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Micellar electrokinetic chromatography–electrospray ionization mass spectrometry for the identification of drug impurities

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Abstract

Previously, we have presented a system hyphenating continuous micellar electrokinetic chromatography (MEKC) with electrospray ionization mass spectrometry (ESI-MS). Here we evaluate this technique for its applicability in impurity profiling of drugs using galantamine and ipratropium as test samples. A background electrolyte (BGE) of 10 mM sodium phosphate (pH 7.5), 12.5–15% acetonitrile and 20 mM sodium dodecylsulfate (SDS) was used for the MEKC–MS analysis of a galantamine sample containing a number of related impurities, and a heat-treated solution of ipratropium containing a number of unknown degradation products. MEKC provided efficient separation of all sample constituents. Despite the presence of non-volatile BGEs, all impurities in the galantamine sample could be detected by ESI-MS in their respective extracted ion traces (XICs) with a detection sensitivity in the sub-μg/ml range (full-scan mode). MS/MS detection provided useful product spectra allowing the structural characterization of the respective galantamine impurities. With the MEKC–MS/MS system, two degradation products could be revealed and identified in the heat-stressed ipratropium sample. The presented method shows good potential for the detection and structure elucidation of minor impurities in drug substances.
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Keywords: Micellar electrokinetic chromatography (MEKC); Mass spectrometry (MS); Impurity profiling

1. Introduction

Micellar electrokinetic chromatography (MEKC) is a powerful mode of capillary electrophoresis (CE) in which charged surfactants, such as sodium dodecylsulphate (SDS), are added to the background electrolyte (BGE) at concentrations above the critical micelle concentration (CMC). As the charged micelles (pseudo-stationary phase) and the BGE (aqueous phase) will move with different velocities when high voltage is applied, differential partitioning of analytes between the phases leads to separation. MEKC actually employs the combined effects of partitioning and electrophoretic mobility of the compounds, and can be used for the analysis of both neutral and charged compounds [1–3]. Like any CE technique, MEKC provides high separation efficiencies. MEKC has a wide applicability and has proven to be a valuable tool for the impurity profiling of drugs where (related and unrelated) impurities and degradation products, which may be partly unknown, have to be separated from the main compound [4–7]. Next to the number of impurities, also the identity of the impurities has to be elucidated when their relative concentration exceeds 0.1%.

Structure elucidation of analytes after separation is most commonly achieved by coupling with mass spectrometry (MS). Unfortunately, direct coupling of MEKC with electrospray ionization MS (ESI-MS) is often considered problematic because of the high concentrations of non-volatile constituents, like buffer salts and surfactants, as commonly applied in MEKC BGEs [8,9]. Consequently, so far coupling of MEKC and MS has been mainly achieved by so-called partial filling techniques in which the surfactant molecules are prevented from entering the ion source of the mass spectrometer [8,10]. However, recently we have demonstrated that direct coupling of MEKC and MS is feasible applying conventional separation conditions [11–13]. MEKC runs of test mixtures of drugs using a BGE of 20 mM sodium dodecyl sulfate and 10 mM sodium phosphate buffer (pH 7.5) could be monitored by ESI-MS [11,13]. Despite ionization

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suppression by the non-volatile BGE constituents, significant analyte signals could still be measured indicating a sensitivity that might be sufficient for drug impurity profiling at the 0.1% level. Furthermore, repeated MEKC–ESI-MS analyses showed that the salts and surfactants did not seriously affect the performance of the mass spectrometer.

In this paper, the potential of the continuous MEKC–ESI-MS approach for impurity profiling is tested using samples of the drugs galantamine and ipratropium. Galantamine (Reminyl®) is both an acetylcholinesterase inhibitor and a nicotine receptor agonist, and is primarily used for the treatment of mild to moderate Alzheimer's disease [14]. Ipratropium is an anticholinergic agent which is used as a bronchodilator in the treatment of asthma [15]. A test sample of galantamine spiked with a number of related compounds at the 0.25% level was analyzed in order to evaluate the usefulness of the MEKC–MS/MS system to separate and identify drug impurities. The MEKC–ESI-MS/MS method was further evaluated by the analysis of a heat-stressed ipratropium sample containing some unknown degradation products. It is demonstrated that despite the presence of non-volatile surfactant and buffer constituents, highly useful structural data can be obtained for the analyzed impurities.

2. Experimental

2.1. Chemicals and materials

Sodium dodecyl sulfate, phosphoric acid, sodium hydroxide, formic acid, triethylamine, and disodium hydrogenphosphate were purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile were from Biosolve (Valkenswaard, The Netherlands). Galantamine and related compounds were from Johnson & Johnson Pharmaceutical Research and Development (Beerse, Belgium). The identity of the related compounds was as follows: (1) galantamine-N-oxide, (2) norgalantamine, (3) dihydrogalantamine, (4) narwedine, (5) epi-galantamine and (6) dehydrogalantamine. Full molecular structures can be found in [16]. Ipratropium was obtained from the Canisius Wilhelmina Hospital (Nijmegen, The Netherlands). Fused-silica capillaries were from Polymicro Technologies (Phoenix, AZ, USA) and flushed with 1 M sodium hydroxide (10 min) and water (10 min) prior to use.

Stock solutions of galantamine (5 mg/ml) and the related compounds (1 mg/ml each) were prepared in methanol. A mixture of galantamine (1 mg/ml) and related compounds (2.5 μg/ml each) was prepared by mixing the stock solutions in the proper ratio and deluting with water, representing 0.25% impurity levels. Accelerated degradation of ipratropium was achieved by storing a solution of 1 mg/ml for 32 days at 80 °C. For the CZE experiments, BGEs of 10–50 mM sodium phosphate (pH 2.5 and 7.5) and 25 mM sodium borate (pH 9.3) were used. The phosphate buffers were prepared by mixing equimolar solutions of phosphoric acid and disodium hydrogenphosphate of the proper concentration to the desired pH. The sodium borate buffer was prepared by adjusting a 25-mM sodium borate solution to pH 9.3 with a 2-M sodium hydroxide solution. For the MEKC experiments, the BGE contained 10 mM sodium phosphate (pH 7.5), 20 mM SDS and 10–15% acetonitrile. The BGE was prepared by adjusting a 15-mM solution of disodium hydrogenphosphate with 15 mM phosphoric acid to a pH 7.5. Subsequently, acetonitrile and water were added to the buffer until a final concentration of 10 mM phosphate and 10–15% acetonitrile. Finally, SDS was dissolved in the proper concentration. The BGEs were sonicated prior to use. The composition of the sheath liquid was formic acid-acetonitrile–water (0.1:75:25, v/v/v), which was shown to provide the best performance in terms of sensitivity and stability.

2.2. CE and LC systems

MEKC with UV absorbance detection was carried out on a P/ACE 5510 system (Beckman Coulter, Fullerton, CA) or an Agilent Technologies HPCE (Agilent Technologies, Waldbronn, Germany), both equipped with a diode array detector. The capillaries had an I.D. of 75 μm and a length of 57 cm (Beckman system) or 48.5 cm (Agilent system) and were thermostated at 20 °C. Sample was injected by applying a pressure of 35 mbar (3500 Pa) for 4 s, and the separation voltage was 30 kV. Detection was performed at a wavelength of 214 nm (bandwidth 10 nm).

MEKC with MS detection was performed using a PrinCE CE system (Prince Technologies, Emmen, The Netherlands) with a 75-μm I.D. capillary of 57 or 85 cm which was not thermostated. The separation voltage was 30 kV and samples were injected using a pressure of 35 mbar for 6 s. During MEKC–MS analyses, a pressure of approx. −20 mbar (−2000 Pa) relative to ambient pressure was applied at the inlet vial to minimize the hydrodynamic flow in the capillary caused by the suction effect of the nebulizer gas. The overall capillary flow rate during a MEKC run was ca. 500 nl/min. Prior to each analysis the capillary was flushed with fresh background electrolyte for 1 min at 2000 mbar (4–7 capillary volumes). During capillary conditioning, the electrospray voltage and the nebulizer gas flow were switched off to prevent excessive contamination of the ion source.

Liquid chromatography (LC) was performed using a Shimadzu LC system (Shimadzu Benelux, 's-Hertogenbosch, The Netherlands) equipped with a UV detector (detection wavelength, 210 nm). A HP Hypersil BDS-C_{18} column (Agilent Technologies, Waldbronn, Germany) was used in combination with a mobile phase consisting of 80% water-triethylamine (79:1, v/v) adjusted to pH 2.5 using phosphoric acid and 20% methanol at a flow rate of 0.5 ml/min.

2.3. MS system

MEKC was coupled to MS using a triaxial sheath liquid interface of Agilent Technologies (Waldbronn, Germany). An Agilent 1100 Series LC/MSD SL ion-trap mass spectrometer (Agilent Technologies) equipped with an ESI source was used. The MS was operated in positive ion mode and the electrospray voltage was optimized and set to +5.0 kV. The sheath liquid was administered using a KDS100 syringe pump (KD Scientific, Holliston, MA, USA) at a flow rate of 5 μl/min in all cases. The nebulizing gas pressure was adjusted to 15 psi (103 kPa). In

full scan mode, the scan range was 150–600 m/z and 3 scans were averaged for one spectrum leading to a data acquisition rate of ca. 3 Hz. The Ion-Charge-Control option of the instrument was enabled to avoid overloading of the ion-trap. In MEKC–ESI-MS/MS analysis, the m/z-values of the target compounds to be fragmented were manually provided to the software, and the fragmentation voltage was self-regulated by the software. Cleaning of the ion source was done weekly according the manual of the MS instrument. During this procedure, the source was removed from the mass spectrometer and thoroughly rinsed with a mixture of methanol–water (50:50, v/v). The spray shield was cleaned daily by wiping it with a tissue wetted with the water–methanol mixture.

2.4. Analysis of MS data

Minor compounds often cannot be distinguished in the total-ion chromatogram (TIC) trace because of the relative strong contribution of BGE constituents to the MS signal. Signals from (unknown) minor compounds such as impurities were revealed from the dataset using a simple home-made program which can handle data as produced by the Agilent acquisition software. Briefly, the program automatically checks each extracted-ion chromatogram (XIC) for relevant peaks using both an intensity and a peak width criterion. The peak intensity should be at least twice the intensity of the average signal (i.e. base line) of the respective XIC, and the peak width should be in the 0.01–0.2 min range (narrower peaks are considered to be spikes, broader peaks do not originate from sample constituents). The resulting XICs are plotted for visual inspection. This way, relevant analyte signals could be revealed effectively.

3. Results and discussion

3.1. MEKC–MS of galantamine

Optimization of the separation of a mixture of galantamine (1 mg/ml) and six related potential impurities was performed using MEKC–UV. A BGE containing SDS, acetonitrile and 10 mM sodium phosphate buffer (pH 7.5) was used, and the concentrations of SDS and acetonitrile were varied. It was found that all constituents could be separated when the SDS concentration was 20 mM or higher, and the BGE contained 10–15% acetonitrile, however optimum resolution was obtained using 15% acetonitrile. In a previous study on continuous MEKC–MS, we showed that significant analyte MS signals could still be recorded under stable electrospray conditions when the SDS concentration was not higher than 20 mM SDS [13]. Therefore, a BGE of 20 mM SDS, 10 mM sodium phosphate (pH 7.5) and 15% acetonitrile was used for the MEKC–ESI-MS analysis of the galantamine sample yielding a nice separation (Fig. 1). Compared to the capillary zone electrophoresis (CZE) analysis using a volatile buffer, as reported in [16], MEKC provides additional selectivity. With MEKC, the separation window is larger leading to an overall improved resolution. For instance, in contrast to CZE, an isomeric impurity of galantamine (peak 5, Fig. 1) could now be separated, and also a neutral compound could be resolved from the system peak caused by the electroosmotic flow (peak 1, Fig. 1).

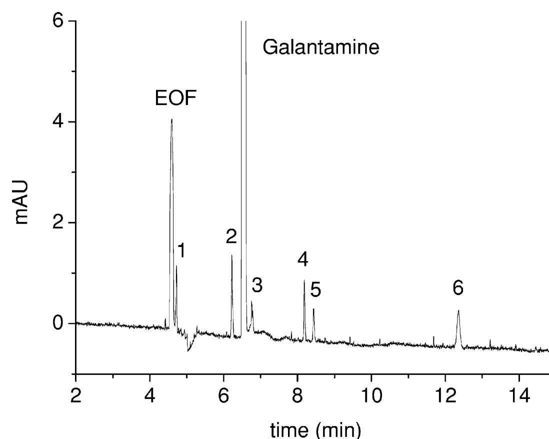


Fig. 1. MEKC–UV of a mixture of 1 mg/ml galantamine and six related compounds (1–6) at 0.25% relative to the main compound, using a BGE containing 10 mM sodium phosphate (pH 7.5), 20 mM SDS and 15% acetonitrile, and the Agilent Technologies CE system. Capillary length, 48.5 cm. Compounds 1–6, see Section 2.

The MEKC system was directly coupled to an ion-trap mass spectrometer equipped with an ESI source using a triaxial sheath liquid interface without preventing the micelles from entering the ion source. Besides a peak for galantamine, no signals of other compounds could be distinguished in the TIC due to the

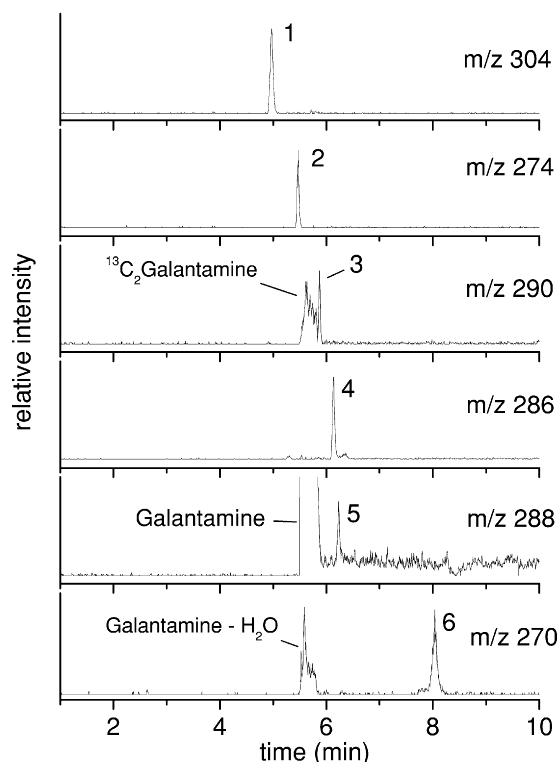


Fig. 2. XICs obtained during MEKC–ESI-MS of a mixture of 1 mg/ml galantamine and six related compounds (1–6) at 0.25% relative to the main compound, using a BGE containing 10 mM sodium phosphate (pH 7.5), 20 mM SDS and 15% acetonitrile. Peak numbers correspond to peak numbers in Fig. 1. Capillary length, 85 cm.

relatively strong contribution of sodium-SDS adducts to the total signal [13]. However, signals originating from the related compounds could clearly be distinguished in the respective XICs without interference of SDS related signals (Fig. 2). The main compound galantamine migrates at about 5.8 min and is detected as its pseudo-molecular ion at *m/z* 288. The same XIC reveals an isomeric potential impurity (peak 5 at 6.2 min). The related compound with *m/z* 290 (peak 3) is just separated from a band caused by galantamine molecules containing two ^{13}C isotopes. The XIC at *m/z* 270 shows a related compound (peak 6), but also an irregular band at 5.8 min which coincides with the galantamine peak. This band is most probably caused by a neutral loss of water occurring during the ESI of galantamine (i.e. in-source fragmentation). The latter underlines the importance of separating impurities from the main compound prior to MS analysis.

In comparison to the MEKC separation using UV detection, the migration window is somewhat compromised, however, the separation of all constituents is fully preserved. The plate numbers are quite satisfactory ranging from 75,000 to 170,000 plates indicating that no serious band broadening is induced by the sheath-liquid interface. Considering the observed signal-to-noise ratios and the concentration of the analyzed related compounds with respect to galantamine (i.e. 0.25%), the overall sensitivity of the method allows detection of the related compounds at a level lower than 0.1% relative to the main compound, being a critical level in analysis of pharmaceutical substances. The detectability for the isomeric compound detected in the XIC of galantamine (*m/z* 288) is somewhat reduced due to the increased background and noise levels after elution of the overloaded main compound.

The *m/z*-values of the related compounds were manually provided to the software and the sample was separated again by MEKC now applying ESI-MS/MS detection. In this way, for all six related compounds information-rich MS/MS-spectra were obtained which can be used to elucidate and/or confirm the identity of impurities. The obtained MS/MS spectra

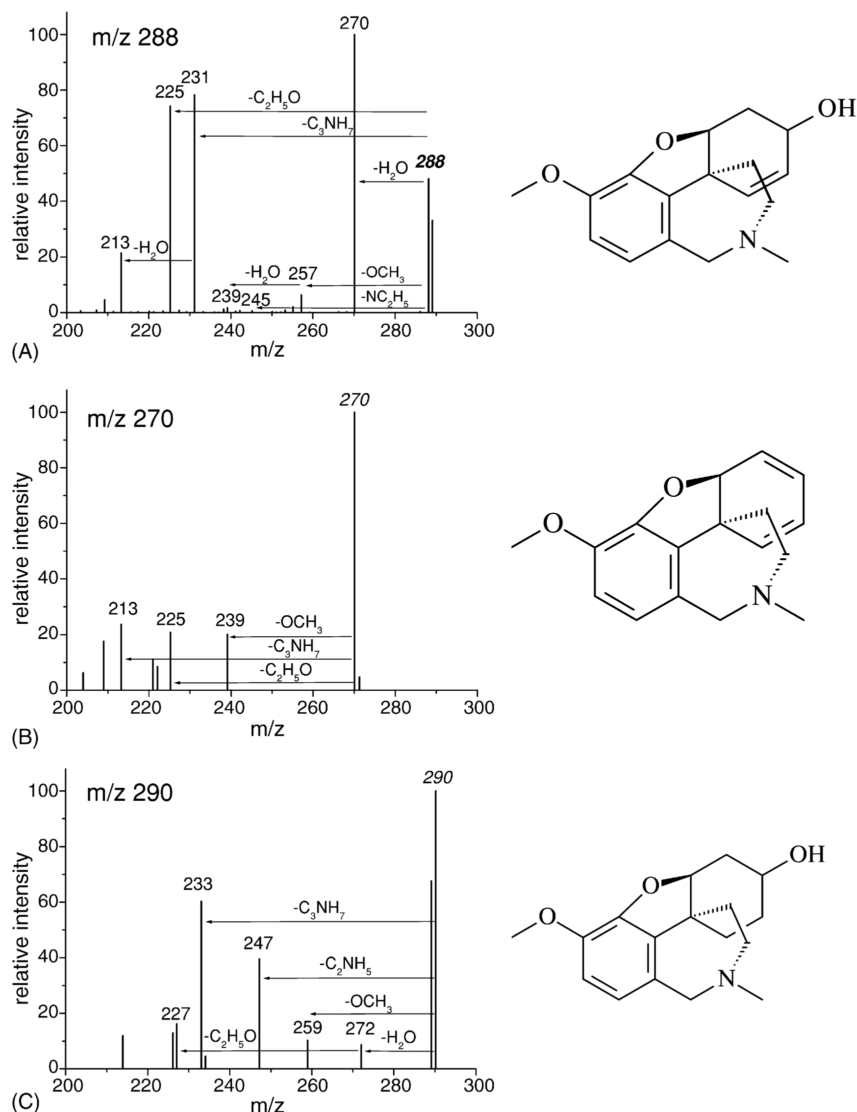


Fig. 3. Product spectra and molecular structures of (A) galantamine (*m/z* 288) and related compounds with (B) *m/z* 270 and (C) *m/z* 290 obtained during MEKC–ESI-MS/MS analysis of the mixture of galantamine and related compounds. BGEs and capillary length, see Fig. 2.

largely correspond to the spectra obtained by Visky et al. [16] who used CE–ESI-MS/MS for the analysis of a corresponding galantamine sample using a volatile BGE. This suggests that representative ESI-MS/MS spectra can indeed be obtained in presence of non-volatile buffer and surfactant, and that relevant and useful data are acquired. Some typical product spectra, i.e. of galantamine (*m/z* 288) and the related compounds with *m/z* 270 and 290, are presented in Fig. 3. Interpretation of the various fragment masses is depicted in the respective mass spectra together with the corresponding molecular structures of the analytes. The related compound with *m/z* 270 is a degradation product of galantamine (a water molecule has been split off). The product spectrum (Fig. 3B) indeed shows that this compound exhibits a similar fragmentation pattern as galantamine, however, no fragments corresponding to a loss of water (18 Da) are observed. The related compound detected at *m/z* 290 corresponds to a galantamine like structure lacking a double bond (Fig. 3C). This is confirmed by MS/MS detection which shows the same fragmentation as galantamine with fragments that are increased with 2 Da when compared to galantamine.

3.2. MEKC–MS of ipratropium

The applicability of the continuous MEKC–ESI-MS system was further evaluated using a solution of 1 mg/ml ipratropium which was stored at 80 °C for 32 days. Initially, the degraded sample was analyzed by LC–UV, CZE–UV and MEKC–UV. LC–UV using a reversed-phase column and a low-pH mobile phase revealed two degradation products at the retention times 11.5 and 26.5 min, respectively, next to the ipratropium main peak (8.0 min). Analysis of this sample using CZE applying BGEs of 10–50 mM sodium phosphate (pH 2.5 and 7.5) and 25 mM sodium borate (pH 9.3), showed only one degradation product. Applying MEKC–UV with a BGE containing 20 mM SDS, 10 mM sodium phosphate (pH 7.5) and 12.5% acetonitrile, again two degradation products were found with migration times of 5.9 min and 14.3 min, with ipratropium migrating at 12.6 min. The peak areas of the two degradation products relative to the main compound as observed in LC–UV and MEKC–UV (detection wavelength, 210 nm) corresponded well. In LC–UV the relative peak areas were 7.1% and 1.9% for the respective degradation products, whereas with MEKC–UV 7.6% and 1.9% was found.

With MEKC–ESI-MS, ipratropium was detected as its molecular ion (*m/z* 332) but no degradation products could be observed in the TIC. Automated checking of all XIC traces (see Section 2) revealed two degradation products at *m/z* 184 and 314, respectively, exhibiting a nice separation (Fig. 4). In order to identify the two unknown degradation products, MS/MS detection was carried out yielding good product spectra without interference of the non-volatile BGE constituents (Fig. 5A). Firstly, we discuss the product spectrum of ipratropium (Fig. 5AI). The most prominent peak (*m/z* 166) in this spectrum is most likely the result of a neutral loss of tropic acid (166 Da). Furthermore, the two minor peaks at *m/z* 124 and *m/z* 290 point at a loss of the isopropyl functionality (C_3H_7) from the quaternary ammonium group. MS/MS of the second degradation product with *m/z*

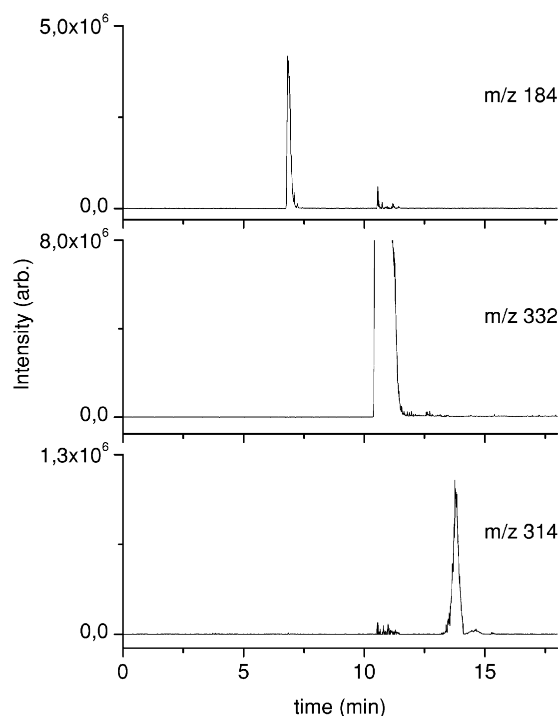
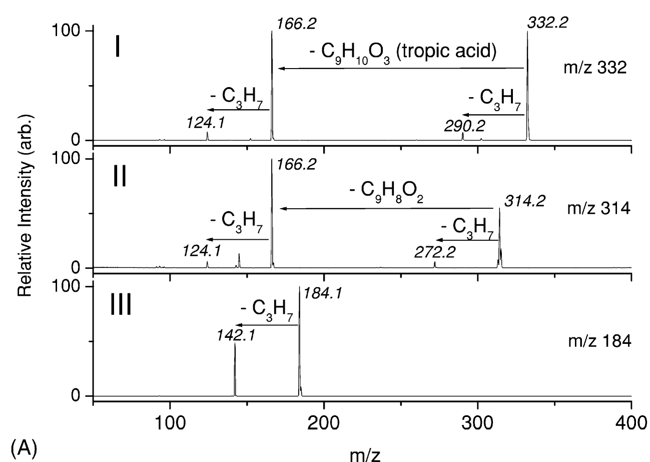


Fig. 4. XICs obtained during MEKC–ESI-MS of a heat-treated solution of ipratropium (1 mg/ml) using a BGE containing 10 mM sodium phosphate (pH 7.5), 20 mM SDS and 12.5% acetonitrile. Capillary length, 57 cm. Peaks: *m/z* 332, ipratropium; *m/z* 184 and *m/z* 314, degradation products.



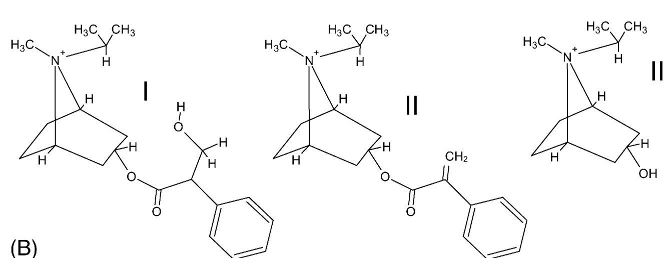


Fig. 5. (A) Product spectra of ipratropium and its degradation products obtained during MEKC–ESI-MS/MS analysis of the heat-treated ipratropium solution: (I) spectrum of ipratropium (*m/z* 332); (II) spectrum of second degradation product (*m/z* 314); (III) spectrum of first degradation product (*m/z* 184). (B) (Proposed) molecular structures of: (I) ipratropium, (II) second degradation product, and (III) first degradation product.

314 results in fragments which partly are identical to the fragments obtained for ipratropium (Fig. 5AII). The observed peak at *m*/*z* 166 and the peaks at *m*/*z* 124 and 272 (loss of isopropyl), together indicate that the double ring structure with the quaternary ammonium group is present in this degradation product. The mass difference between the molecular ions of ipratropium and this degradation product (i.e. 18 Da) can be explained by the formation of a double bond in the tropic acid moiety accompanied by the split off of a water molecule resulting in the formation of apo-ipratropium (Fig. 5BII). This degradation product was also reported by Simms et al. [17].

The first eluting degradation product with *m*/*z* 184 is most probably the result of the hydrolysis of the ester bond in ipratropium leading to the loss of the tropic acid moiety (Fig. 5BIII). The loss of isopropyl observed in the MS/MS spectrum of this compound (Fig. 5AIII) confirms the presence of the double ring structure containing the quaternary ammonium group. Interestingly, the proposed molecular structure (Fig. 5BIII) has no UV chromophore and, thus, cannot be observed with MEKC–UV. Therefore, we suspected the first degradation product we observed in MEKC–UV to be tropic acid (see above) which shows significant UV absorbance, but probably no or a poor response in ESI-MS when positive-ion mode is used. Analysis of a standard solution of tropic acid by MEKC–UV (clear peak at 5.9 min) and MEKC–MS (no peak) confirmed our assumption. This example nicely demonstrates the usefulness of employing different detection techniques in impurity profiling.

4. Conclusions

Continuous MEKC–ESI-MS was successfully applied for the analysis and characterization of impurities in drug substances. Despite ionization suppression by non-volatile buffer and surfactant, MS detection of impurities present around the 0.1% level was still possible. The resulting TIC was less useful for impurity detection due to background signals by surfactant molecules, but automated checking of all XIC traces revealed the MS signals of minor constituents. Furthermore, representative MS/MS spectra could be obtained which were demonstrated to be useful for identification of unknown sample constituents.

The presented method has shown to be useful for the qualitative analysis of various impurities. However, quantitation using this approach will be less straightforward due to the potentially non-linear ion suppression by non-volatile BGE constituents. We are now studying the use of internal standards to overcome this limitation and will test the use of continuous MEKC–ESI-MS(/MS) for quantitative analysis.

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