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Identification of unknown degradation products in a new cholesterol-reducing drug by ion-chromatography coupled to mass spectrometry

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Abstract

The combination of suppressed and non-suppressed cation-exchange chromatography with electrospray ionization mass spectrometry was demonstrated for the structural elucidation of unknown by-products (mostly quaternary ammonium compounds) in a new cholesterol-reducing drug. The suppressed mode using methanesulfonic acid and acetonitrile turned out to be unsuitable because of regenerant (tetrabutylammonium hydroxide) passing through the membrane of the suppressor into the eluent which led to a significant increase of spectral background in the mass spectrometer. Employing a mobile phase consisting of 200 m*M* formic acid and 60% (v/v) acetonitrile, the separation and detection of 8 unknown compounds was possible in the non-suppressed mode. The three most prominent compounds were selected for structural elucidation utilizing collision induced dissociation experiments. In a series of experiments the fragmentation behavior was investigated for different fragmentation voltages finally leading to structure proposals. Using gas chromatography hyphenated with mass spectrometry, additional information for the structure of the unknowns was collected and a possible way of their formation was proposed.

Keywords: Structural elucidation; Quaternary ammonium compounds; Colesevelam

1. Introduction

Since the mid-1980s, elevated cholesterol (hypercholesterolemia) has been widely recognized as a significant risk factor for coronary heart disease. As a result of the increased awareness and the broad prevalence of elevated LDL cholesterol, cholesterol-reducing drugs have emerged as one of the largest and fastest-growing pharmaceutical product categories.

Recently, a new bile acid sequestrant with the active substance colesevelam hydrochloride has been developed by GelTex Pharmaceuticals [1]. Colesevelam hydrochloride is a crosslinked polyallylamine alkylated with 6-bromohexyltrimethylammonium bromide and decylbromide (Fig. 1). This nonabsorbed hydrogel binds the bile acids and their conjugates through an anion-exchange mechanism combined with a hydrophobic interaction between the hydrophobic portion of the bile acids and the hydrophobic binding site of the polymer [2]. Within the quality control of the active substance different parameters such as bile acid binding, allylamine content, volatile and non-volatile impurities are

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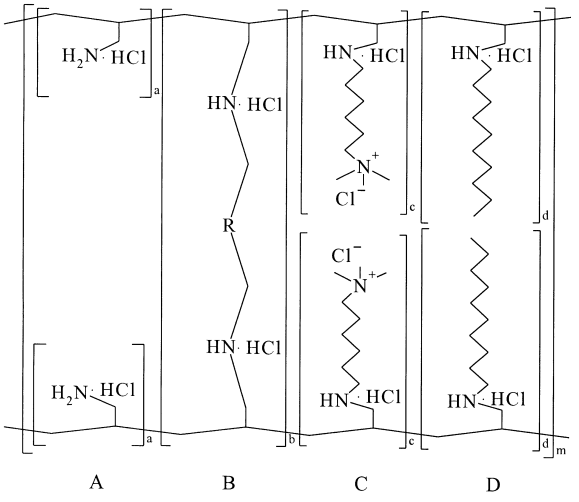

Fig. 1. Structure of the colesevelam hydrochloride gel.

determined by reversed-phase high-performance liquid chromatography (RP-HPLC) with UV- and fluorescence detection, gas chromatography (GC) and ion chromatography (IC) with suppressed conductivity detection. IC is mainly used for determination of various alkyltrimethylammonium compounds present as by-products in colesevelam hydrochloride. Unfortunately, up to now it has not yet been possible to identify all peaks of the IC chromatogram (some at very low concentrations). Therefore, it seemed promising and interesting to combine IC with mass spectrometry.

In general quaternary ammonium compounds like those expected as impurities in colesevelam hydrochloride can be analyzed either by GC after derivatization [3], by RP-HPLC [4–8], ion-exchange chromatography [9,10] or ion-pair chromatography [11–14]. During the last years mass spectrometry (MS) has gained importance for the determination of quaternary ammonium compounds in various contexts. Besides GC–MS [15], especially the combination of HPLC or ion chromatography (IC) with MS using an appropriate interface proved to be a powerful technique for many analytical problems. A thermospray interface for example was successfully employed for the determination of quaternary ammonium drugs [16] and electrospray interfaces have frequently been used for the combination of liquid-phase separation techniques and MS for the analysis of a variety of organic quaternary ammonium compounds [17–19]. Electrospray ionization mass spectrometry (ESI-MS) offers the advantage of a sensitive detection even at rather high HPLC flow-rates (up to 1 ml/min) but on the other hand it usually lacks the possibility to obtain fragmentation spectra which are essential for structure elucidation of unknown compounds. Nevertheless, by increasing the ion extraction voltage in the ESI-interface a certain degree of fragmentation can be inforced; this process is called collision induced dissociation (CID). Using single quadrupole instruments this technique has been successfully employed for the determination and identification of drugs or their metabolites respectively [20,21].

The aim of the present work was to demonstrate strategies for the structure elucidation of unknown compounds in a new pharmaceutical substance which cannot be analyzed by GC–MS. IC–MS offers an alternative approach for that kind of compounds. Unfortunately, the mobile phases commonly used in IC are not always compatible with MS. Therefore, this study should also contribute to the general exploration of suitable conditions of suppressed and non-suppressed IC for combination with MS and a critical comparison of detection limits in the different separation modes.

2. Experimental

2.1. Instrumentation

IC with conductivity detection was performed using a DX-300 quaternary gradient pump, a DX-100 conductivity detector, an AS-40 autosampler and a CMMS II suppressor (all Dionex, Sunnyvale, CA, USA). For the combination with MS, an HP 1100 HPLC System equipped with a vacuum degasser, quaternary pump, UV–Vis diode array detector and an HP 1050 autosampler (all Agilent, Palo Alto, CA, USA) were employed; MS measurements were done on a quadrupole system HP 5989B using a pneumatically assisted electrospray interface HP 59987A (Agilent) equipped with a RF-only hexapole (Analytica of Branford, Branford, CT, USA). For atmospheric pressure chemical ionization (APCI) experiments the APCI interface from Analytica of Branford was employed. The drying gas (nitrogen)

temperature was 300°C using the ESI-interface and 350°C for the APCI interface; the flow-rate was adjusted to 7 l/min in both cases. The nebulizing gas pressure (nitrogen) was 5 bar for both the ESI and APCI interface and a APCI vaporizer temperature of 350°C was selected. GC–MS experiments were performed on an HP 5890 II gaschromatograph coupled with an HP 5989A mass spectrometer (both Agilent).

2.2. Chemicals

Analytical grade acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany), methanesulfonic acid and hydrochloric acid from Fluka (Buchs, Switzerland) and a tetrabutylammonium hydroxide solution (40%) from Riedel-de-Haen (Seelze, Germany). High-purity water was prepared by a Milli-Q water purification system (Millipore, Milford, USA).

The reference substances 6-hydroxyhexyltrimethylammonium (hydroxyquat), 6-aminohexyltrimethylammonium (aminoquat), aminodihexyltrimethylammonium (aminodihexylquat) and decyl-6-aminohexyltrimethylammonium (decylaminoquat) were either supplied by DSM Fine Chemicals Austria (Linz, Austria) or GelTex Pharmaceuticals (Waltham, MA, USA) as their bromide salts with a purity better than 99%.

2.3. Columns and mobile phases

IC was performed on an IonPac CS 14, 250×4 mm column (Dionex) using a gradient of methanesulfonic acid, diaminopropionic acid and acetonitrile in combination with suppressed conductivity detection. For IC in combination with MS detection, isocratic conditions were employed with eluents containing either methanesulfonic acid (suppressed mode), trifluoroacetic acid or formic acid (non-suppressed mode) and acetonitrile.

2.4. Sample pretreatment

10 ml of 10 m*M* hydrochloric acid were added to an amount of sample corresponding to 1 g dry active substance weighed accurately into a centrifuge tube. After shaking the tube for a minimum of 30 min with a wrist action shaker, the sample was centrifuged. A portion of the clear solution was additionally filtered through a syringe-driven filter unit (pore size: 0.22 μm) and was used for the experiments.

3. Results and discussion

3.1. Optimization of the IC separation

The existing method for the determination of quaternary ammonium compounds employed for the quality control of colesevelam hydrochloride is based on suppressed IC with conductivity detection using an IonPac CS14 column with a mixture of methanesulfonic acid, 2,3-diaminopropionic acid and acetonitrile as the mobile phase. The methanesulfonic acid concentration is increased from 3.6 m*M* to 27 m*M* within 40 min and the acetonitrile content is increased from 14% (v/v) to 45% (v/v) within the same period of time; the concentration of 2,3-diaminopropionic acid is kept constant at 4 m*M*. With this method it was possible to separate the possible impurities hydroxyquat (6.5 min), aminoquat (14.0 min), aminodihexylquat (28.5 min) and decylaminoquat (38.2 min) besides other cations such as sodium, ammonium and trimethylammonium. Nevertheless, unknown compounds of interest, eluting within the first seven min, could not be sufficiently separated from hydroxyquat. Therefore the elution strength of the eluent was lowered step by step to improve the resolution of the unknowns. Additionally, the diaminopropionic acid was omitted with regard to mass spectrometric detection. In a series of experiments isocratic conditions using methanesulfonic acid at concentrations of 30, 20 and 10 m*M* and an acetonitrile content of 40, 30 and 9% (v/v) respectively were used for the separation of the early eluting species. As a result two unknown components could be separated from hydroxyquat with an eluent consisting of 10 m*M* methanesulfonic acid and 9% (v/v) acetonitrile. The mass spectrometric detection was performed after suppression using 100 m*M* tetrabutylammonium hydroxide (TBAOH) at a flow-rate of 3 ml/min as the regenerant. Scanning from m/z 50 to 400, a prominent peak appeared at m/z 242 which could be attributed to tetrabutylammonium that passed through the mem-

brane of the suppressor module and caused a high background signal of the MS detection [17]. Despite the presence of TBA^+ in the eluent, which is assumed to exhibit suppressing effects on the electrospray process, the two unknown compounds could be detected after background subtraction showing a molecular mass of 174 (named unknown 1 in the following) and 176 (named unknown 2 in the following). For further experiments (collision induced dissociation) the sensitivity of the suppressed system seemed to be too poor because of the high spectral noise; the detection limit for the standard material hydroxyquat was 0.05 mg/ml for the total ion current chromatogram (scan range 50 to 400) using a signal-to-noise criterion of 3. Therefore non-suppressed IC–ESI-MS systems based on a volatile organic acid as the eluting species were investigated to overcome the problem of high spectral noise.

3.2. Non-suppressed IC–ESI-MS

The difficulty in non-suppressed IC was to find a suitable eluent which offered both a reasonable separation and MS compatibility. Trifluoroacetic acid (TFA), one of the most acidic organic acids (pK_a 0.23) being compatible with MS in terms of volatility, was successfully employed for the separation of three unknown compounds using conductivity detection. Because of suppressing effects as described previously [22] and ion pair formation with cations in the ESI-interface, TFA led to a strong decrease of sensitivity in MS detection which made it unsuitable for that purpose.

Formic acid has frequently been used for liquid chromatographic separations in combination with electrospray MS but was rarely employed for cation-exchange chromatography because of its comparably high pK_a-value of about 3.7. To compensate for this drawback, the employment of a rather high concentration (200 m*M*) was investigated and found to be suitable for the separation of the early eluting species. Additionally, the influence of the acetonitrile content in the mobile phase on sensitivity was studied but it hardly affected the signal-to-noise ratios. As a result, the detection limit for hydroxyquat could be remarkably improved (0.01 mg/ml, $S/N=3$). The sensitivity for unknown 1 could be increased by a factor of 3 and by a factor of 2.3 for unknown 2 using the signal-to-noise criterion (compared to the suppressed system) with an eluent consisting of 200 m*M* formic acid and 60% (v/v) acetonitrile. For further sensitivity enhancement an injection volume of 50 μl was applied instead of 10 μl. With this improved method finally 8 unknown compounds could be detected with the MS. For structure elucidation the five most prominent peaks were selected exhibiting S/N ratios not smaller than 4 for the extracted molecular ion (scan mode m/z 50–400). Fig. 2 shows the extracted ion chromatograms of these 5 unknown compounds in a real sample acquired in the SIM mode.

3.3. Structure elucidation of selected unknown compounds

3.3.1. ESI-MS fragmentation spectra

The applicability of ESI-MS for the identification of unknown compounds is limited compared to electron impact ionization (used in GC–MS) or some other types of ionization sources. Nevertheless, if a liquid phase separation technique is the only one which can be reasonably employed for an analytical problem, the combination with MS by an atmospheric pressure ionization interface offers some information for a final structure elucidation; an

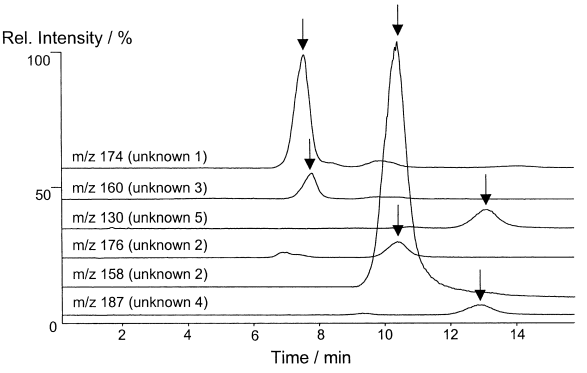

Fig. 2. Extracted ion chromatograms of a real sample containing unknown compounds. The arrows indicate the peaks corresponding to the unknown compounds 1 to 5. IC-conditions: Column: IonPac CS14 (250×4 mm). Mobile phase: 200 m*M* formic acid containing 60% (v/v) acetonitrile. Flow rate: 1 ml/min (split: 100 μl/min into the MS). Injection volume: 50 μl. MS-conditions: Positive ion mode (SIM). Nebulizing gas pressure: 70 p.s.i. (N_2). Drying gas flow-rate: 7 l/min (N_2). Drying gas temperature: 300°C.

important tool in this context are collision induced dissociation (CID) experiments. By increasing the potential difference between the sampling orifice (in this case a transfer capillary with metallized ends) and the first skimmer a certain degree of fragmentation depending on the voltage is observed. In the present analytical problem, CID experiments were performed for five unknown compounds varying the potential at the capillary exit between 75 and 225 V; the resulting curves are shown in Fig. 3. As can be seen, compound 1 shows only one major fragment ion besides the molecular ion (m/z 174) which is at a

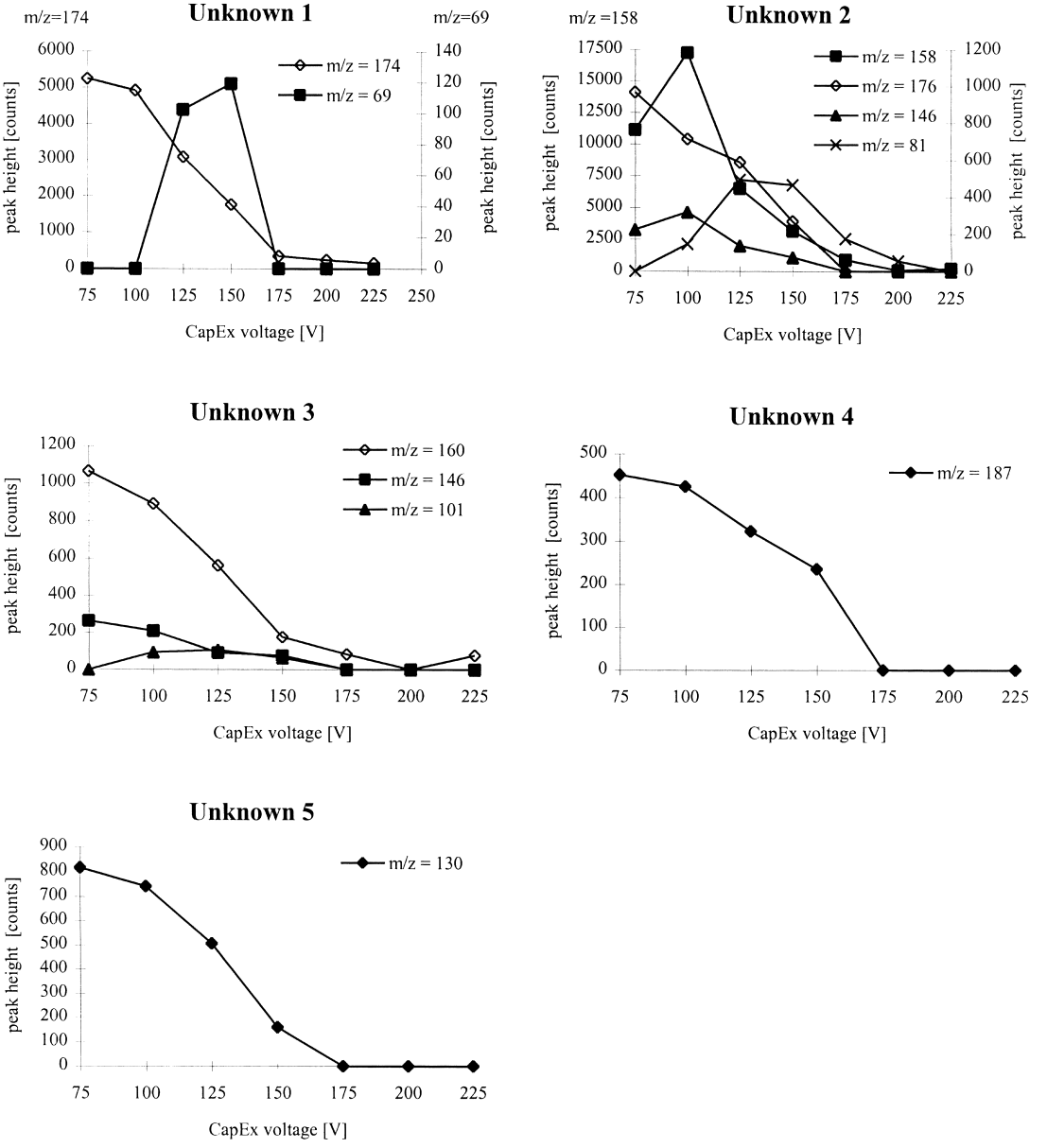

Fig. 3. Fragmentation behavior of five unknown compounds (CID experiments). IC-conditions as in Fig. 2. MS-conditions: Positive detection mode (Scan m/z 50–400). Other conditions as in Fig. 2.

m/z value of 69. The abundance of the molecular ion steadily decreases with increasing capillary exit voltage whereas the intensity of the fragment ion reaches a maximum at a voltage of approximately 150 V. The same effects can be observed for unknown 2 which shows a decreasing abundance of the molecular ion (m/z 176) and maxima for fragments at 100 V (m/z 158 and 146) and at 125 V for the fragment at m/z 81 respectively. Compound 3 (m/z 160) shows two fragments (m/z 146 and 101); for compound 4 and 5 obviously no fragments can be detected which may be partly attributed to a rather low concentration of these unknowns in the sample and therefore the abundance of possibly occurring fragments may be too low for detection.

In addition to ESI-MS also APCI-MS was investigated in a series of experiments for the current analytical problem but unfortunately no additional information was obtained by this technique.

3.3.2. Structure proposals for the unknown compounds 1 to 3

It is assumed that the unknown compounds show structures similar to the already known impurities because of their similar separation behavior in IC. Furthermore the existence of a quaternary ammonium group is likely since the mass spectrometric response using an ESI-interface is at least for compounds 1, 2 and 3 rather high. The possibility of the existence of an amino group instead of the quaternary ammonium can be excluded since previous GC-experiments to determine these compounds failed most probably because of the low volatility which is the case for quaternary ammonium compounds. Nevertheless, a final structure assignment is difficult since ESI-MS is known as a very mild ionization source and even CID-experiments are commonly restricted to the identification of compounds which can easily undergo fragmentation, for example by the elimination of a neutral fragment like water from a hydroxyl group.

Taking into account the structure of the original gel (see Fig. 1) and assuming the presence of a trimethylammonium group the conclusion seems to be justified that the unknown compounds most probably are derivatives of hexyltrimethylammonium. For the unknown compound 1 with a molecular mass of 174 the only reasonable structure proposal is 5-methoxyhexyltrimethylammonium; the 6-methoxyhexyltrimethylammonium isomer has been suggested as a possible by-product before. Another indication of this theory is the fact that the unknown compound 1 has also been found as a by-product in the 6-methoxyhexyltrimethylammonium standard.

Unknown 2, exhibiting a molecular mass of 176, obviously contains at least one hydroxyl group because in the CID-experiments the loss of water from the molecule (m/z 158) can be observed. The fact that 6-hydroxytrimethylammonium, which is available as a standard material, does not show the loss of water although it is containing a hydroxyl group, leads to the assumption that the unknown compound 2 contains another functional group nearby the concerning hydroxyl group, which facilitates the split off of water. Finally the only reasonable structure of the unknown compound 2 is 5,6-dihydroxyhexyltrimethylammonium. Based on similar considerations as for compound 1 and 2, unknown compound 3 showing a molecular mass of 160 was finally assigned to 5-hydroxyhexyltrimethylammonium. For the confirmation of these results it might be advisable to synthesize these compounds and inject each of them into the IC/MS. As the present work focussed on IC/MS aspects, such a chemical synthesis has not yet been carried out.

3.3.3. GC–MS experiments

Additionally to IC–MS measurements also GC–MS experiments were performed with the aim to find further indications for the correctness of the proposed structures previously revealed by ESI-MS. In the sample the following compounds could be identified: 1-chlorodecane, 1-methoxydecane, 1,1-dimethoxydecane, methyldecanoate and 2-decanol. While the origin of most of these compounds can be explained, the presence of the 2-decanol is surprising, since no decane derivative with a reactive group in position 2 has ever been employed. A possible explanation for the occurrence of this compound may be its formation due to Hofmann elimination. Looking at the region D in Fig. 1, the Hofmann elimination can either take place between the chain of the gel and the secondary amine giving decylamine (which is normally present in real samples and can be analyzed by GC) and a carbon–carbon double

bond to the chain of the gel, or the degradation takes place between the secondary amine and the decyl rest giving 1-decene and leaving an amino group at the chain of the gel. In the second case the 1-decene can react with bromide being present in the gel forming 2-bromodecane which may undergo hydrolyses forming 2-decanol.

The same reaction scheme is also possible for the region C of the gel; in this case the cleavage between the secondary amine and the hexyltrimethylammonium rest during the Hofmann elimination leads to aminoquat (generally present in real samples and detectable by IC), while the cleavage between the chain of the gel and the secondary amine leads to 5-hexenyltrimethylammonium, finally reacting to 5-hydroxyhexyltrimethylammonium. The two reaction paths are schematically depicted in Fig. 4.

The reaction of 5-hexenyltrimethylammonium with bromine leading to 5,6-dibromohexyltrimethylammonium is assumed to be responsible for the formation of 5,6-dihydroxyhexyltrimethylammonium. The presence of bromine can be explained by the oxidation of bromide which was still determined in the gel (exhibiting strong anion-exchange properties) even after intense washing procedures. Another hint for the formation of bromine is the change of the gel's color (from white to yellow) during storage.

4. Conclusions

CID experiments could be successfully employed for structure proposals of unknown compounds in colesevelam hydrochloride gel. Together with other data obtained by GC–MS three unknowns could be identified as methoxy- and hydroxy-derivatives of hexyltrimethylammonium. The formation of the hydroxy-derivatives could be explained from different pathways of the Hofmann elimination of the gel; the presence of bromide in the gel was attributed to be responsible for the final conversion of the degradation products to the hydroxy-derivatives.

Although other MS-techniques using a triple quad-

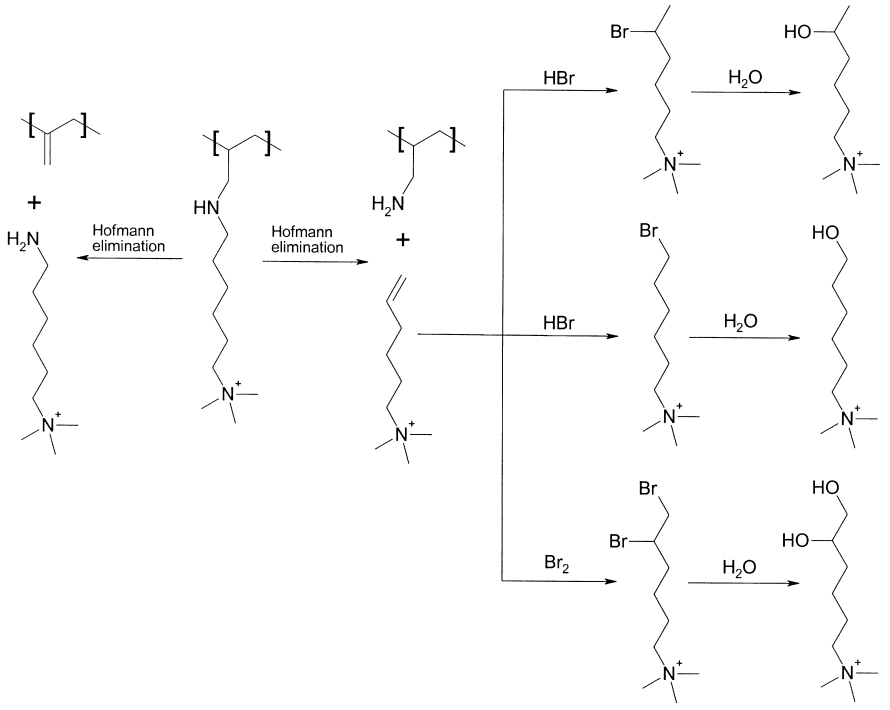

Fig. 4. Possible reaction paths of the Hofmann elimination of colesevelam hydrochloride resulting in three different hydroxy derivatives of hexyltrimethylammonium.

rupole or ion trap instrument may produce a higher degree of fragmentation leading to more expressive mass spectra, the single quadrupole instrument used in the present work together with the results from other experiments was sufficient for the proposal of the unknown's structures.

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