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Analysis of quaternary ammonium drugs by thermospray liquid chromatography–mass spectrometry using a resin-based stationary phase

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Abstract

The analysis of quaternary ammonium drugs by thermospray liquid chromatography–mass spectrometry (LC–TSP-MS), using a resin-based stationary phase, is described. The use of such a stationary phase eliminates the need of using an ion-pairing agent, which often is incompatible with mass spectrometric detection. Gradient elution has been used to improve the separation and to reduce the analysis time. Detection limits in selective ion monitoring mode as well as in selective reaction monitoring, are in the order of 50 pg for each compound. The usefulness of the method has been demonstrated with the bioanalysis of antrenyl in plasma, using mepenzolate as an internal standard, showing a limit of determination of 1 ng ml^{-1}.

Keywords: Stationary phases, LC; Thermospray mass spectrometry; Quaternary ammonium drugs; Antrenyl

1. Introduction

The analysis of strong organic bases like quaternary ammonium drugs is mainly based on reversed-phase liquid chromatography with UV absorbance detection, using various additives to the mobile phase. Additives such as organic amines [1], alkylsulphonates [2], a mixture of both organic amines and alkylsulphonates [3,4] or electrolytes [5,6], like ammonium nitrate or ammonium formiate, improve the separation and resolution in comparison with unmodified reversed-phase systems. Coupling of ion-pair chromatography with mass spectrometry is unfavourable due to the high concentration of involatile ion-pairing agents. Part of these problems can be overcome via phase-system switching with an ion-exchange trapping column as described by Vreeken et al. [7] and Barcelo et al [8]. Separation methods, especially suited for ionic species, like capillary zone electrophoresis (CZE) have been described more recently [9]. Although coupling of CZE with mass-spectrometry (MS) is of growing interest, this technique still lacks for sensitivity.

Resin-based stationary phases like XAD-2 and PRP-1, are apolar copolymers and therefore lack any influence of residual silanol groups, as present in, for example, octadecyl-silica (ODS).

Mass spectrometric detection of quaternary ammonium compounds has been described using sur-

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face ionization (SI) [10,11], particle-beam ionization (PBI) in combination with SI [12], thermospray (TSP) [7,8,13] and electrospray ionization (ESI) [14]. With all these ionization techniques mass spectra are characterized by molecular cations, R_4N^+, in positive ionization mode. Rafaelli et al. [14] described that under TSP conditions dealkylation or Hoffmann-type elimination reactions can occur. This has been described for esters of choline and carnitine and isopropamide [13]. Isopropamide was also included in our study, and was found to be the only quaternary ammonium compound that shows significant fragmentation under TSP conditions.

In LC the quaternary ammonium compounds will be present in solution as cations, independent of pH. Ionization in TSP-MS is based on ion evaporation of the molecular cations. The mechanism of ion evaporation is discussed thoroughly in literature [15–17].

In this paper the possibility of coupling of TSP-MS with a resin-based stationary phase has been investigated. Various quaternary ammonium drugs have been studied using gradient as well as isocratic elution with acetonitrile–0.05 *M* ammonium acetate mixtures. The MS–MS characteristics of these compounds have been used for detection at low concentration levels as needed in bioanalysis.

Antrenyl (oxyphenonium bromide), which has been extensively studied due to its anticholinergic potency, has been used as a model compound, using mepenzolate as internal standard, to demonstrate the potential of the method. For pharmacokinetic studies a limit of quantification of 1 ng ml^{-1} is required. Due to the low molar absorptivity of antrenyl, UV absorbance detection is not the method of choice. To obtain acceptable detection limits laborious extraction methods and derivatization have to be carried out prior to GC with electron capture detection (ECD) [18,19]. However, with TSP-MS–MS employing selective reaction monitoring (SRM) 1 ng ml^{-1} antrenyl in plasma can be analyzed.

Although sample pretreatment is still necessary in TSP-MS–MS, the extraction procedure is less laborious. In principle, a single ion-pair extraction with perchloric acid, or a solid-phase isolation, is sufficient to extract the parent drug and the internal standard from plasma.

2. Experimental

All experiments were performed on a Finnigan MAT (San Jose, CA, USA) TSQ-70 equipped with a Finnigan MAT thermospray interface in discharge-off mode. Vaporizer and ion-source temperature were optimized and kept at 90 and 250°C, respectively. The repeller was optimized daily to investigate ion-source contamination and kept at the optimum voltage, which was between 50 and 100 V. Model 2150 LC pumps (LKB, Bromma, Sweden) were used for solvent delivery and a Model 2152 HPLC controller was used to program gradient elution. During all the experiments a PRP-1, 5 μm 150×4.1 mm column (Hamilton, Reno, NV, USA) and a Rheodyne 7125 injector (Berkeley, CA, USA) were used. The loop volume was 20 μl for all the experiments, except for the analysis of antrenyl in plasma. In that case a Spectra-Physics 8880 autosampler (Spectra-Physics, San Jose, CA, USA) with a loop volume of 100 μl was used. To investigate the retention factor of the different quaternary ammonium drugs, the mobile phase in isocratic mode consisted of acetonitrile–0.05 *M* ammonium acetate mixtures with acetonitrile percentages up to 50%. In gradient elution a gradient has been programmed from 10 to 50% of acetonitrile in 30 min. For the chromatographic system a flow-rate of 0.5 ml min^{-1} was used. To meet best performance in TSP, higher flow-rates are required. This was achieved with the use of a "make up" flow-rate of 1.0 ml min^{-1}, which consist of acetonitrile–0.05 *M* ammonium acetate (10:90, v/v). The introduction of the "make up" flow-rate was done directly after the PRP-1 column via a zero low dead T-piece. MS data were collected in selective ion monitoring (SIM) mode. For the analysis of mixtures of quaternary ammonium drugs SIM procedures were made with an individual scan time of 0.5 s/cation, resulting in a total scan time of 4.5 s. Mixtures of 10 or 100 ng ml^{-1} (200 pg or 2 ng/injection), of each compound, were analyzed.

Experiments in MS–MS mode were performed in full scan mode, at a level of 2 ng/injection, for optimization of the collision gas pressure and the collision energy. Optimum values were 0.5 Pa and 35 eV, except for isopropamide, which has an optimum collision energy of 20 eV.

For the analysis of antrenyl in plasma calibration curves were generated in the range from 1 to 100 ng ml^{-1} antrenyl, together with 100 ng ml^{-1} of mepenzolate as internal standard. To 1.0 ml of spiked plasma, 100 μl of 1 *M* perchloric acid and 5 ml of dichloromethane were added. The tubes were vortexed for 30 s and the phases were separated by centrifugation at 3000 *g* for 10 min. The water layer containing the denaturated proteins was decanted. The dichloromethane was evaporated to dryness with a vortex evaporator (Haake Büchler, Lenexa, KS, USA), and the residue was dissolved in 300 μl of water. A 100-μl aliquot of each sample was injected onto the chromatographic system, which consists of acetonitrile–0.05 *M* ammonium acetate (30:70, v/v), with a total flow-rate, including the make-up flow, of 1.5 ml min^{-1}. MS–MS data were collected in SRM, alternatively scanning at *m/z* 130 and 132, the product ions of mepenzolalte and antrenyl, respectively.

2.1. Chemicals

Acetonitrile, dichloromethane and perchloric acid (Baker, Deventer, Netherlands) were of HPLC grade. Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Ammonium acetate was purchased from Merck (Darmstadt, Germany). Neostigmine bromide, propanthaline bromide, pipenzolate bromide, mepenzolate bromide, clidinium bromide, isopropamide iodide, oxyphenonium bromide and valethamate bromide were all obtained from Sigma (St. Louis, MO, USA). Methylbenactyzine iodide was kindly donated by the academic hospital.

The structures of the quaternary ammonium compounds are listed in Table 1.

3. Results and discussion

3.1. TSP-MS

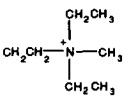
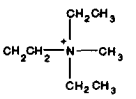
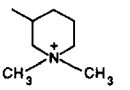
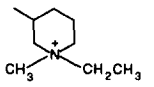
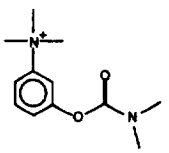
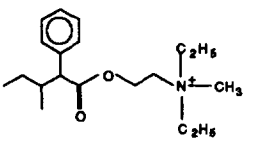
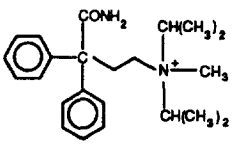
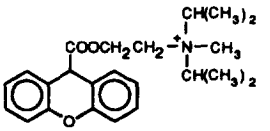
The problem of using silica-based reversed-phase stationary phases for the determination of quaternary ammonium drugs is caused by the residual silanol groups. Eliminating the influence of residual silanol groups can be performed by end-capping, although even after thorough end-capping procedures, residual silanol groups are still present. Addition of an ion-pairing agent is a suitable way to overcome this problem. Unfortunately, ion-pair chromatography is not compatible with MS. An alternative is the application of non-silica based stationary phases, which still have reversed-phase properties. Copolymers of styrene and divinylbenzene do have these qualities, can be used over a wide pH range, and resist high percentages of organic modifier without significant swelling. Such materials are commercially available as XAD-2 and PRP-1, whereas PRP-1 consists of spherical particles.

Optimization of TSP-MS conditions was performed in the so-called flow-injection analysis mode (FIA) using antrenyl at a concentration level of 2 ng/injection as a model compound. Mass spectra of all the quaternary ammonium drugs are characterized by the appearance of the intact cations, without fragmentation. The only exception to this rule is isopropamide, showing some fragmentation, already described by Vicchio et al. [13]. Experimental parameters such as ion-source temperature, vaporizer temperature and repeller potential were optimized at several eluent compositions. It appeared that best results were obtained at low percentages of organic modifier, 0.05 *M* ammonium acetate and a flow-rate of 1.5 ml min^{-1}, as is also observed in the so-called buffer-ionization mode. Use of the discharge electrode should be avoided, because it decreases the signal drastically, by disturbing the ion evaporation process. Under optimized conditions using selective ion monitoring (SIM) at *m/z* 348 detection limits of 50 pg could be obtained for antrenyl.

Using these conditions all nine quaternary ammonium drugs were checked on their sensitivity in the FIA mode, using SIM at each cation. Only isopropamide was analyzed using the molecular cation plus the main fragment at *m/z* 238. Sensitivities were found to be in the same order of magnitude as for antrenyl.

Unfortunately, a PRP-1 based chromatographic system using optimal MS conditions, i.e., a low percentage of organic modifier, resulted in unacceptably long retention times for all quaternary ammonium drugs, except for neostigmine. On the other hand, for an optimal PRP-1 based chromatographic

Table 1
Structures of quaternary ammonium drugs

Compound	Cation	Structure	
		R_1	R_2
Antrenyl $C_{21}H_{34}NO_3$	348		
Methylbenactyzine $C_{21}H_{28}NO_3$	342		
Mepenzolate $C_{21}H_{26}NO_3$	340		
Pipenzolate $C_{22}H_{28}NO_3$	354		
Clidinium $C_{22}H_{26}NO_3$	352		
Neostigmine $C_{12}H_{19}N_2O_2$	223		
Valethamate $C_{19}H_{32}NO_2$	306		
Isopropamide $C_{23}H_{33}N_2O$	353		
Propantheline $C_{23}H_{30}NO_3$	368		

system using high percentages of organic modifier mass spectrometric sensitivity was poor. One way to solve this problem is to apply a make-up flow after the chromatographic separation, which was performed at 0.5 ml min^{-1}. With a make-up flow-rate of 1.0 ml min^{-1}, acetonitrile–0.05 *M* ammonium acetate (10:90, v/v), mixtures up to 50% of acetonitrile can be used in the chromatographic system and still remain at acetonitrile percentages acceptable for buffer ionization.

The influence of the percentage acetonitrile on the retention factor for neostigmine, mepenzolate, antrenyl, valethamate and propanthaline is depicted in Fig. 1. These compounds differ widely in their apolar

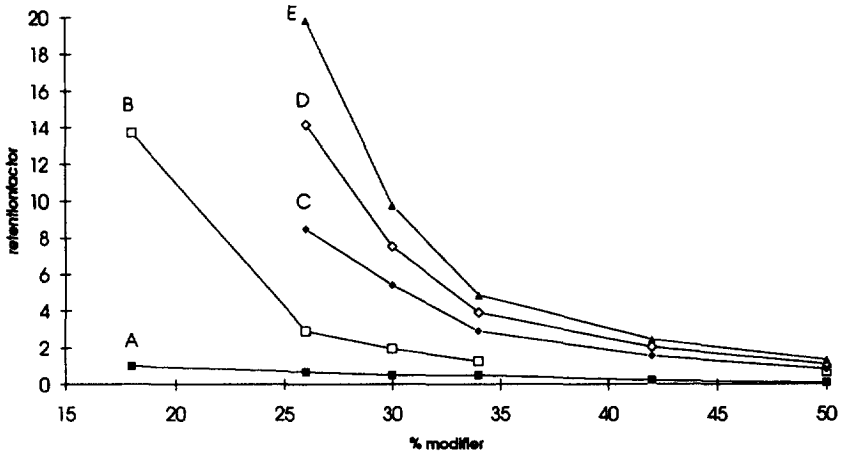


Fig. 1. Retention factor as a function of percentage acetonitrile for: (A) neostigmine; (B) mepenzolate; (C) antrenyl; (D) valethamate; (E) propanthaline.

moiety. Neostigmine is hardly retained, even at 50% acetonitrile, whereas propanthaline is already strongly retained at acetonitrile contents above 30%.

Isopropamide, methylbenactyzine, clidinium and pipenzolate are not included in Fig. 1, because of the great similarity of the retention factor with mepenzolate, caused by the two phenyl groups.

In comparison to ion-pair chromatography using sodium octylsulphonate, to enhance the capacity, and dimethyloctylamine, to enhance resolution, the elution order of propanthaline and antrenyl is reversed. The addition of dimethyloctylamine and octylsulphonate influences the retention factor and elution order of quaternary ammonium drugs after a certain period of time. To avoid this phenomenon Schutter et al. [4] described a method of on-column silylation with N-trimethylsilylimidazole (TMSIM). Compared to that method, the use of PRP-1 material is much more straight-forward, without seriously disturbing the retention factor.

Gradient elution, starting at a percentage of 10% acetonitrile increasing to 50% in 30 min results in a good combination between separation efficiency and speed of analysis. Propanthaline, having the longest retention time, elutes at 22 min, whereas mepenzolate and pipenzolate, isocratically hard to separate, are almost baseline separated.

The ion chromatograms of these three quaternary ammonium drugs at a level of 200 pg/injection for pipenzolate and propanthaline and 400 pg/injection for mepenzolate, in SIM mode, is shown in Fig. 2. Detection limits corresponds to about 50 pg for each quaternary ammonium compound.

3.2. TSP-MS–MS

The MS–MS characteristics of all the quaternary ammonium drugs were studied in the FIA mode at a concentration level of 2 ng/injection. Optimization was carried out for the collision pressure as well as the energy. It was found that with a collision pressure of 0.5 Pa, best sensitivity could be obtained using an energy of 35 eV, except for isopropamide, which has an optimum energy of 20 eV. In principle, quaternary ammonium compounds can undergo several fragmentation reactions. Two of the most important reactions are C–N cleavage to tertiary amines and alkyl compounds and hydrolysis of the ester linkage, forming alcohols. Valethamate, isopropamide, neostigmine and propanthaline do show C–N cleavage, whereas all the other compounds presented in Table 1 show hydrolysis of the ester linkage. The fragmentation pattern and the formed ions are presented in Table 2a. The loss of water out

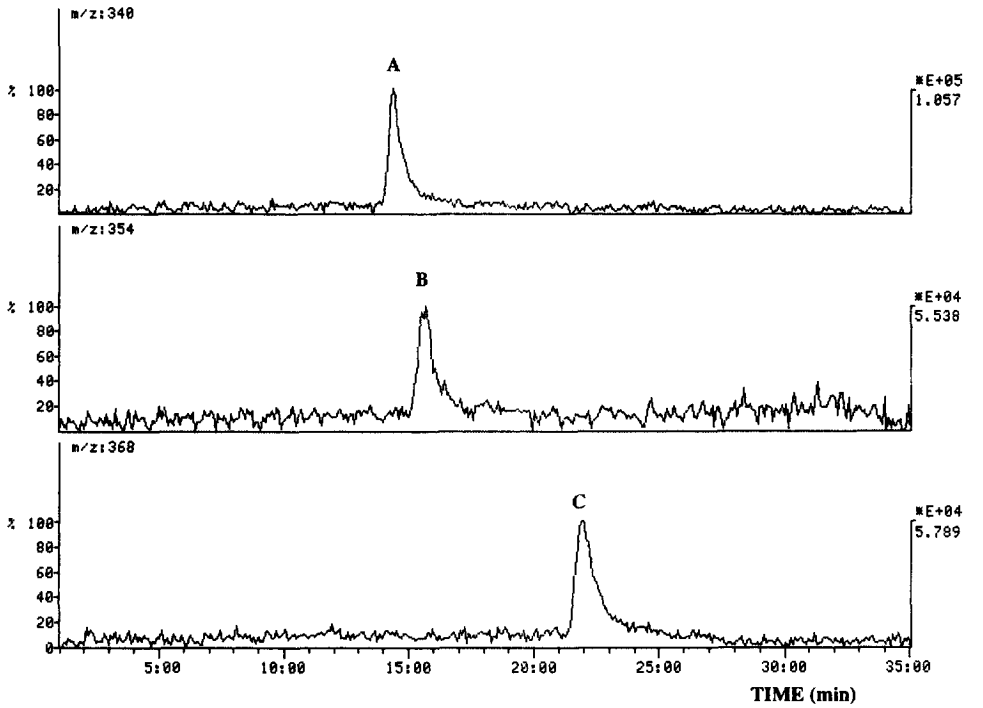


Fig. 2. Ion chromatogram of (A) 400 pg mepenzolate, (B) 200 pg pipenzolate and (C) 200 pg propanthaline in SIM mode, using a gradient, starting at 10% acetonitrile increasing this percentage to 50% in 30 min.

of the formed alcohol is a minor fragment in those quaternary ammonium compounds having a quaternary ring structure. The appearance of the benzophenones and *m/z* 105 is typically more abundant in antrenyl and methylbenactyzine, because of the absence of the quaternary ring structure.

The fragmentation for isopropamide, obtained in MS–MS, resembles the fragmentation induced by the vaporizer temperature, major fragmentation due to C–N cleavage forming an alkene at *m/z* 238, and a quaternary ammonium at *m/z* 116. However, the minor losses of the three alkyl groups attached to the quaternary amine, as described by Vicchio et al. [13] are not observed using MS–MS. As a consequence, those fragments are most likely to be formed by thermal induction.

Neostigmine shows, besides a small C–N cleavage ($-CH_3$), a cleavage of the ester linkage. Instead of mainly forming the alcohol fragment, the charge is retained at the dimethylamine side of the molecule, capable of forming a rather stable ion at *m/z* 72 [$O{=}C{=}N^+(CH_3)_2$].

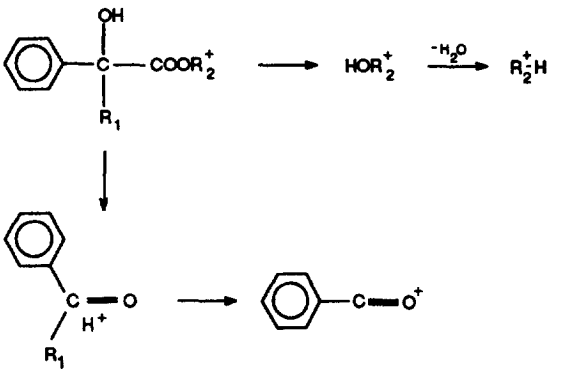
Valethamate shows a clear C–N cleavage forming an alkene at *m/z* 219. The opposite quaternary ammonium at *m/z* 87 is totally absent. A second major fragment is observed at *m/z* 163, due to cleavage of C_4H_8 out of the fragment at *m/z* 219. Some minor fragmentation is observed at *m/z* 149 and 91, the first due to cleavage left of the carboxyl, leaving the charge at the phenyl part, the second due to a McLafferty rearrangement, forming a tropylium ion.

Propanthaline shows two main fragments, at *m/z* 181 and 100, and a smaller fragment at *m/z* 326, due to C–N cleavage. By cleavage of the carboxyl from the ring structure *m/z* 181 is formed, with charge retention on the ring. The other fragment, *m/z* 100, is probably formed by cleavage of the O–CH_2 bond forming an alkene and executive loss of C_3H_6 .

The MS–MS data of neostigmine, isopropamide, valethamate and propanthaline are presented in the bottom part of Table 2b.

LC–MS–MS techniques are extremely suitable working with plasma and urine samples. Due to the

Table 2
(a) Proposed structures of fragments created with collision induced dissociation for mepenzolate, pipenzolate, clidinium, antrenyl and benactyzine



Compound	Precursor m/z (%)	Fragments m/z (%)	
Antrenyl	348 (15)	132 (100), 189 (20),	105 (30)
Methylbenactyzine	342 (10)	132 (100), 183 (35),	105 (50)
Mepenzolate	340 (15)	130 (100), 183 (2), 112 (5)	
Pipenzolate	354 (40)	144 (100), 183 (4), 126 (3)	
Clidinium	352 (15)	142 (100), 183 (1), 124 (4)	

(b) MS–MS data for neostigmine, isopropamide, valethamate and propanthaline

Neostigmine	223 (8)	208 (25), 151 (12), 72 (100)
Isopropamide	353 (6)	238 (100), 116 (8)
Valethamate	306 (4)	219 (85), 163 (100), 149 (5), 91 (8)
Propanthaline	368 (3)	326 (6), 181 (100), 100 (30)

specificity of the fragmentation reaction the signal-to-noise ratio can be improved considerably. Not only in metabolism studies, but also in studying group specific fragmentation reactions, LC–MS–MS can be rather important.

Methylbenactyzine, mepenzolate, pipenzolate and clidinium show a common loss of 210, caused by the cleavage of the ester bond. Using a so called neutral loss of 210 all compounds with this specific reaction will be identified. Fig. 3 shows the ion chromatograms obtained from the injection of a mixture of these quaternary ammonium drugs at a level of 200 pg, except for mepenzolate, which was at a level of 400 pg.

As a consequence of the parallel scanning of the two quadrupoles, the scanning range should cover all involved masses. In this case the scanning was performed from m/z 339 to 355. The response for methylbenactyzine is somewhat lower, because besides a neutral loss of 210 it also shows two other strong fragments.

3.3. Analysis of antrenyl in plasma using TSP-MS–MS

Antrenyl is an interesting quaternary ammonium drug due to its anticholinergic activity. Plasma concentration-time curves after therapeutic administration vary from the low ng ml^{-1} range up to 200–500 ng ml^{-1}, depending on the dose and the way of administration. Typical values after 10 mg orally administered are 1–5 ng ml^{-1}, during 7 h of sampling. Ensing [18] and Greving et al. [19] have described a double ion-pair extraction before hydrolysis of antrenyl and the internal standard followed by derivatization to the pentafluorobenzyl derivative. With this extraction method it was pos-

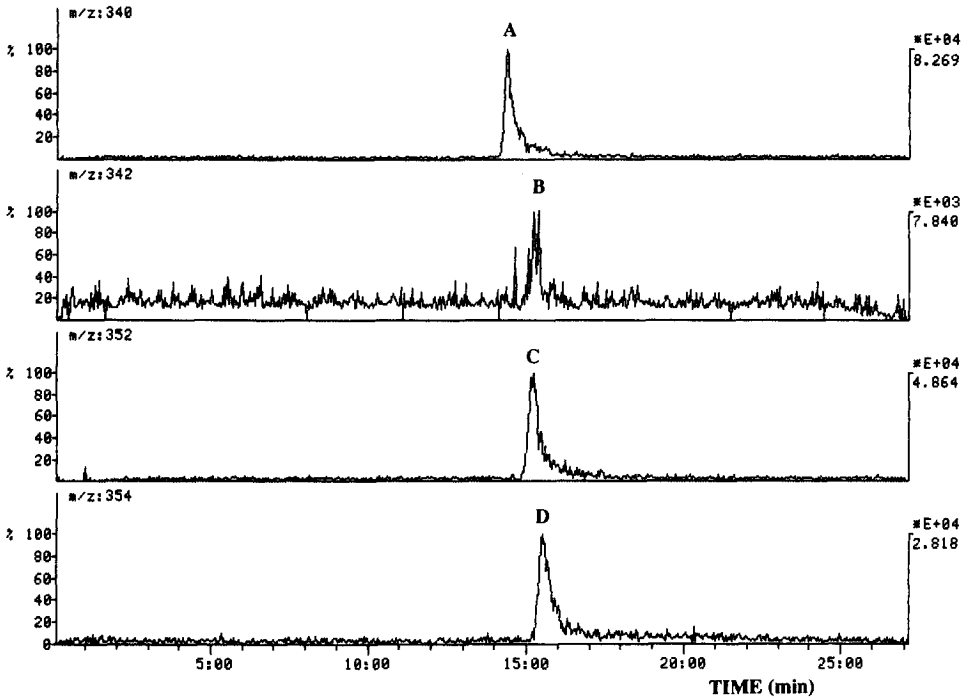


Fig. 3. TSP-MS–MS ion chromatograms of (A) 400 pg mepenzolate, (B) 200 pg methylbenactyzine, (C) 200 pg clidinium and (D) 200 pg pipenzolate, performing a neutral loss of 210, using air as collision gas (0.5 Pa) and a collision energy of 35 eV.

sible to detect antrenyl in the low ng ml^{-1} range using GC with ECD.

Of course, most accurate data are obtained using isotope dilution. Still, the use of compounds with similar structure characteristics as an internal standard has been investigated, because the isotope-labelled compounds are not always readily available. Methylbenactyzine, mepenzolate, pipenzolate and clidinium were checked as candidates for the internal standard. Mepenzolate turned out to be the best one, with respect to extraction efficiency, and was used in all the following experiments.

Using TSP-MS–MS, detection limits below 50 pg can be obtained for antrenyl in standard solutions. Due to its ionic character a single ion-pair extraction, with perchloric acid acting as ion-pairing agent, is sufficient to isolate the drug from plasma.

With this method fatty acids, which can interfere, are also extracted from plasma. This problem can be overcome by a back-extraction of antrenyl using 10^{-3} *M* tetrapentylammonium (TPA). The fatty acids and acidic compounds will remain in the organic phase. However, due to the excess of TPA, such a back-extraction is not desirable with respect to compatibility with TSP-MS–MS. Another way to solve the problem of co-extraction of fatty acids is dissolving the residue, after evaporation to dryness of the dichloromethane, in water. In this way recoveries up to 80% could be obtained, with standard deviations within 10%.

For the calibration curve based on peak area ratios a good linearity was obtained over the range from 1 to 100 ng ml^{-1}. In Fig. 4 the ion chromatogram of an extract of blank plasma spiked with 1 ng ml^{-1} antrenyl and 100 ng ml^{-1} mepenzolate as internal standard is shown. The total analysis time is 9 min. To check whether there are compounds co-eluting with antrenyl or the internal standard, full scan experiments with blank plasma are performed, but no interferences could be observed. From Fig. 4 it can be seen that the peaks tend to tail to a certain extent. This phenomenon is probably induced by the deposition of analyte to the wall of the vaporizer, and adsorption of antrenyl and mepenzolate to it. After

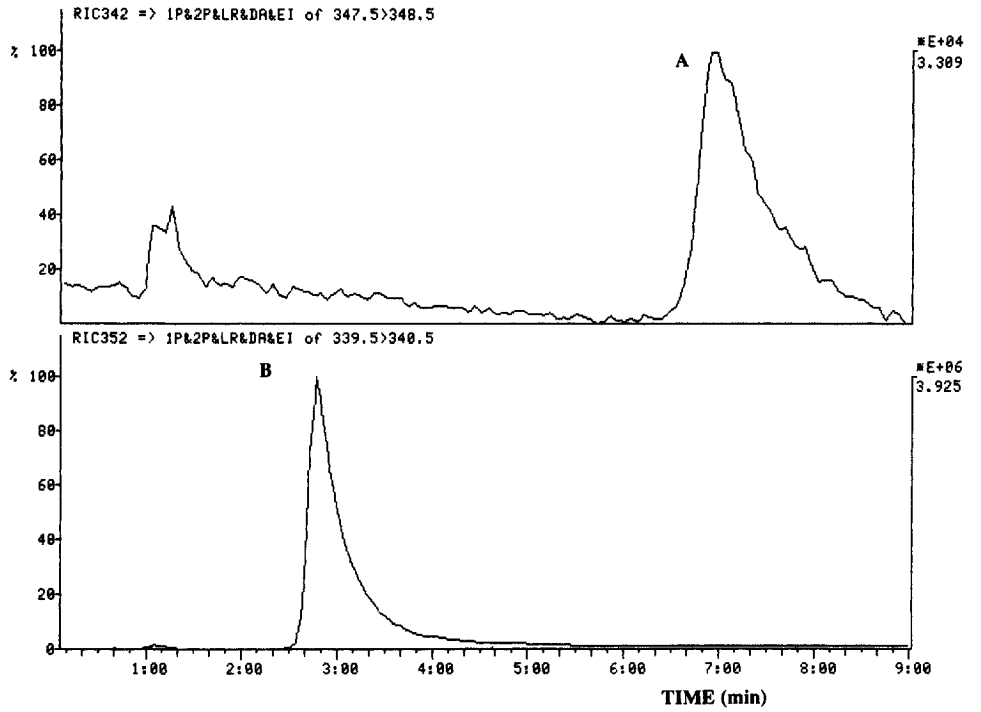


Fig. 4. TSP-MS–MS of a spiked plasma sample: (A) 1 ng ml^{-1} of antrenyl and (B) 100 ng ml^{-1} mepenzolate, using an isocratic system, consisting of acetonitrile–0.05 *M* ammonium acetate (30:70, v/v).

either re-installing a new vaporizer or flushing the vaporizer with acetonitrile–acetic acid in water (95:5, v/v) overnight, a good peak shape could be obtained again.

4. Conclusions

It appeared that PRP-1 is a suitable stationary phase for the analysis of a wide variety of hydrophobic quaternary ammonium drugs. Direct coupling with TSP-MS is possible, because no involatile additives are used like in ion-pair chromatography. Detection limits for all the used quaternary ammonium drugs are in the order of 50 pg. MS–MS is used to characterize the collision induced fragmentation. The analysis of antrenyl in plasma in shown with mepenzolate as internal standard, down to a level of 1 ng ml^{-1}, using TSP-MS–MS. Further quantitative work needs improvement with respect to extraction reproducibility and chromatographic peak shape.

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