it accounts for just a few percent of the heparin mass (Saad & Leary, 2003).

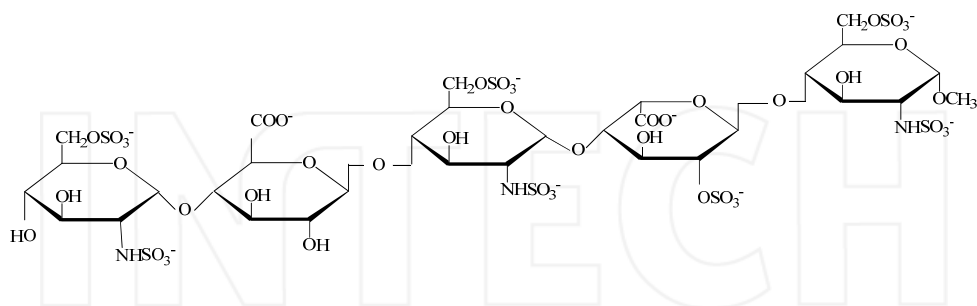
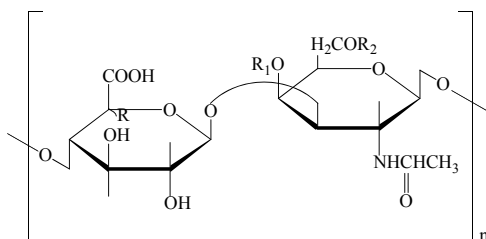


Fig. 1. Main sequence structure of heparin/heparan sulphate.

The *dermatan/chondroitin sulphate* are the main components of connective tissues and the chemical structure comprises alternating uronic acid and N-acetyl-galactosamine residues; depending on their composition 3 main types of chondroitin sulphates have been identified (Gunay & Linhardt, 1999). The structures of these types of chondroitin sulphate are presented in Figure 2.



Chondroitin Sulphate Type A: R=H, R₁=SO₃H, R₂=H, R₃=COOH

Chondroitin Sulphate Type B: R=COOH, R₁=SO₃H, R₂=H, R₃=H

Chondroitin Sulphate Type C: R=H, R₁=H, R₂=SO₃H, R₃=COOH

Fig. 2. Structure of chondroitin sulphate.

Chondroitin sulphate A and chondroitin sulphate C contain D-glucuronic acid, N-acetylgalactosamine and sulphate residues in equimolar quantities but differ in the position of the sulphate ester group (Saito et al., 1968). Dermatan sulphate formerly called chondroitin sulphate B is similar to chondroitin sulphate A but instead of D-glucuronic acid it consists mainly of L-iduronic acid.

Hyaluronic acid has alternating repeating units of the structure, (1→3) β-N-acetyl-D-glucosamine (1→4) β-D-glucuronic acid, but typically it is not sulphated and the molecules are larger than the other glycosaminoglycans, often several million Da, with special rheologic characteristic (Mao et al., 2002).

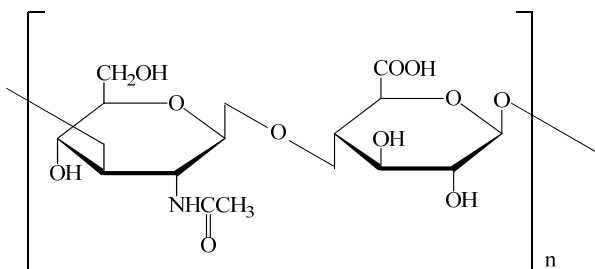


Fig. 3. Structure of the repeating units of Hyaluronic acid.

2.2 Special problems of these molecules and potential approach to solve them

GAGs are extremely difficult to analyze because of their polydispersity and microheterogeneity of chemical structure combined with high polarity. The first two aspects (polydispersity and microheterogeneity) push to get an optimal separation of all species presented in a sample in order to accurately characterize each component. It is in fact important to consider that these molecules are closely related polymers with common disaccharides/oligosaccharides and if not well separated it is also impossible to get detailed structure information.

In the meantime the high polarity of GAGs make them difficult compounds for optimal chromatographic separation and an adequate separation of all components present in a GAGs sample is virtually impossible.

In such conditions two approaches are feasible:

1. Application of analytical techniques able to gather structure data also of unfractionated or partially fractionated complex mixtures
2. Structure specific degradation of the GAGs in order to reduce such complex samples with almost infinite variant of similar polymers to more discrete mixtures of oligomers corresponding to their “building blocks” that can be properly analyzed and quantified obtaining quite detailed information of the polymers themselves intended as a mean structure or a fingerprint.

Regarding the first approach, only NMR permits to get in-depth chemical structure information of almost unfractionated materials but the instrumental sensitivity is a major drawback when studying limited biological samples and in any case the information obtained are practically a mean of the individual structures of the components present in the sample. Despite the fact that hyphenation of NMR and HPLC (as separation method) is feasible, due to sensitivity problems its application on such complex samples is more theoretical than practical so far.

Before to close this parenthesis on NMR, outside the scope of this chapter, it is evident that NMR spectroscopy is also a very effective technique for the characterization of the oligosaccharides (Yung, 2011). It gives valuable information on monosaccharide composition, glycosidic linkage, uronic acid type and sulfation patterns (Bo et al., 2011), but it requires always relatively large amount of material and is not suitable for the analysis of very small samples, like most biological samples. By coupling capillary isotachopheresis with on-line microcoil NMR detection (CITP-NMR) the sensitivity for the characterization of heparin - derived oligosaccharides has been improved (Korir & Larive, 2007).

Considering the limitations related to this first analytical approach, based on intact molecules, the focus in the next paragraphs will be on presenting methods to analyze GAGs following depolymerization; clearly that in rare cases of small oligosaccharide (i.e. from synthesis) or highly purified fractions such step can be avoided.

2.3 Overview on methods of GAGs depolymerization

As previously pointed out most often the structure characterization of GAGs involves degradation steps to obtain disaccharides and/or a range of oligosaccharide fragments allowing an efficient disaccharide/oligosaccharide mapping (Bo et al., 2011). These structural analyses of GAGs may involve complete or partial chemical degradation, as well as enzymatic degradation permitting a quite selective cut of the polysaccharide chains (Ruiz-Calero et al., 2003; Gatti et al., 2010). Highly specific enzymes have been isolated and they are now commercially available making their application viable (Johnson, 1982; Jandik et al., 1996; Cohen & Linhardt, 1990). In figure 4 the main used enzymes are shown.

An important aspect of these enzymes is the high specificity for the substrate, fact that if it is welcomed in biochemical studies can be critical sometimes when analyzing semisynthetic derivatives.

Chemical degradation is a very interesting alternative to enzymatic degradation giving also quite specific structure information. The degradation with nitrous acid and the periodic oxidation followed by Smith degradation are two classical tools for GAGs analysis.

In several cases depolymerization methods are followed by derivatization procedures generally to enhance the analytical detection when using conventional HPLC or CE detectors.

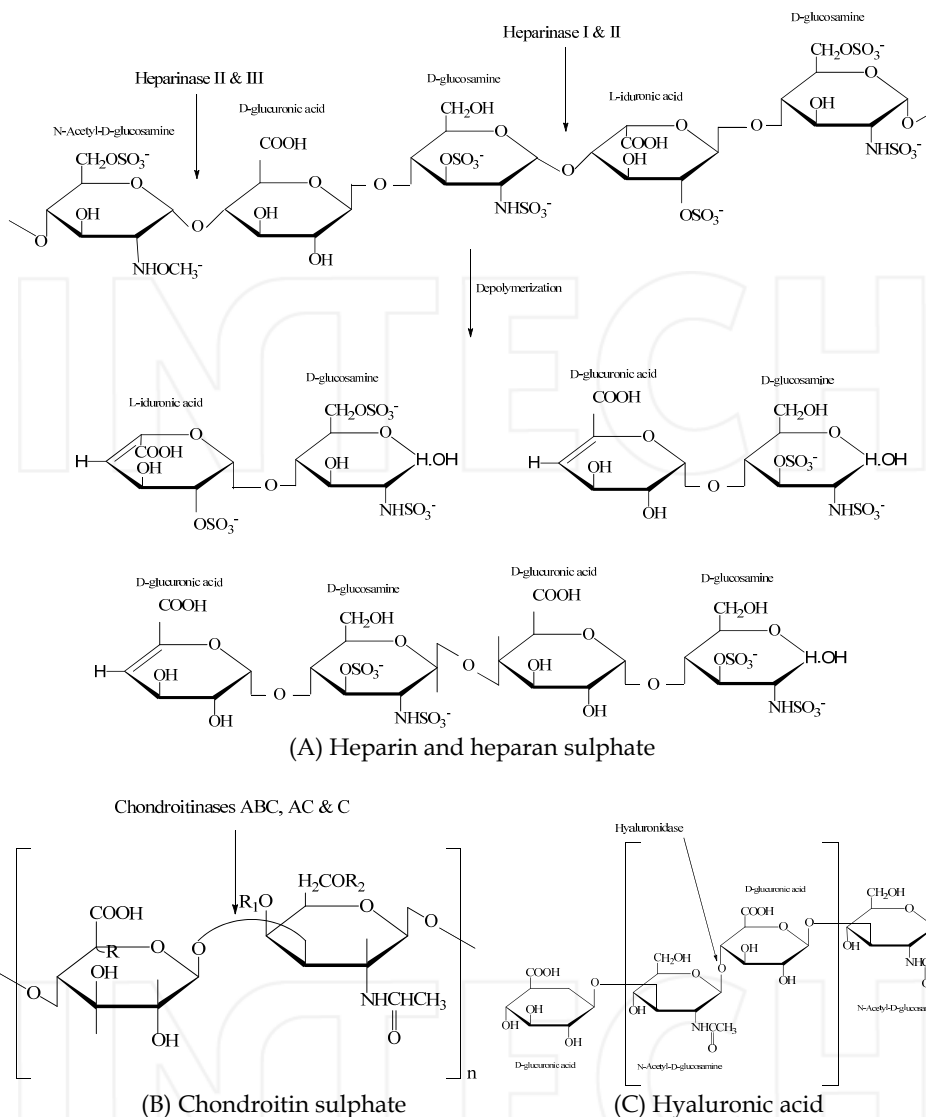


Fig. 4. The enzymatic cleavage of GAGs; A) heparin, heparan sulphate and the specific enzymes: Heparinase I, Heparinase II and Heparinase III; B) Chondroitin sulphate and the specific enzymes: Chondroitinase ABC for Chondroitin sulphate A, B and C, Chondroitinase AC for Chondroitin sulphate A and C and Chondroitinase C for Chondroitin sulphate A and C; C) Hyaluronic acid and the specific enzyme: Hyaluronidase

2.4 Overview on HPLC analysis of GAGs

For the analysis of intact GAGs, a variety of chromatographic techniques have been developed mainly to separate different group of GAGs and/or to characterize the molecular

weight distribution. Concerning this last aspect gel permeation chromatography (both as HPLC or low pressure chromatography) is the preferred approach.

Ion exchange chromatography and reversed phase ion-pair chromatography are the most employed techniques to separate the main classes of intact GAGs, which present differences in polarity, both as low pressure chromatography (Patel et al., 2009; Jin et al., 2009) or high performance liquid chromatography (Shao et al., 2004; Trehly et al., 2009). More recently also capillary electrophoresis (CE) (Malsch & Harenberg, 1996; Malsch et al., 1996) have been used successfully. The main interest is, however, on methods for the separation of depolymerisation fragments of GAG, obtained by different digestion methods (chemical or enzymatic), by HPLC (Du & Eddington, 2002; Plaas et al., 1996; Toyoda et al., 2000) or CE (Malavaki et al., 2008; Ruiz-Calero, et al., 2003) followed by detection with conventional spectral system (UV or fluorescence in case of adequate derivatization), as in the previous papers, but in the last years often by mass spectrometry (Oguma et al., 2007; Yang et al., 2011; Barosso et al., 2005; Silvestro et al., 1992; Da Col et al., 1993; Hemstrom & Irgum, 2006; Silvestro et al., 1996).

The peculiar aspects of separation in connection with MS detection will be detailed in the following paragraph.

2.5 Overview on mass-spectrometric analysis of GAGs

Methodologies using fast atom bombardment (FAB) were first employed; however a direct coupling to HPLC separation was not achieved, as in the case of peptides, due to limitations in mobile phase composition (Dell et al., 1988; Mallis et al., 1989). The introduction of soft ionization methods (ESI), permitting an easy interfacing to HPLC has first provided an on-line separation and structure elucidation of complex oligosaccharide mixtures (Henriksen et al., 2006; Barosso et al., 2005; Thanawiroon et al., 2004). As a complement, matrix-assisted laser desorption ionization (MALDI) has proved to be a valuable tool for the analysis of protein/peptide-heparin/HS oligosaccharide complexes (Ori et al., 2009; Venkataram et al., 1999).

The first important point to consider is the kind of oligosaccharides and disaccharides to be analyzed in particular if they are sulphated or not sulphated.

2.5.1 Sulphated oligosaccharides

As discussed previously (*Chapter 2.4*), sulphated oligosaccharides and disaccharides are separated, when using conventional detectors (UV, fluorescence, electrochemical), employing strong ion exchange columns with gradients of molarity of salts from strong acids (sulphates, phosphates, chloride etc.). It is evident that such mobile phases, being non-volatile, cannot be used in case of MS interfacing and alternative solutions have been developed.

An option is the application of weak anion exchange columns permitting the elution of sulphated derivatives also with gradients of ammonium acetate, compatible with an ESI source; nonetheless it cannot be excluded that highly sulphated large oligomers are not eluted from this kind of columns.

Another possibility, probably the most widely used, is the separation on a reversed phase column in presence of an ion-pairing reagent. In an early paper by our group (Da Col et al., 1993) tetrapropylammonium (TPA) was selected as ion pair reagent for this kind of separation; smaller quaternary ammonium derivatives (tetramethylammonium and tetraethylammonium) didn't improve at all the chromatographic separation while larger

derivative (tetrabutylammonium and higher) showed critical problems of volatility in the ionization source without offering other advantages. It is important to note that the choice of the stationary phase can be quite critical: we observed in fact that in case of reversed phase columns with longer aliphatic chain (C_8 or longer) the sulphated oligosaccharides couldn't be eluted being probably retained by an HILIC-like retention mechanism. Best conditions were obtained on $C_4 - C_6$ columns with gradients of acetonitrile water (from 100% to 50% water) in presence of 3.3 mM tetrapropylammonium at pH 4.5 with formic acid. To prepare the mobile phase most common tetrapropylammonium salts like the sulphate (common in conventional HPLC) were avoided due to volatility problems and preparations were made starting from tetrapropylammonium hydroxide. An example of TIC chromatograms recorded on a digest of heparin is presented in Figure 5.

More recently, several groups have successfully introduced the use of tertiary aliphatic amines (tributylammonium acetate in acetonitrile as ion pair reagents) claiming that a better volatility can be obtained (Thanawiroon et al., 2004). It must be observed that short chain amines like triethylamine, widely used in HPLC, are not able to form ion-pair with these sulphated oligosaccharides.

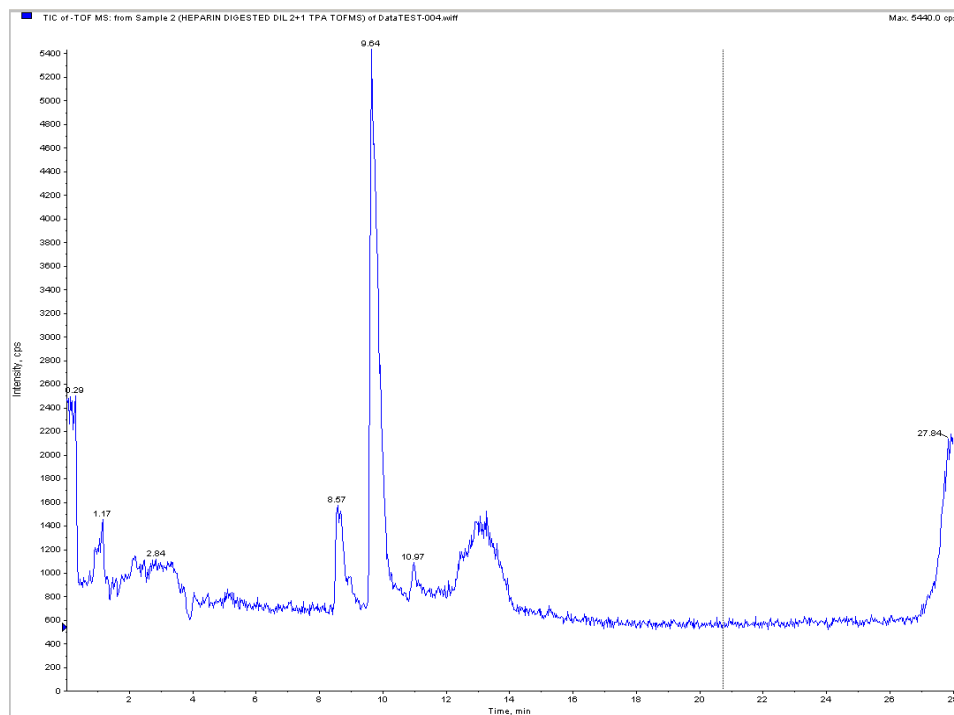


Fig. 5. Total ion chromatogram obtained in TOF full scan after injecting a sample of heparin digested with heparinase II. Column: Kromasil C4 (100x2.1mm, 3 μ m); mobile phase: (A) TPA 3.3 mM in water, pH 4 and (B) TPA 3.3 mM in acetonitrile/water (90/10, v/v), pH 4. Mass spectrometer: QTOF Qstar XL, operated in negative ESI mode.

The addition of an ion pair reagent to sulphated oligosaccharides brings to the formation of complexes stable enough to be ionized as such. Complex pseudomolecular ions with one or more molecules of the ion-pair reagent, sometimes multiple charged, are predominant in spectra of chromatographic separations of such di- and oligo-saccharides; 2 examples of similar spectra are presented in Figure 6 and Figure 7.

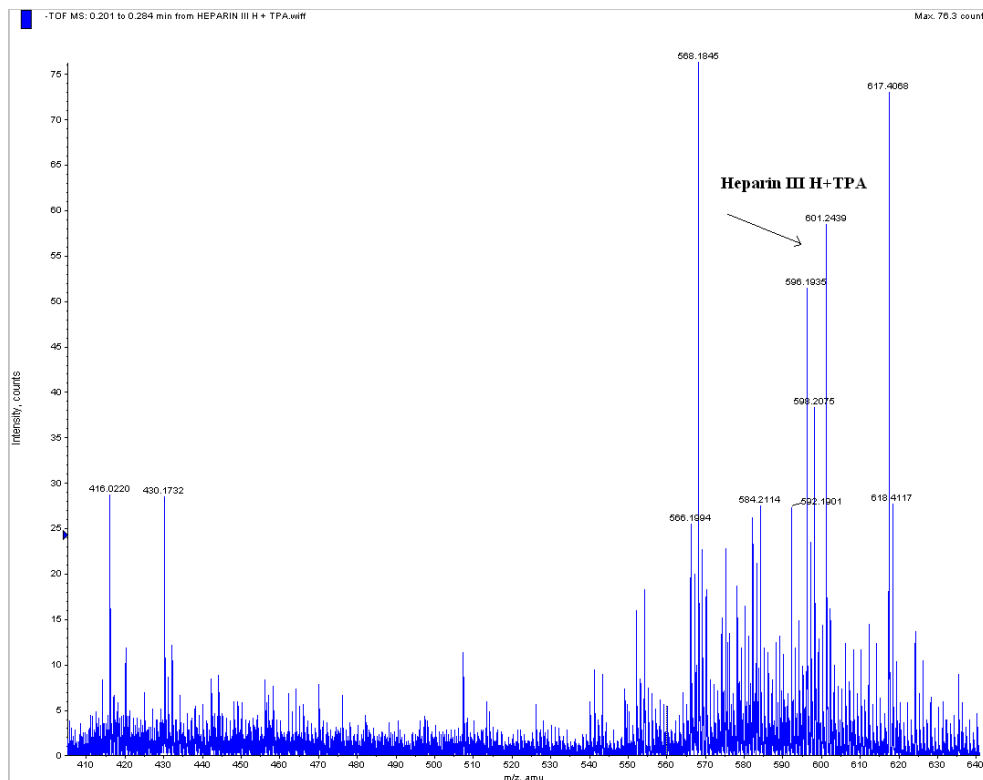


Fig. 6. The molecular ion of the adduct formed by the disaccharide Heparin III H and TPA $[M+TPA-H]^- = 601.2439$. Spectrum recorded by direct infusion with the syringe of a solution diluted at 1 $\mu\text{g}/\text{mL}$. Mass spectrometer: QTOF Qstar XL, operated in negative ESI mode.

It is evident that the use of high resolution instruments is a valuable tool to clarify the charge state of these ions while to clarify the stoichiometry of ion-pair reagent/oligosaccharide ratio the utilization of a labelled ion-pairing reagent is very interesting (Silvestro et al., 1996).

In an effort to obtain more easily interpretable spectra an attractive approach is also the use model MMPC of the ion suppressor (i.e. Dionex...) to remove the tetrapropylammonium from the chromatographic mobile phase. The experiments carried out have really showed an efficient removal of tetrapropylammonium (an example is shown in Figure 8). Anyway, the oligosaccharides as free acidic compounds are much more prone to non-sequence related in source fragmentation during ionization (mainly desulphation), making sometimes very difficult to understand the oligosaccharide real structure.

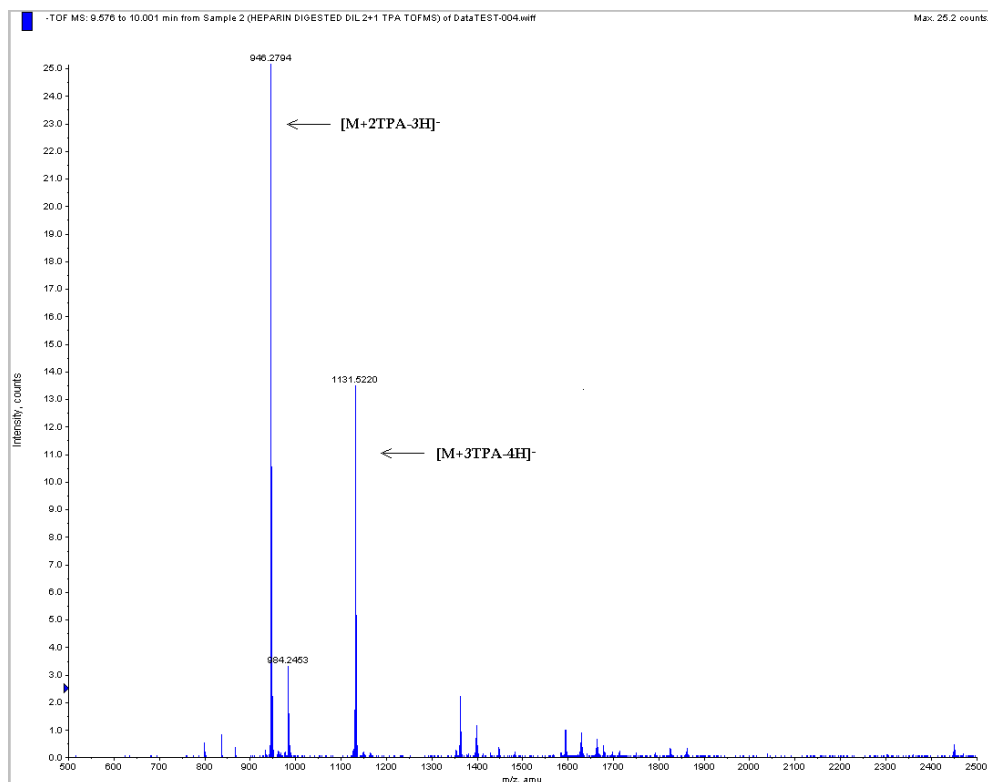


Fig. 7. Extracted spectrum obtained after injecting a sample of heparin digested with heparinase II. Column: Kromasil C4 (100x2.1mm, 3 μ m); mobile phase: (A) TPA 3.3 mM in water, pH 4 and (B) TPA 3.3 mM in acetonitrile/water (90/10, v/v), pH 4. Mass spectrometer: QTOF Qstar XL, operated in negative ESI mode. M: trisulphated disaccharide obtained from heparin by enzymatic digestion.

The MS/MS properties of these di- and oligo-saccharides are also noteworthy: in case of parent ions (single or multiple charged) the desulphation fragments are generally the most abundant being easily formed and generally very few weak signals coming from fragmentation of the sugar back bone can be observed. The situation is similar in case of TPA complexes with the tendency to an even less favourable fragmentation.

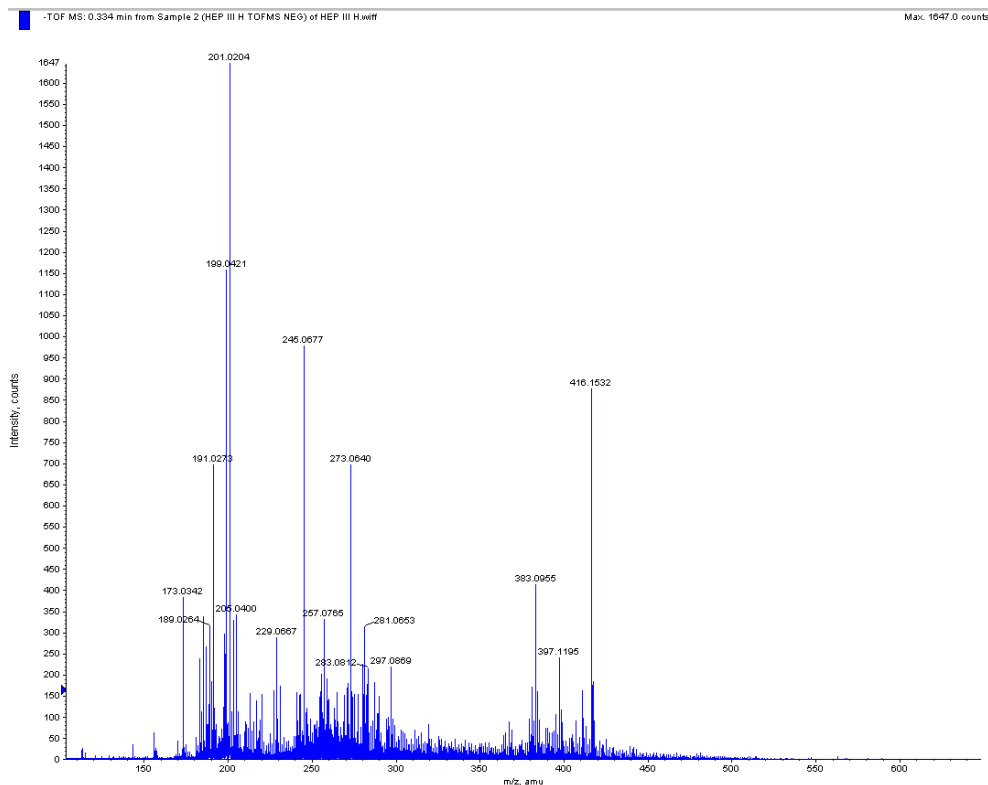


Fig. 8. The molecular ion of the oligomer Heparin III H after ion suppression; $[M-H]^- = 416.1532$ can be easily seen. Spectrum recorded by direct infusion with the syringe of a solution diluted at $1 \mu\text{g/mL}$. Mass spectrometer: QTOF Qstar XL, operated in negative ESI mode.

2.5.2 Non sulphated oligosaccharides

The analytical separations of these molecules are less challenging than those of the more charged sulphated products. In this case the use of ion exchange columns eluted with gradients of volatile buffers (i.e. ammonium acetate) at high ionic strength is effective and permits a good separation of oligomers with different chain length. The application of ion-pair chromatography is practically not needed and in general the formation of ion-pair complexes having only carboxylic groups is not effective.

Deprotonated molecular ions, sometimes multiple charged, are typically observed, as shown in Figure 9.

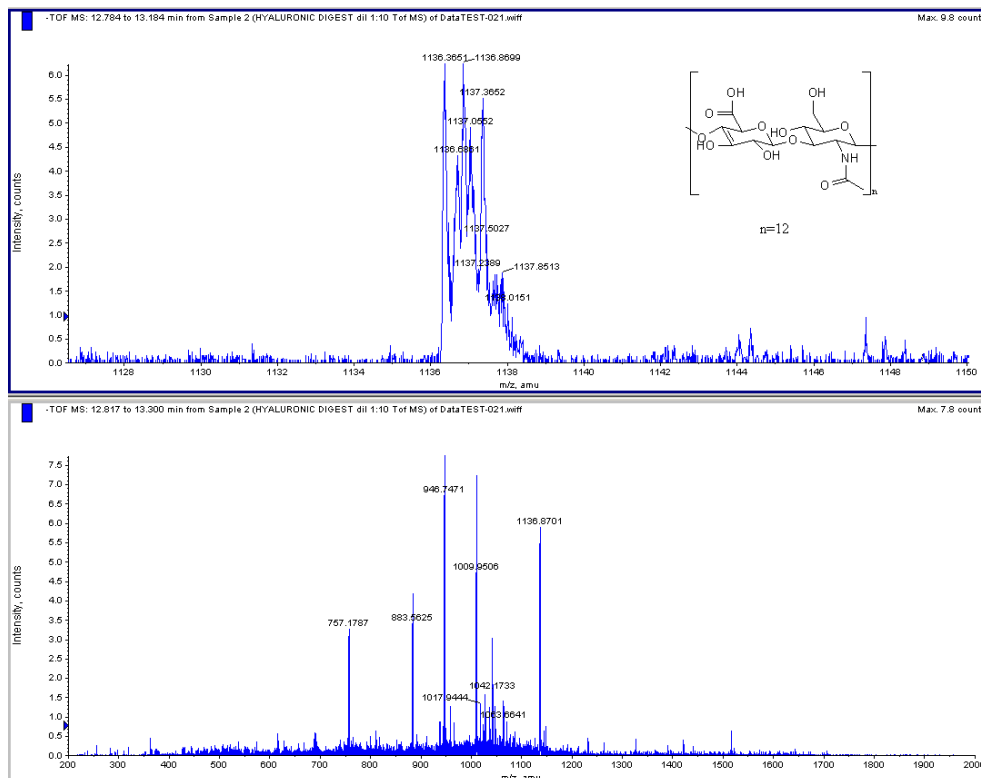


Fig. 9. Mass spectrum obtained in TOF full scan mode after injecting a sample of a hyaluronic acid digested with hyaluronidase. Column: Supelcosil LC-SAX 1 (250x4.6mm, 5 μ m); mobile phase: (A) water/methanol (1/1, v/v) (B) ammonium acetate 0.5 M in water/methanol (1/1, v/v). Mass spectrometer: QTOF Qstar XL, operated in negative ESI mode. The main ions in the spectrum are multiple charged; they all correspond to disaccharide oligomers resulted from hyaluronic acid degradation. A detail can be seen in the spectra above for $[M-2H]^{-2}=1136.8$, a doubly charged ion corresponding to a dodecasaccharide.

Daughter spectra can be obtained as it is shown in Figure 10. However, it can be seen, these kinds of molecules are hard to break and only scarce structural information can be gathered.

3. Bisphosphonates analysis

3.1 Chemical structure and main features of bisphosphonates

Bisphosphonates, polar structures with two phosphonate groups covalently bonded to carbon (called P-C-P bridge, Fig. 11) are a class of drugs used in the treatment of osteoporosis, osteolytic metastasis, Paget's disease (osteitis deformans), and other disorders involving bone fragility. They have also non-therapeutic usage, based on their chelating properties (as water softeners, in agriculture, in paper, detergent and cosmetics industries).

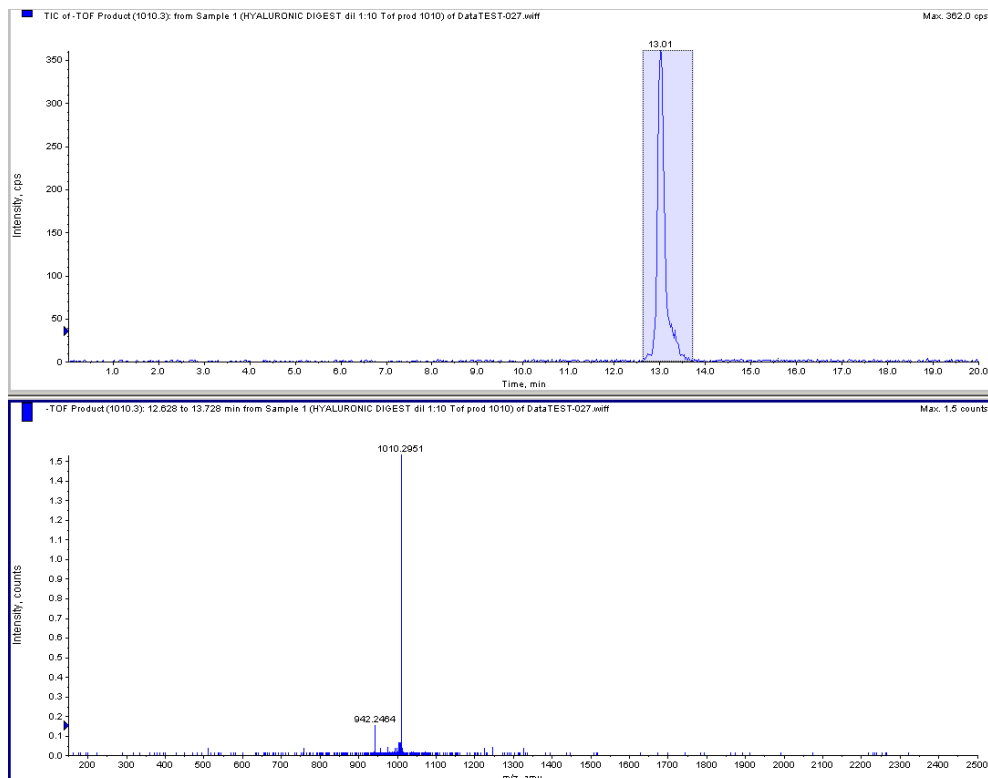


Fig. 10. Product ion spectrum of one of the oligomers presented in Figure 9 - $[M-2H]^{2-} = 1010$. Column: Supelcosil LC-SAX 1 (250x4.6mm, 5 μ m); mobile phase: (A) water/methanol (1/1, v/v) (B) ammonium acetate 0.5 M in water/methanol (1/1, v/v). Mass spectrometer: QTOF Qstar XL, operated in negative ESI mode; collision energy: -30V.

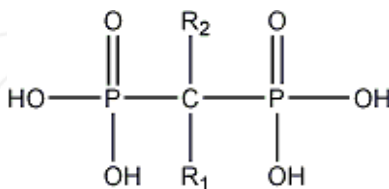


Fig. 11. General structure of bisphosphonates.

The long-chain substituent (R_2 in figure) determines the chemical properties and mechanism of action, while the short-chain substituent (R_1) influences mainly the pharmacokinetics of the bisphosphonate drug (Fleish 2004). Obviously, bisphosphonates are extremely hydrophilic and their structures similar to endogenous phosphorylated compounds. They can be grouped in two classes: nitrogen (N) - containing (pamidronate, neridronate, olpadronate, alendronate, ibandronate, risedronate, zoledronate) and non-N-containing (etidronate, clodronate, tiludronate) bisphosphonates.

3.2 Special problems of these molecules and potential approach to solve them: derivatization or not

Analysis of bisphosphonates from biological matrices is very challenging, due to the aforementioned features of these molecules: high polarity and similarity with endogenous phosphates. Finding appropriate conditions for their extraction from plasma or urine is very difficult; chromatographic separation raise typical problems of hydrophilic compounds and last but not least, bisphosphonate molecules often lack chromophores or fluorescent groups needed for sensitive UV or fluorescence detection in liquid chromatography. As an approach to facilitate either the isolation from biological samples, or the chromatographic separation, or the detection, or all of them, derivatization is widely used in the analysis of bisphosphonates. Mass spectrometric detection was used more recently to develop sensitive quantification methods, but also most of the times in combination with derivatization procedures. In gas-chromatography (GC), derivatization is needed to increase the volatility and thermal stability of the molecules. The N-containing bisphosphonate undergo reaction of the amino- group, and for all compounds the derivatization of the hydroxyls from the phosphonate group can give good results.

3.3 HPLC analysis of bisphosphonates

Various analytical procedures for the determination of bisphosphonates from biological samples or pharmaceutical formulations have been presented so far; review papers were previously published by Sparidans and den Hartigh (1999), Zacharis and Tzanavaras (2007). One of the first methods, developed for dichloromethylene bisphosphonates, was described by Chester et al (1981). The separation is based on ion-exchange chromatography, without derivatization, using an AG1 X8 resin column and a mobile phase containing hydrochloric acid and a flame-photometric detector. The coprecipitation with calcium phosphate was used to extract the target compounds from urine. This method has a limit of detection of 2 $\mu\text{mol/l}$.

Separation procedures without derivatization are performed on ion-exchange columns like Waters IC-Pak HR (Tsai et al, 1992) or Dionex OmniPac PAX-100; the mobile phases contain mineral acids (mostly diluted nitric acid without or with small percentage of organic modifier) and the detection is realized through flame-photometry (Chester et al, 1981), conductivity (Tsai et al, 1992) or inductive coupled plasma mass spectrometry (ICP-MS) (Kovacevic et al, 2004).

Mineral acids are replaced with organic acids (especially formic acid) in researches that use ion-chromatography in tandem with mass-spectrometry (Ip et al, 1994). An alternative to ion-exchange chromatography is ion-pairing reversed-phase chromatography. Xie et al (2006) used ion-pair chromatography with evaporative light-scattering detection to determine ibandronate in pharmaceutical formulations. The C8 Inertsil column was eluted in isocratic conditions with a mobile phase consisted of ammonium acetate containing amylamine/acetoneitrile/methanol. Tetrabutyl ammonium hydrogen sulphate (Rao et al, 2005), tetrabutyl-ammonium phosphate (Aluoch et al, 2005), 1-octyltrimethylammonium phosphate (Vallano et al, 2003) are among the ion-pair reagents employed in separations without derivatization.

As mentioned above, a pre- or post-column derivatization step is often needed to introduce in the molecule a chromophore group, in order to increase the sensitivity of the detection by UV or fluorescence. The pre-column derivatization, if possible directly in biological samples, is also very important for the isolation of bisphosphonates, introducing in the hydrophilic

molecule a large non-polar group that facilitates their subsequent extraction in organic solvents.

Most of the published methods involve therefore a derivatization approach, either of the amino, or of the hydroxyl groups, or of both of them in some cases.

Fluorescamine was one of the most used derivatization reagents of the amino group (Flesh et al, 1991, Wong et al, 2004). Aminoalkylphosphonic acids were isolated from urine or plasma by coprecipitation with calcium phosphate; then the precipitate was dissolved in Na₂EDTA pH10 and reacted with fluorescamine. The reaction products were chromatographed on an octadecyl stationary phase (Nucleosil C18) and detected by fluorescence.

9-Fluorenylmethyl-chloroformate was also widely applied in the determination of alendronate from different matrices like pharmaceutical preparations, plasma or urine. The derivative was chromatographed on C18 columns, with mobile phases consisting of acetonitrile, methanol and pyrophosphate buffer, and detection in UV or fluorescence (de Marco et al, 1989, Ptacek et al, 2002, Yun et al, 2006, Apostolou et al, 2007). As an example, Ptacek et al (2002) obtained with this derivatization a limit of quantification as low as 5 ng/ml from urine, with reproducible results that could be used for bioavailability studies. The reaction was conducted at alkaline pH after bisphosphonates isolation from biological sample by coprecipitation with calcium phosphate and the calcium ions removed on ion-exchange resins; the chromatography was carried out on a C18 stationary phase, in gradient conditions, with fluorescence detection.

The derivatization of the amino group with isobutyl chloroformate was proposed by Sakiyama et al (1996) prior to GC separation. A fused-silica capillary column HP-1 (5m x 0.53 mm i.d., 2.65µm film thickness) was used and flame photometric detection. The same reaction was later used by our group directly on biological samples (urine); this reduced the polarity of the molecule and allowed the extraction with organic solvent (Tarcomnicu et al, 2007).

An interesting reaction described by Kline et al (1992) uses 2,3 naphthalene-dicarboxaldehyde, in presence of cyanide ions as nucleophiles. The substituted cyanobenzof[*f*]indole obtained was measured by fluorescence. Alendronate was determined from urine samples using this technique. The same authors studied also the use of acetylpenicillamine as nucleophile for improved sensitivity; electrochemical detection was preferred over fluorescence.

Introducing a chromophore in order to get sensitive UV or fluorescence measurements is also possible post-column, after the separation by ion-exchange chromatography. Daley-Yates et al (1989) described such a derivatization with ammonium persulphate and molybdenum ascorbate; a phosphomolibdate detected in visible (820 nm) was obtained.

Nevertheless, in the LC methods above presented, although the separation is performed on octadecyl stationary phase, the mobile phases consisted of phosphate, pyrophosphate, citrate buffers in combination with various percentages of organic modifiers like methanol or acetonitrile. These inorganic buffers, as well as mineral acids, are not suitable for LC-MS.

3.4 Overview on mass spectrometric analysis of bisphosphonates

When coupling gas chromatography with mass spectrometry, derivatization is needed in order to obtain volatile compounds. In this case the hydroxyls of the phosphonate group are silylated or alkylated. Sakiyama et al (2005) described a method for alendronate analysis in urine that employs double derivatization: first the amino group was reacted directly in the

biological matrix with isobutyl chloroformate and extracted with organic solvent, then diazomethane was used to methylate the phosphonate groups. A fused silica capillary column containing cross-linked OV-1 stationary phase (Quadrex 12m \times 25mm id, 0.25 μ m film thickness) eluted with helium at 1 ml/min was employed for chromatographic separation. The limit of detection was 20 ng/ml.

The studies conducted for (aminoalkyl)phosphonic acids, widely used either as pesticides or as pharmaceuticals, are good starting points also for bisphosphonate analysis. A large variety of derivatization approaches is described in the literature.

Silylation with N-methyl N-(tert-butyltrimethylsilyl)trifluoroacetamide (MTBSTFA) was proposed by Moye and Deyrup (1984) for GC/MS analysis of the herbicide glyphosate and its metabolite, aminomethylphosphonic acid. In order to obtain high yield of the reaction, coating of the glassware by exposing it to a diluted solution of phosphoric acid in ethanol was necessary; however authors reported problems at low ppb levels.

Also in GC/MS pentafluorobenzyl bromide (Palit et al, 2004) and trimethyl orthoacetate (Kudzin et al, 2003) were used. The two techniques were applied in the determination of alkyl and aryl bisphosphonic drugs and in trace analysis of organophosphorous pesticides or nerve agents and their metabolites from biological or environmental samples. Palit et al (2004) studied the derivatization of methyl phosphonic and pinacolyl methyl phosphonic acid, degradation products of organophosphorous compounds, with pentafluorobenzyl bromide (PFBBBr) at alkaline pH, in different conditions. They further compared the stability of the pentafluorobenzyl phosphonic esters with that of the silylated derivatives, the later more widely used in GC/MS, but more sensitive to humidity.

The same reaction was tested by our group on two drugs from the bisphosphonate class, and the obtained compounds were separated by LC-MS/MS (unpublished data). A phenyl stationary phase (Supelcosil LC-DP 15cm \times 3mm, 5 μ m) was used, eluted in gradient with a mobile phase containing 50mM ammonium acetate and acetonitrile. High resolution mass spectrometric detection on a quadrupole-time of flight (QTOF) instrument model Qstar Pulsar i (AB Sciex), equipped with an electrospray ionisation source (ESI) operated in negative ions mode was employed. Di- and triesters resulted mainly from the derivatization of clodronate, as shown in figure 12; further optimisation is needed to increase the yield of one of the derivatives in order to use this approach for quantitative purposes.

Zhu et al (2006) reported a methodology for the quantification of risedronate and alendronate by LC-MS/MS in biological samples after derivatization of the phosphonate groups with diazomethane. As a general approach, the urine or serum samples were loaded onto Bond-Elut SAX solid-phase extraction (SPE) columns that were washed with water prior to on-column derivatization with diazomethane. The methylated compounds were eluted with methanol and separated on an ion-exchange Zorbax 300-SCX column, with ammonium formate pH 2.5 and acetonitrile (75/25, v/v) as mobile phase. Triple quadrupole mass-spectrometers model API 4000 or 5000 were used, operated in ESI positive mode. Low limits of quantification (LLOQs) as of 0.2 ng/ml for risedronate and 1 ng/ml for alendronate in urine were achieved using this technique.

A general drawback of the methods using diazomethane is the toxic and explosive nature of the reagent; special care needs to be taken to ensure safety of all operations.

Alternative alkylations of aminoalkyl phosphonic acids could be performed with fluorinated alcohols/perfluorinated anhydrides mixtures (Deyrup et al, 1985) or trimethyl orthoacetate (Roy et al, 2000, Kudzin et al (2003).

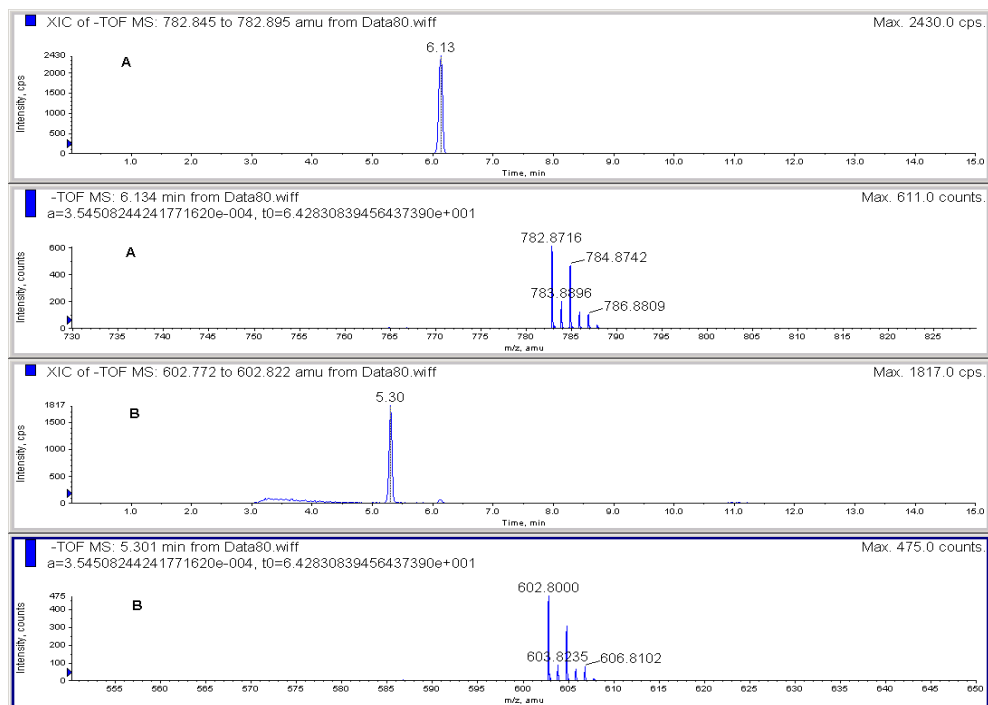


Fig. 12. Extracted chromatogram of the negative molecular ions corresponding to the triester - $[MH]^- = 782.8716$ (A) and diester - $[MH]^- = 602.800$ (B) obtained in the reaction of clodronate and PFBBBr. Column: Supelcosil LC-DP 15cmx3mm, 5 μ m, mobile phase: ammonium acetate and acetonitrile, in gradient. Mass spectrometer: QTOF Qstar Pulsar i, operated in negative ESI mode.

Deyrup et al (1985) analysed glyphosate (N-phosphonomethylglycine) and its major metabolite, aminomethylphosphonic acid, from water samples by GC with flamephotometric, electron capture or mass spectrometric detection, after derivatization with trifluoroethanol (TFE) and trifluoroacetic anhydride (TFAA). With this approach the amino and hydroxyl groups are derivatized in the same step. No special glass coating is needed, as in the case of MTBSTFA, and no particular safety issues are raised, as in the case of diazomethane derivatization. An LLOQ of 0.5 μ g/L in deionized water was obtained with electron capture detection.

Trimethylorthoacetate (TMOA) was preferred by Royer et al (2000) for the derivatization of another herbicide, gluphosinate (DL homoalanine-4-yl (methyl)phosphonic acid), and two of its metabolites, 3-methylphosphono-propionic and 2-methylphosphono-acetic acids, prior to analysis by GC-MS/MS with chemical ionization. This method was applied for the quantification of gluphosinate and its metabolites in water samples, over a range of 10-150 μ g/L.

Kudzin et al (2003) combined anhydride and TMOA approaches for the derivatization of a series of biologically active aminoalcanphosphonic acids. Briefly, the neat standards or dried water extracts were dissolved in 0.1 ml of trifluoroacetic acid/trifluoroacetic anhydride

mixture (1/1, v/v), incubated for 90 min at 40 °C, then 0.4 ml TMOA were added, followed by another incubation step at 100 °C for 120 min. Both amino and hydroxyl groups were derivatized and the reaction mixtures were analyzed after preconcentration under vacuum, by ^{31}P NMR or GC-MS. The derivatization products were stable for several weeks at ambient temperature and the quantifiable levels by GC-FID (flame ionization detection) were in the low $\mu\text{mol/L}$ range.

More recently our group studied the application of TMOA derivatization in the analysis of bisphosphonate drugs. The hydroxyl groups reacted with TMOA in acidic conditions independently from the presence of amino groups and all tested drugs (alendronate, pamidronate, etidronate, clodronate, risedronate, ibandronate) were successfully derivatized. Triethylorthoacetate (TEOA), trimethylorthoformiate (TMOF), trimethylorthobutirate (TMOB) were also tested as alkylation reagents and found effective (unpublished data). All reagents do not require special safety measures which is an important advantage.

Studying these reactions, the expected alkylation products were found, but also other compounds with higher molecular mass were obtained in the case of bisphosphonates containing a hydroxyl group at the carbon in the P-C-P bridge. This hydroxyl is also involved in the reaction probably resulting in cyclic structures. In the case of clodronate, the carbon in the bridge is substituted with two chlorine atoms and only the tetraalkylated structure is formed, as it can be seen in Figure 13. When the reagent was TMOF, an increase of 28 mass units was observed; for TMOA and TEOA the shift was 42, and for TMOB 70 Da. Based on these results, we have further developed a double derivatization method for the quantification of alendronate in urine by LC-MS/MS (Tarcomnicu et al, 2007). First, the amino group was reacted with isobutyl chloroformate (IBCF), to facilitate the extraction of the polar drug from biological matrix with organic solvent. The dried extract was redissolved in acetic acid, and incubated with TMOA for 1 h at 100 °C. The methylation of the phosphonate groups significantly reduced the polarity of the molecule permitting the separation on a reversed-phase column (Supelco Discovery HSC18) with a gradient of mobile phase containing formic acid 0.1% in water/formic acid 0.1% in acetonitrile, which was suitable for LC/MS and enhanced the ionization in positive mode. The cyclic compounds, as described above, were obtained with highest yield for alendronate and pamidronate; a product ion spectrum of the alendronate derivative is presented in Figure 14.

The method was fully validated according to FDA guidelines over a range of concentrations of 6.667 - 4860 ng/mL alendronate in urine, and it was applied to a bioequivalence study. With the help of a switching valve 2 columns were run in parallel, resulting in an analysis time of 5 min/sample, that was very short compared with HPLC-fluorescence or with GC methods.

Trimethylsilyl diazomethane (TMSD) was another derivatization reagent successfully used in LC-MS/MS, in order to analyze ibandronate from plasma samples for pharmacokinetic applications (Tarcomnicu et al, 2009). A high-throughput method with an LLOQ of 200 pg/mL was optimized and fully validated according to FDA guidelines. Owing to its structure (N-substituted aminobisphosphonate), ibandronate was isolated from plasma extracts with the same technique previously proposed for alendronate (Tarcomnicu et al, 2007). To reduce the polarity of the molecule and make it suitable for reversed-phase LC, in the next step we decided to methylate the phosphonic groups. First, TMOA was tried as derivatization reagent, but in the case of plasma extracts this was found not as effective as for urine extracts. TMSD proved to be very reactive also with plasma extracts, as already

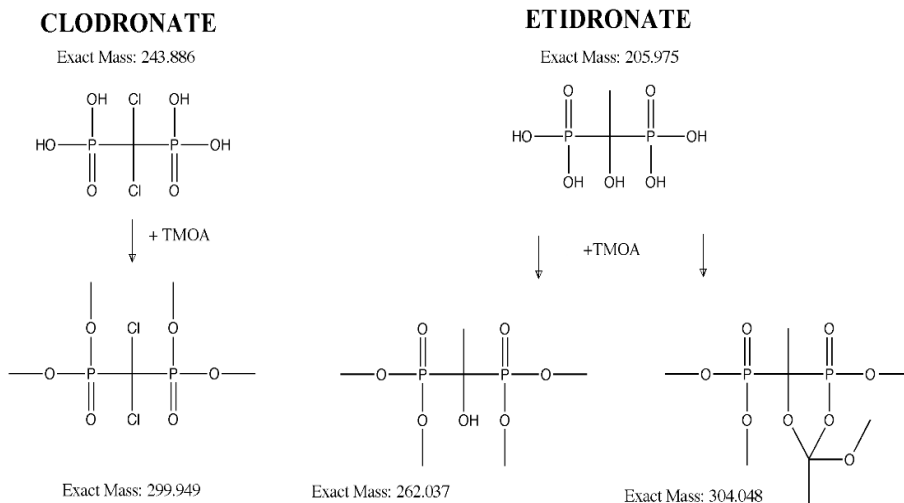


Fig. 13. Clodronate and etidronate derivatization with TMOA; for the latter both hypotheses (methylation and methylation plus cyclization) are shown.

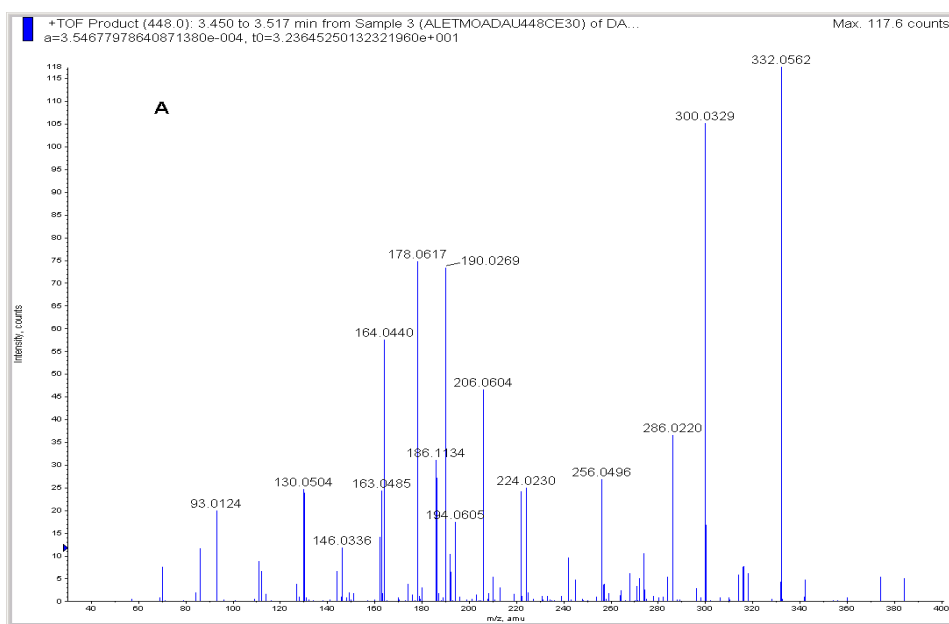


Fig. 14. Product ion spectrum of the alendronate derivative with IBCF and TMOA ($[M+H]^+ = 448$), during chromatographic separation. Column: Supelco Discovery HSC18, 10cmx2.1mm, 5 μ m, mobile phase: (A) formic acid 0.1% in water and (B) formic acid 0.1% in acetonitrile; gradient conditions. Mass spectrometer: QTOF Qstar Pulsar i, operated in positive ESI mode.

studied by another group (Ranz et al 2008) and compared to diazomethane it is a stable and safe reagent. The alkylation reaction was carried out for 30 min at 70 °C and the fully methylated compound was obtained. The derivatization scheme is presented in Figure 15, the product ion spectrum of the ibandronate derivative in Figure 16, and Figure 17 shows selected chromatograms obtained on a blank, calibrator 1 (LLOQ) and calibrator 8 extracted from plasma. The separation was performed on a Discovery HSC18 column (10cm×2.1mm i.d., 5µm particle size) and a mobile phase consisting of formic acid 0.1% in water and formic acid 0.1% in acetonitrile, with a composition gradient. An alternative satisfactory approach using alkaline mobile phase (ammonium hydroxide 0.05% in water/ammonium hydroxide 0.05% in acetonitrile) on a Purospher Star RP-18e (3cm×2.1mm, 2.7µm) was also tested; good peak intensities, similar to the ones with acidic mobile phase, were achieved. Elution at acidic pH was preferred for this study due to better background, but the basic mobile phase can be also considered.

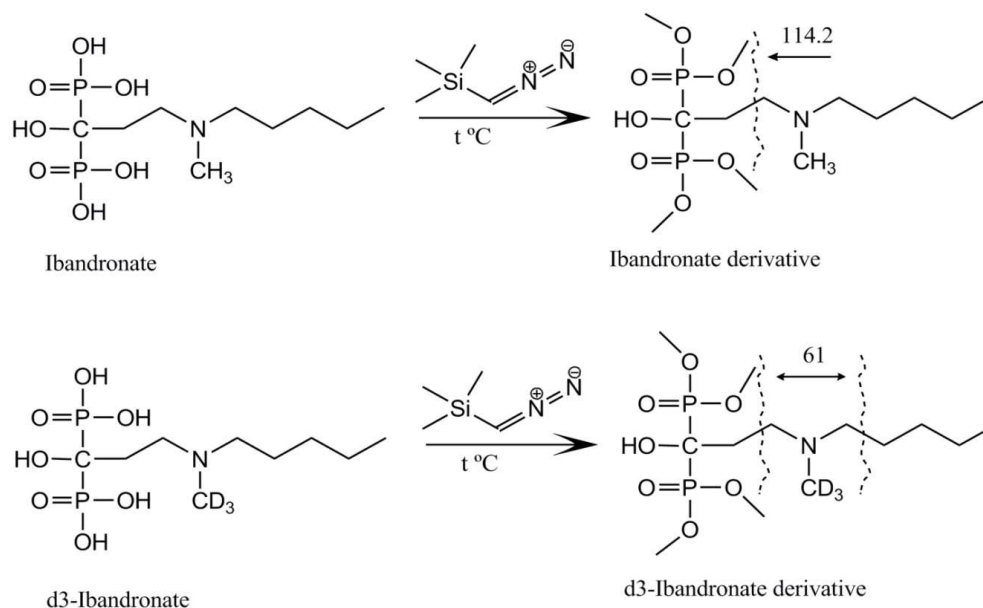


Fig. 15. Derivatization scheme of ibandronate and its internal standard, d3-ibandronate.

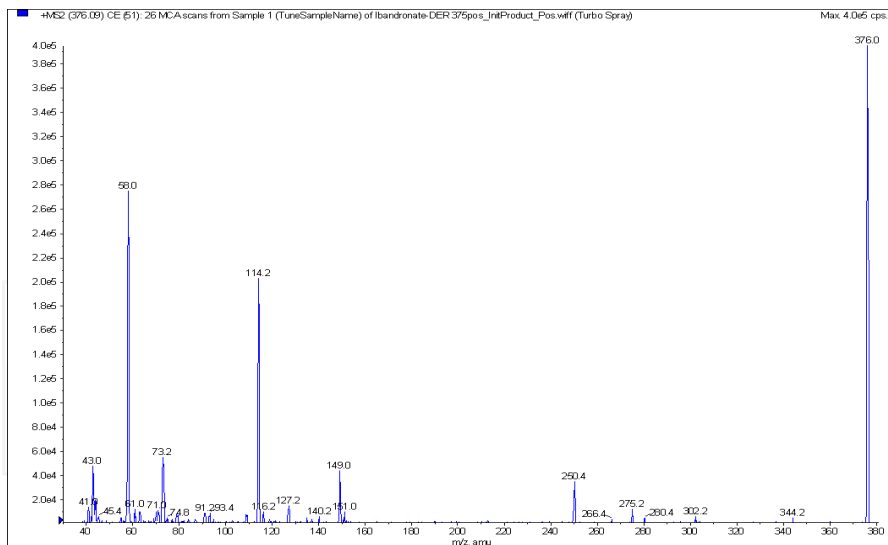


Fig. 16. Product ion spectrum of the ibandronate derivative with TMSD - $[M+H]^+ = 376$ obtained with direct infusion via syringe of a $1\mu\text{g}/\text{mL}$ solution water/methanol (1/1, v/v). Mass spectrometer: quadrupole-linear ion trap API4000QTrap, operated in positive ESI mode; collision energy: 25V.

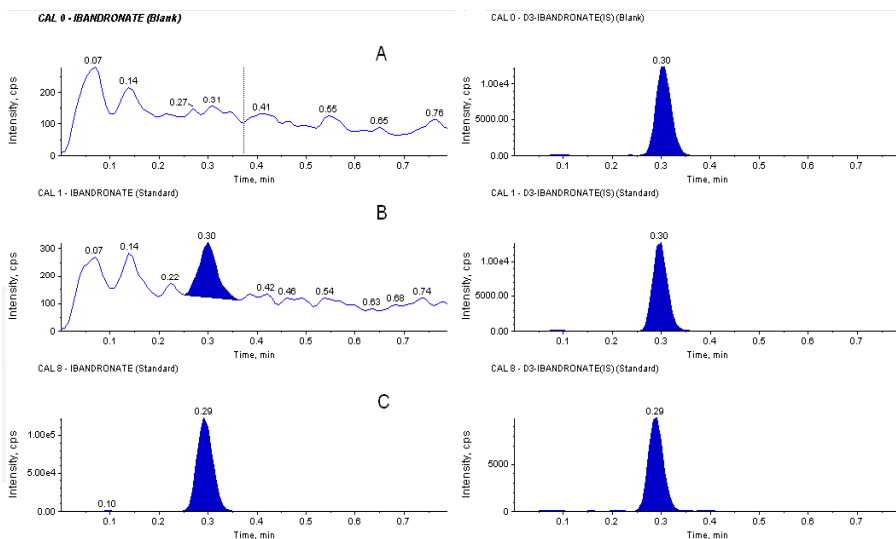


Fig. 17. HPLC traces of the MRM transitions selected for ibandronate (left) and d3-ibandronate (right) recorded on a blank plasma sample (A), plasma spiked with ibandronate for CAL1 – 0.2 ng/ml (B) and CAL8 – 175.0 ng/ml (C) concentrations. Note: The retention time shown in the figure is relative, as the analyses were performed in overlapping mode; 1.5 min must be added to get the total retention time.

3.5 Remarks

Considering existing data for the analysis of bisphosphonates, especially with MS detection, the derivatization approach has given so far the best results. Nevertheless, the new developments in column manufacturing could open possibilities for the analysis of underivatized bisphosphonates. A promising direction is represented by HILIC (hydrophilic interaction liquid chromatography) which is gaining interest in the analysis of polar compounds, with applications in various fields (A.J. Alpert, 2011, Van Nuijs et al, 2011). HILIC is a version of normal phase liquid chromatography; the typical mobile phase consists of acetonitrile with a small percentage of water, while the stationary phases are polar surfaces (silica, diol, amino, amide, zwitterionic bonded phases). The retention of polar compounds is not based on liquid-solid partition like in reversed phase separations, but on a liquid-liquid partition mechanism between the water-deficient bulk eluent and the water-enriched layer immobilized on the HILIC stationary phases (Alpert et al, 1990). Therefore polar analytes early eluting in reversed-phase are strongly retained in HILIC conditions. HILIC is particularly useful in the analysis of aminoglycoside antibiotics (more details will be provided in the following chapter), other various pharmaceuticals (e.g. metformin, salicylic and acetyl salicylic acid, tetracyclines), nucleosides, organic phosphates, peptides and proteins, sugars, warfare agents or drugs of abuse (cocaine, benzoyllecgonine) etc. The use of mobile phases with high percentage of organic solvent in HILIC separation is beneficial for MS detection, because of enhanced ionization which results in an increased sensitivity. Methanol, ethanol, 2-propanol, tetrahydrofuran can be used for some applications. The most common buffers used to control pH and ionic strength are ammonium acetate or ammonium formate, at low molarities, also favouring the ionization in MS; only rarely high molarities or non-volatile buffers are required. Another advantage is the simpler sample preparation, being no need for derivatization. The functional groups on the phase surface influence the selectivity of the separation, thus the capacity to differentiate similar compounds. Other interactions which could be involved in HILIC are electrostatic interaction, hydrogen-bonding, dipole-dipole interaction, molecular shape selectivity, and even hydrophobic interaction.

The retention mechanisms have been studied by several groups in order to characterize and classify HILIC stationary phases (Dinh et al, 2011, Kawachi et al, 2011).

4. Analysis of aminoglycoside antibiotics

4.1 Chemical structure and main features

Aminoglycosides are antimicrobial agents used in the treatment of both animals and humans against aerobic gram-negative bacteria. These molecules are natural products or semisynthetic derivatives of compounds produced by different varieties of actinomycetes isolated from soil (Higgins and Kastner, 1967); the class include very important antibiotics as gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin and neomycin.

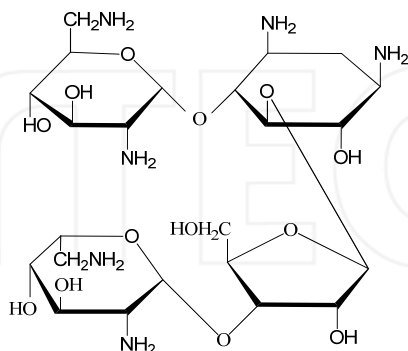
The first drug of the class, Streptomycin, was isolated from a strain of *Streptomyces griseus* by Waksman and coworkers in 1944 and it is an important agent for the treatment of tuberculosis. Gentamicin and netilmicin are derived from species of the actinomycete *Micromonospora*. Tobramycin is produced by *S.tenebrarius* (Chambers, 2006). Amikacin, a derivative of kanamycin, and netilmicin, a derivative of sisomicin, are semisynthetic products.

The antimicrobial activity of aminoglycosides is based on their ability to selectively inhibit protein synthesis, in bacteria; the most important pathogens treated with aminoglycosides are pseudomonas, enterococci, coliforms, and salmonellae.

The molecule of aminoglycosides presents two or more amino sugars linked by a glycosidic bridge to a hexose nucleus (aminocyclitol) that is either streptidine (found in streptomycin) or 2-deoxystreptamine (in all other available aminoglycosides).

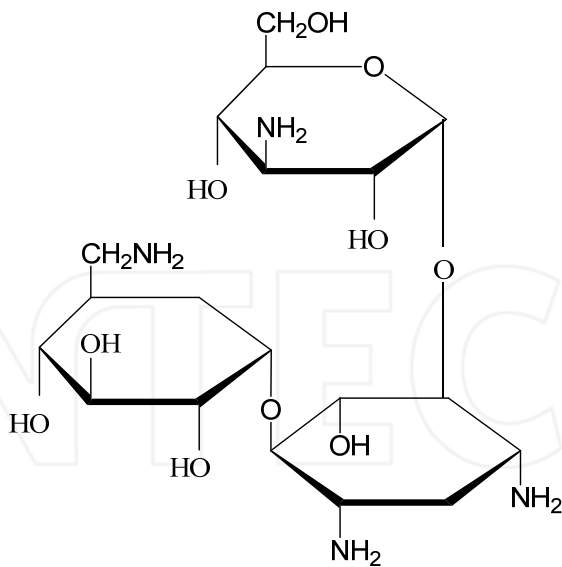
The aminoglycosides are divided into the following subclasses (Chambers, 2006):

- a. *The neomycin family*, which includes neomycin B and paromomycin, presents three amino sugars attached to the central 2-deoxystreptamine.



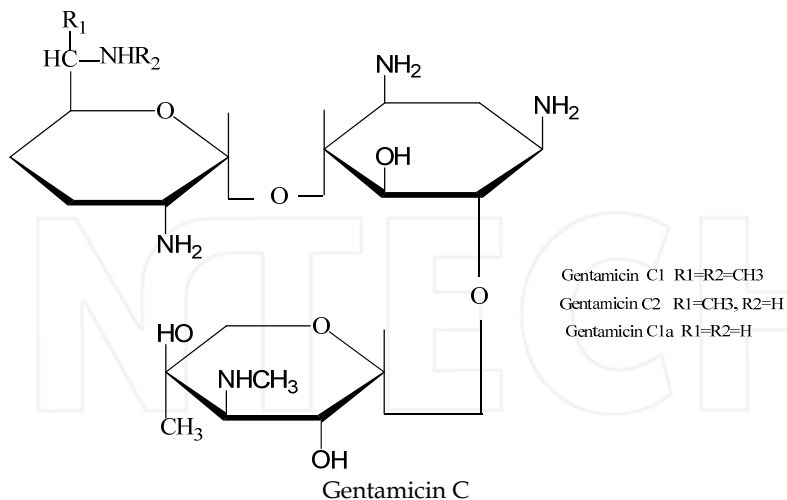
Neomycin B

- b. *The kanamycin family* (kanamycin A - B, amikacin and tobramycin) presents two amino sugars attached to the central 2-deoxystreptamine moiety; one of these being a 3-aminohexose.

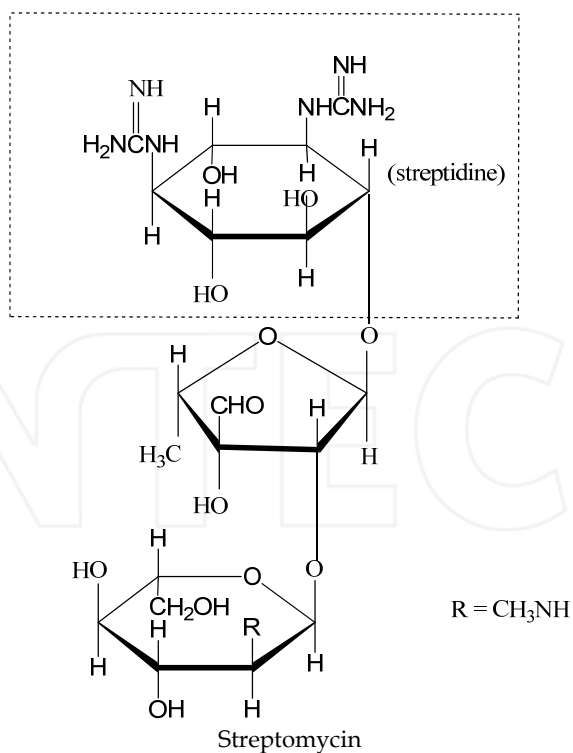


Kanamycin A

- c. *The gentamicin family*, including gentamicin, sisomicin and netilmicin, contains two amino sugars attached to the central 2-deoxystreptamine, one of these being garosamine.



- d. *Streptomycin* contains streptidine that is not in the central position like in the other aminoglycosides.



4.2 Chromatography of aminoglycosides

Aminoglycoside antibiotics are very polar compounds, highly water soluble, practically insoluble in hydrophobic organic solvents, lacking chromophores for UV absorption or fluorescent groups. They have been determined by bioassays, GC-MS and HPLC, in this case often following derivatization (to improve detection and/or separation), adding ion-pair agents in the mobile phase or by ion-exchange techniques. More recently, HILIC was also proposed as worthwhile approach and there is an increasing number of applications; in literature brief reviews about the application of these methods for the separation of aminoglycoside antibiotics can be found (Tawa et al, 1998, Isoherranen and Sobak, 1999, Stead, 2000).

Because of the nonvolatile and very polar nature of aminoglycosides the gas chromatographic analysis invariably involves derivatization of the amino and hydroxyl groups of these molecules. trimethylsilylimidazole (TMSI) and heptafluorobutyrylimidazole (HFBI) have been used as derivatizing agents for the analysis of gentamicin, tobramycin, netilmicin, amikacin and paromomycin in serum by Mayhew et al (1978). Trimethylsilyldiethylamine (TMSDEA) was also tested as derivatization reagent for both amino and hydroxyl groups but with poor results as repeatability and linearity (Margosis and Tsuji, 1978).

Owing to their hydrophilic properties, aminoglycosides are not adequately separated also by reversed-phase liquid chromatography, and therefore derivatization with non polar agents or mobile phase containing counter-ions reagents, forming ion pairs, have been widely employed in HPLC.

As derivatization reagents *o*-phthalaldehyde (OPA), 1-fluoro-2,4-dinitrobenzene (FDNB) and dansyl chloride are the most commonly used for the analysis of aminoglycosides (Isoherranen and Sobak, 1999). OPA reacts in the presence of mercaptan or other strong reducing agents with primary amines to form fluorescent derivatives. The reagent is stable in different buffer solutions and the reaction has a fast rate at room temperature allowing the derivatization to be performed either pre- or post- column. FDNB easily reacts with both primary and secondary amines, in basic conditions, producing UV absorbing derivatives with maximum absorption wavelength at 365 nm.

The separation columns used for the derivatized aminoglycoside generally contain C8 or C18 silica as stationary phase; the mobile phase consist of acidic buffers with methanol and/or acetonitrile.

Gentamicins C1, C1a and C2 were determined as their 2,4-dinitrophenyl derivatives in plasma and urine using a C18 RP column and as mobile phase 680ml/l acetonitrile-320ml/l Tris buffer (8.3mmol/l titrated to pH 7.0) (Isoherranen and Sobak, 2000). A HPLC method for the analysis of tobramycin in urine samples used pre-column derivatization of tobramycin with fluorescein isothiocyanate followed by fluorescence detection; the chromatographic separation was carried out on a Phenomenex Luna C18 column and the limit of quantitation of the method in urine was 250 ng/mL (Mashat et al, 2008).

Another derivatization agent used for the RP-HPLC analysis of aminoglycoside antibiotics was phenylisocyanate in the presence of triethylamine. Phenylisocyanate groups easily react with amino groups of aminoglycosides like kanamycin, neomycin and gentamicin (Kim et al, 2001 and 2003); the complex structures were confirmed by ESI-MS.

Clarot et al (2005) described a method for the determination of neomycin sulphate, framycetin sulphate and other related compounds by evaporative light scattering detection (ELSD) thus avoiding the need of derivatization for detection. The chromatographic separation was performed on a Polaris C18 column using a mobile phase with 170 mM

trifluoroacetic acid; in this case trifluoroacetate ions act like counter-ions forming ion pairs with aminoglycosides. The ELSD elution of the complexes with antibiotics was confirmed by MS.

Ion-pair chromatography is probably the most popular approach for aminoglycosides analysis; when using standard detectors mainly alkyl sulphonates (pentane, heptane and hexane sulphonates) in acetate or phosphate buffers are selected as counter-ions. For HPLC-MS applications the volatile fluorinated carboxylic acids are preferred. Pre-column and post column derivatization with *o*-phthalaldehyde are often used for the detection of these molecules also when separated by ion-pair HPLC. As an example, Aubin et al separated nine aminoglycoside antibiotics (streptomycin, dihydrostreptomycin, neomycin, sisomicin, gentamicin C1, C1a, C2, C2a, netilmicin) on an Atlantis C18 column eluted with 0.02M potassium dihydrogen phosphate (adjusted to pH 3.0 with phosphoric acid) containing 35 g/l sodium sulfate, 500mg/l sodium octanesulfonate and 15ml/l tetrahydrofuran; the detection was achieved with a pulsed electrochemical detector. Tobramycin and colistin sulphate were simultaneously determined from pharmaceutical formulations using a Zorbax SBC18 column eluted with acetonitrile / water / trifluoroacetic acid and evaporative light scattering detection (Clarot et al, 2009).

Pulsed electrochemical detection was applied also for the analysis of underivatized tobramycin (Shruti et al, 2010) and kanamycin by ion-pairing with octanesulfonate in phosphate buffer (Manyanga et al, 2010). A system for the determination of gentamicin by ion chromatography using pulsed amperometric detector after isocratic elution on a polystyrene column was developed by Metrohm in 2005.

4.3 Analysis of aminoglycosides by mass spectrometry

The concentrations usually monitored in plasma or serum of patients treated with aminoglycoside antibiotics are in the low ng/mL range. Due to their non negligible toxicity in biological systems and to side-effects like inducing resistance in bacteria or alteration of the normal microbial flora (thus influencing various biological systems), antibiotics are also carefully monitored in environmental or food and feed samples. Maximum residue limits (MRLs) have been set in most countries and they are in the low ppb ranges, too. Therefore, mass spectrometry, being a very sensitive and selective analytical technique is extremely important in the analysis of these antibiotics. With respect to aminoglycosides, the GC/MS methods are based on complex and lengthy derivatizations as above mentioned; Preu et al, 1998 adapted the method using trimehtylsilylimidazole and heptafluorobutyrylimidazole for capillary GC/MS.

LC-MS/MS methods have been preferred recently owing to their high sensitivity and ease of use compared to GC/MS. Two non-derivatization approaches have been developed in parallel, reversed phase ion-pair chromatography and HILIC.

Aminoglycoside antibiotics are among the best candidates to be analyzed with HILIC and such methods were applied for difficult matrices like plasma, kidney or meat. Oertel et al (2004) described an automated method for the simultaneous quantification of amikacin, gentamicin, kanamycin, neomycin, paromomycin, and tobramycin in human serum by HILIC-MS/MS. Separation was carried out on a zwitterionic SeQuant ZIC-HILIC column, 100mm × 2.1mm with a Phenomenex SecurityGuard C18, 4mm × 2mm i.d. The mobile phase was composed of acetonitrile, 2mM ammonium acetate and formic acid, in gradient conditions, and data acquisition was performed with a Quattro Micro triple quadrupole

mass spectrometer equipped with ESI source, operated in positive ions mode. Low limits of quantification (LLOQ) of 100ng/mL were obtained using 500µL serum extracted by solid phase extraction (SPE).

Ishii et al (2008) reported a quantification method for seven aminoglycosides from swine and bovine meat and kidney that also employed a SeQuant ZIC-HILIC column (100 mm × 2.1 mm, 5 µm) eluted with a mobile phase composed of (A) ammonium acetate 150 mM + 1% formic acid in water and (B) AcN, at a flow rate of 0.3 mL/min. MS detection was performed in positive mode. LOQs in swine bovine kidney were 25 ng/g for gentamicin, 50 ng/g for spectinomycin, dihydrostreptomycin, kanamycin and apramycin, and 100 ng/g for streptomycin and neomycin, well below the existing FAO MRLs.

Beside HILIC the most common separation technique for aminoglycoside antibiotics is by ion-pair chromatography, performed mostly on octyl (C8) or octadecyl (C18) columns, eluted with mobile phases containing trifluoroacetic (TFA), pentafluoropropionic (PFPA), heptafluorobutyric (HFBA) or nonafluoropentanoic (NFPFA) acids as ion-pair reagents. Good peak shape and high sensitivities are obtained, thus compensating the negative aspect that the ion-pair reagents are serious contaminants for the MS interface.

Shen et al (2008) studied the separation of amikacin, streptomycin, spectinomycin and gentamicin by ion-pair and HILIC. The ion-pair chromatography was performed on an Agilent Zorbax SB-C8 (30 X 2.0 mm, 5 µm column), eluted with a mobile phase consisting of (A) 10 mM NFPFA with 10 mM ammonium hydroxide in water and (B) 5 mM NFPFA acid with 5 mM ammonium hydroxide in a 10:90 mixture of water:acetonitrile, with a gradient from 20% to 90% (B). HILIC was carried out on a SeQuant Zic-HILIC (50 X 2.0 mm, 5 µm) column, with a mobile phase composed of (A) 5 or 25 mM ammonium formate in water with formic acid (pH ≈2.5) and (B) acetonitrile with 1% (v:v) formic acid; the gradient started from 90% B and was decreased to 10%B. A simple protein precipitation with cold acetonitrile was used for clean-up of the different samples (mouse, rat or Guinea pig plasma) and triple quadrupole (API 4000 or 5000) mass spectrometers were used for detection. An LLOQ of 20ng/mL for all four antibiotics was achieved with the ion pair method, while the HILIC method was highly sensitive only for spectinomycin, needing further optimization for the other analytes.

Amikacin, neomycin and gentamicin in plasma or tissue samples were also analyzed by Zimmer et al (2008) using a similar approach. They tested HILIC on a Varian Inertsil 5 Si (50X2.0mm column) with a mobile phase composed of acetonitrile /ammonium acetate 100 mM in water, pH 3, and ion pair separation on a Discovery HS-C18 (2.1x50mm, 3µm) column eluted with 0.1% HFBA in water / 0.1% HFBA in acetonitrile (all methods were carried out using a composition gradient). LLOQs of 5 ng/mL were obtained using ion-pair LC, which also in this case has proven to be more sensitive than HILIC.

Granja et al (2009) described a method for the determination of streptomycin residues in honey that employs ion-pairing with HFBA in water/acetonitrile (85:15, v/v) as a mobile phase (isocratic conditions), on a C18 column (Gemini 5 micron C18, 50x2mm). Also Hammel et al (2009) presented a multi-screening approach for 42 antibiotic residues in honey and 3 aminoglycosides where among them. HPLC analyses were run on a Zorbax SB-C18 reverse phase column (2.1×50 mm, 1.8µm). The mobile phases were constituted with solvent A:water phase containing 1mM NFPFA mixed with 0.5% formic acid (v/v) and solvent B: acetonitrile/methanol (50/50, v/v) containing 0.5% formic acid (v/v).

The following paragraphs present the practical experiments of our group on aminoglycoside antibiotics by LC-MS/MS (unpublished data). The first method was developed for residues of streptomycin in honey, by ion-exchange chromatography, on a Grom-Sil 300WCX

(100x4.6mm, 7 μ m) eluted in gradient with (A) methanol/water (60/40, v/v) and (B) ammonium acetate 500mM in methanol/water (60/40, v/v); the initial mobile phase was 100% A. The honey sample was simply diluted 1/1 with water and injected; detection was achieved with an API 4000 triple quadrupole mass-spectrometer operated in positive ESI ionization. The calibration curve in honey was built over a range of 10 - 250 ng/mL and gentamicin was used as internal standard. In order to improve the sensitivity and robustness of the determination, later we switched to ion-pair chromatography on a pentafluorophenylpropyl column (Discovery HSF5 100x2.1 mm, 5 μ m), using a gradient of PFPA 0.2% in water/PFPA 0.2% in acetonitrile as mobile phase. An LLOQ of 3.3 ng/mL was obtained with the same instrument set-up and same sample preparation; gentamicin was replaced as internal standard by cimetidine. Figure 18 shows two examples of chromatograms, recorded on honey samples.

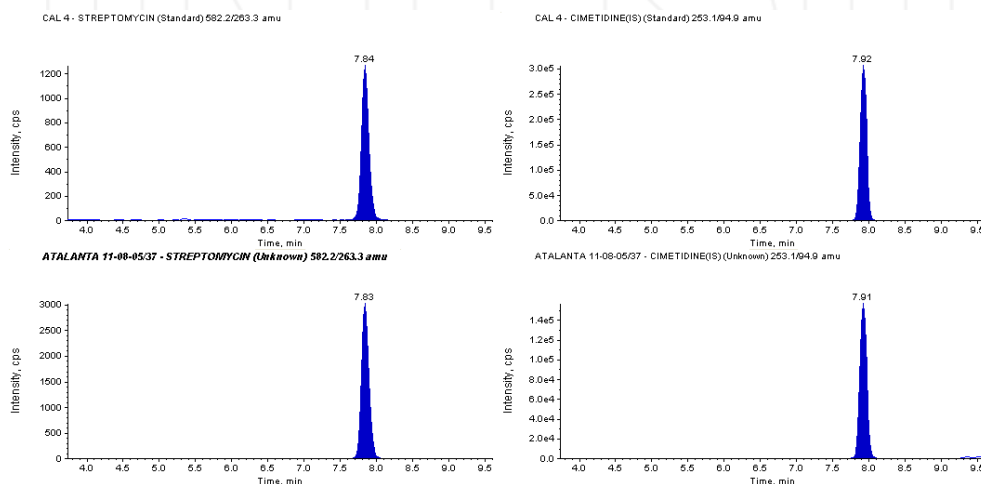


Fig. 18. HPLC traces of the MRM transitions selected for streptomycin and its internal standard cimetidine, recorded on a spiked honey sample - concentration 90 ng/mL (top), and on an unknown sample (bottom chromatograms) that was obviously contaminated with streptomycin. Column: Discovery HSF5 (10cmx2.1mm, 5 μ m), mobile phase: A) 0.2% PFPA in water and B) 0.2% PFPA in acetonitrile; flow: 0.3 ml/min; injection volume: 50 μ L. Mass spectrometer: Triple quadrupole API4000, operated in ESI positive mode.

The same method was later adapted for the analysis of neomycin in ophthalmic solutions, and streptomycin was used as internal standard. The samples were just diluted 1:1000 with the initial mobile phase (water/acetonitrile (95/5, v/v) + 0.2 % pentafluoropropionic acid) and injected in the LC-MS system; being the concentrations quite high (1-5 μ g/mL) no further optimization was needed. An example of chromatogram is presented in Figure 19. Another approach in aminoglycoside analysis by HPLC remains pre-column derivatization, aiming to reduce polarity and make the compounds suitable for reversed-

phase separations. Based on a previous research of Lauser and Bergner-Lang (1995), we tested in our laboratory the derivatization of neomycin with FMOC. The reaction needs to be optimized, because partial and fully substituted derivatives (all six amino groups reacted with FMOC) are formed and in order to obtain a better sensitivity the equilibrium should be ideally directed towards one product. We performed the chromatographic separation on a C8 column eluted with water, acetonitrile and formic acid and preliminary tests on plasma extracts have given promising results. The chromatographic traces corresponding to the fully substituted neomycin and paromomycin derivatives (the selected internal standard) on plasma samples are shown in Figure 20, while the product ion spectrum of neomycin derivative in Figure 21.

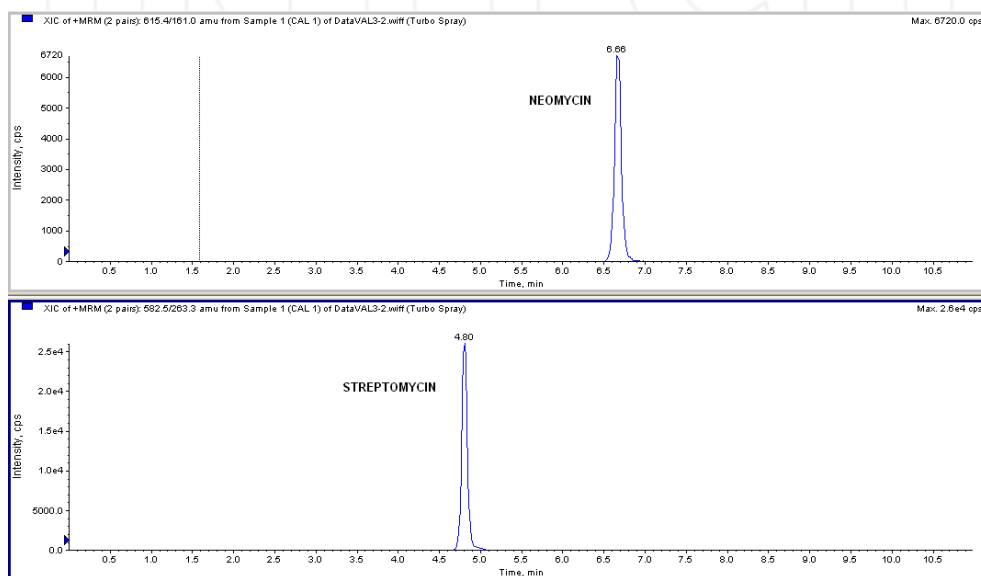


Fig. 19. Chromatographic traces of neomycin and its internal standard streptomycin, recorded on spiked sample (concentration 1 $\mu\text{g}/\text{ml}$). Column: Discovery HSF5 (10cmx2.1mm, 5 μm), mobile phase: A) 0.2% PFFA in water and B) 0.2% PFFA in acetonitrile; flow: 0.3 ml/min; injection volume: 10 μL . Mass spectrometer: quadrupole-linear ion trap API4000 QTrap, operated in ESI positive mode.

Last but not least, we have studied HILIC for the determination of vancomycin in plasma, using neomycin as internal standard. The column employed was an Ascentis Express HILIC (100x2.1mm, 2.7 μm), the separation being carried out in isocratic conditions with acetonitrile/water (90/10, v/v) containing 0.1% formic acid. A good sensitivity in the range of few ng/mL , adequate for therapeutic drug monitoring, was obtained.

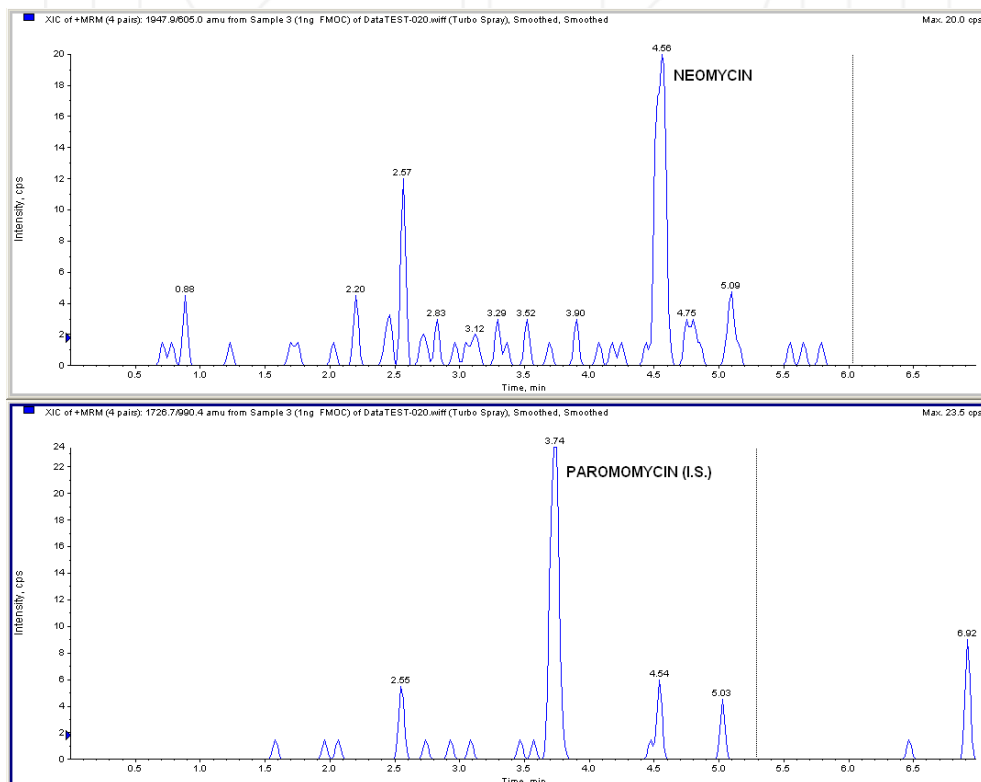


Fig. 20. The MRM transitions of neomycin and paromomycin (internal standard) derivatives with FMOC, obtained after injection of a derivatized plasma extract (spiked concentration 1ng/mL). Column: Ascentis Express C8 (100x2.1mm, 2.7 μ m); mobile phase: A) water/acetonitrile (90/10, v/v) and B) acetonitrile, with composition gradient; injection volume: 50 μ L. Mass spectrometer: triple quadrupole API 4000 operated in ESI positive mode.

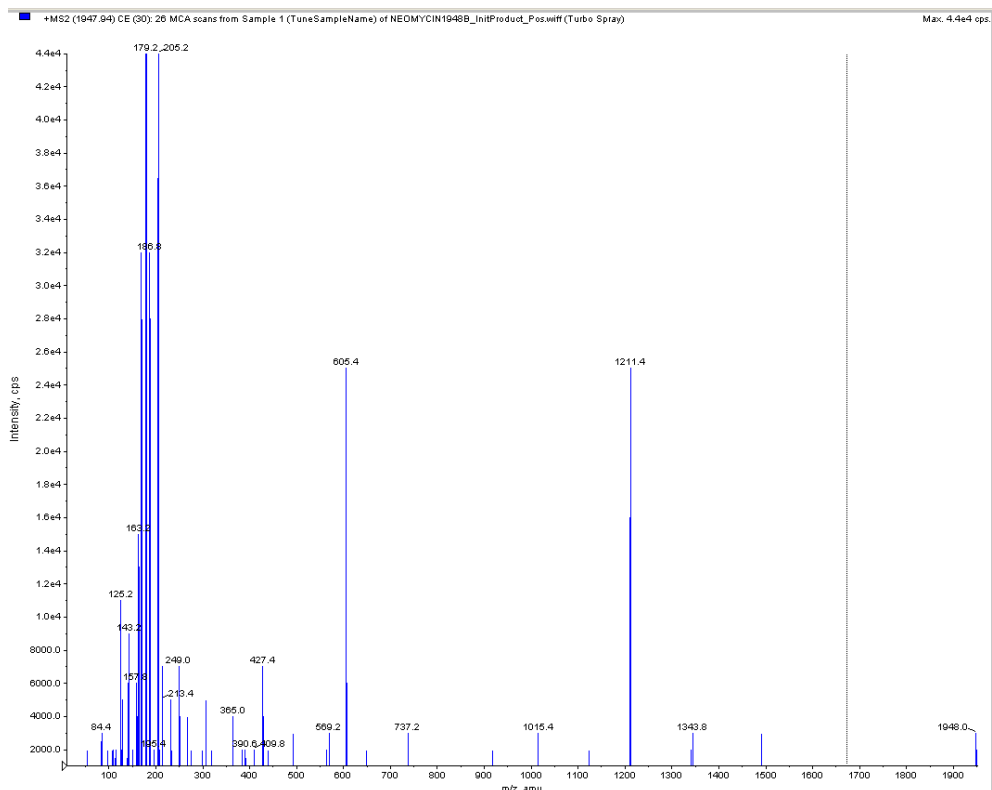


Fig. 21. Product ion spectrum of the neomycin derivative with FMOF - $[M+H]^+ = 1948$ obtained with direct infusion via syringe of a $1\mu\text{g}/\text{mL}$ solution in water/methanol (1/1, v/v). Mass spectrometer: triple quadrupole API4000, operated in positive ESI mode; collision energy: 30V.

5. Conclusions

The data presented above clearly show that, despite the fact that highly polar compounds present optimal characteristics to be analyzed by MS, they are difficult molecules due to critical separation problems generated by the need to employ mobile phases compatible with the ionization source and process. It is therefore essential, when developing a new method for such compounds, to first consider the available options to get an optimal separation compatible with the MS interfacing.

Between the various options HILIC is gaining a lot of interest as a separation for highly polar compounds; the development of several columns dedicated to this technique are widening a lot its application and the growing mass of publications in the field is a prove. HILIC employs high percentage of organic solvent in the mobile phase usually leading to enhanced ionization and lesser contamination of the ion source.

Ion exchange remains an interesting alternative especially when considering weak exchanger stationary phases; in comparison to HILIC however the technical development is moving much slower. Mixed mode columns (ion exchange + reversed phase) are an interesting opportunity to get improved separation methods.

The use of ion-pair reagents in connection with reversed-phase columns is another opportunity but it presents a few pitfalls: restrictions on ionization polarity (in general ion-pair suppress ionization in the same polarity, i.e. quaternary ammonium salt and positive ionization), often complex spectra, reduced analytical sensitivity and finally a tendency to contaminate the ionization source and quadrupoles. It is however interesting, see the GAGs examples, that ion-pair reagents can, in some cases, positively help the ionization reducing source fragmentation.

Globally the previously indicated strategies are preferable whenever possible. As a final option derivatization has to be considered, without meaning that it is the last desirable approach. It has to be considered especially in case of amphoteric compounds bearing both positive and negative charges; such compounds are generally difficult to extract and the ionization is not optimal; aminobisphosphonates are a good example in this context. The development of derivatization methods can be time consuming but often very solid methods and a quite good analytical sensitivity can be obtained. It is interesting to see a continuous development for obtaining optimal derivatization reagents for LC-MS detection. GC derivatization agents are indeed aiming to get non-polar and volatile compounds, aspects not always desirable in LC-MS, while fluorescence and UV detection reagents can be chemically very complex without favoring the ionization process.

The analytical approach described can be usefully applied as well to other classes of highly polar compounds (for examples polynucleotides, phosphorylated carbohydrates, several pharmaceuticals).

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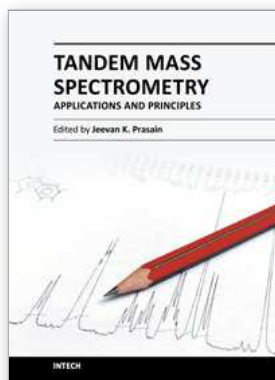
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