Journal of Chromatography, 647 (1993) 167–181
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2812

Determination of penicillin G, ampicillin, amoxicillin, cloxacillin and cephapirin by high-performance liquid chromatography–electrospray mass spectrometry

Rolf F. Straub and Robert D. Voyksner*

Analytical and Chemical Sciences, Research Triangle Institute, P.O. Box 12194, Research Triangle Park, NC 27709 (USA)

ABSTRACT

This report contributes to a preliminary investigation of high-performance liquid chromatographic (HPLC)–mass spectrometric (MS) methods for confirming β-lactam antibiotic residues in bovine milk. Initial work for each antibiotic evaluated the collisional activated dissociation (CAD) spectra that could be generated between the capillary and skimmer in the electrospray (ESP) interface. The drugs show various characteristic fragmentation, mostly within the β-lactam ring and the amide group. Response for a particular compound in a given solvent can vary drastically. Usually, the more organic component in the solvent, the higher the ESP response. In many cases use of acetonitrile also results in slightly better ion currents than for methanol when comparing equal percentages of either organic solvent in water. The ESP response of most of the tested antibiotics can be enhanced by the addition of formic acid or acetic acid to the mobile phase methanol–water (1:1). In general, the negative ion spectra are lower in intensity, exhibiting an $[M-H]^-$ ion and producing less fragmentation at higher CAD voltages as compared to positive ion spectra. An isocratic reversed-phase HPLC method for the separation of a mixture of five common β-lactam antibiotics was developed using acetic acid as a mobile phase additive and optimized for detection with a new ESP HPLC–MS interface. A post-column split ratio of 70:1 for the eluent from a 150 × 2 mm I.D. column was chosen to provide the required lower flow-rate (approximately 4 μl/min). The limit of detection for the simultaneous determination of these antibiotics was estimated to be 100 ppb. Electrospray HPLC–MS could be used to confirm these antibiotics for quantities down to about 100 pg entering the mass spectrometer. Multiresidue analysis with microbore HPLC–ESP-MS has the advantage that no post-column splitting of the eluent is required and all of the analyte (on-column injected) will be transferred into the ESP interface. Preliminary work showed good mass spectrometric sensitivity down to the level of regulatory interest, but chromatographic separation efficiency must be improved.

INTRODUCTION

Some penicillins and cephalosporins were produced semi-synthetically and represent an interesting class of β-lactam antibiotics because of their broad antimicrobial activity against both gram-positive and gram-negative organisms [1,2]. Penicillines contain bulky side-chains attached to a 6-aminopenicillanic acid nucleus (sulfur-containing thiazolidine ring fused to a β-lactam ring) and are degradable in presence of solvents [3] or by heat [4]. Acid labile penicillins are rapidly inactivated at an acid pH. Additionally, all penicillins are rapidly inactivated at an alkaline pH in the presence of carbohydrates. Cephalosporin structures are based on the 7-aminocephalosporinic acid nucleus (condensed dihydrotiazole ring in its skeleton) and are generally stable in acidic media as well as in presence of penicillinase [5]. Many efforts have been made to synthesize cephalosporins with different physico-chemical properties (mainly liposolubility) by variation of substituents [6]. Table I lists the chemical structures of β-lactam antibiotics discussed in this paper. They are commonly used as bacterial agents in excessive livestock farming and bovine milk production. The Food and Drug

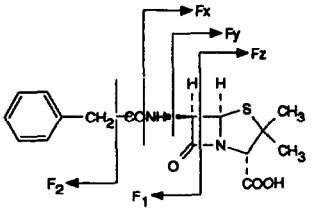
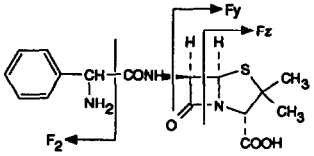
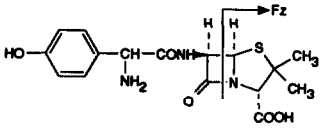
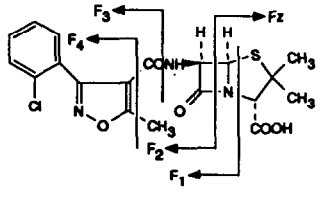
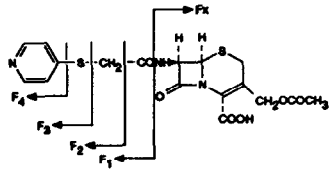
* Corresponding author.

0021-9673/93/$06.00

TABLE I

IDENTITY OF CAD FRAGMENTS–POSITIVE ION MS OPERATION

M_r = Molecular mass of the free acid drug; CAD voltage = voltage measured at the end of the capillary (Analytica QBV 25-AL electrospray interface); m/z = mass-to-charge ratio (relative intensity of the peak in %, type of fragment); F_x, F_y, F_z, F_1, F_2, F_3, and F_4 = different collisional activated dissociation (CAD) fragments seen in the ESP spectra.

Drug (M_r)	CAD voltage	m/z (relative intensity and tentative identification of CAD fragments)
	80	392 (54); 391 (51, $[M + K + H_2O]^+$); 367 (93, $[M + H + MeOH]^+$); 353 (41, $[M + H + H_2O]^+$); 335 (41, $[M + H]^+$); 309 (8); 279 (25); 176 (43, $[F_1 + H]^+$); 167 (33); 160 (49, $[F_z + H]^+$); 149 (14); 114 (25, $[F_z - COOH]^+$); 65 (43, $[2MeOH + H]^+$)
	160	367 (27, $[M + H + MeOH]^+$); 335 (7, $[M + H]^+$); 217 (4, $[F_x + H]^+$); 202 (3, $[F_y + H]^+$); 176 (21, $[F_1 + H]^+$); 160 (37, $[F_z + H]^+$); 149 (100); 121 (14); 114 (4, $[F_z - COOH]^+$); 93 (19); 91 (15, $[F_2]^+$); 70 (14); 57 (18)[a]; 52 (36)[a]
Penicillin G (334.4)		
	80	382 (18, $[M + H + MeOH]^+$); 350 (100, $[M + H]^+$); 65 (16, $[2MeOH + H]^+$)
	160	350 (89, $[M + H]^+$); 192 (24); 174 (20, $[F_y + H - CO]^+$); 160 (22, $[F_z + H]^+$); 114 (89, $[F_z - COOH]^+$); 108 (8); 106 (100, $[F_2]^+$)
Ampicillin (349.4)		
	80	398 (12, $[M + H + MeOH]^+$); 367 (29); 366 (100, $[M + H])^+$; 349 (19, $[M + H - NH_3]^+$); 65 (19, $[2MeOH + H]^+$)
	160	366 (62, $[M + H]^+$); 349 (85, $[M + H - NH_3]^+$); 208 (41); 160 (6, $[F_z + H]^+$); 114 (100, $[F_z - COOH]^+$); 70 (14)
Amoxicillin (365.4)		
	80	481 (28, $[M + 2Na]^+$); 437 (84, $[M + 2H]^+$); 436 (100, $[M + H]^+$); 148 (20); 74 (46); 65 (95, $[2MeOH + H]^+$)
	160	468 (56, $[M + H + MeOH]^+$); 454 (11, $[M + H_2O]^+$); 436 (36, $[M + H]^+$); 321 (<0.1, $[F_1 + H^+]^+$); 277 (64, $[F_2]^+$); 222 (19, $[F_3 + H]^+$); 220 (11); 178 (56, $[F_4 + H]^+$); 160 (100, $[F_z + H]^+$); 114 (68, $[F_z - COOH]^+$)
Cloxacillin (435.9)		
	40	446 (63, $[M + Na]^+$); 424 (25, $[M + H]^+$); 413 (100, $[M - MeOH]^+$); 234 (32); 95 (8); 59 (33)[a]; 55 (36)[a]
	160	424 (45, $[M + H]^+$); 292 (41); 226 (15, $[F_x - COOH]^+$); 193 (21); 181 (26); 152 (62, $[F_1]^+$); 141 (23); 124 (59, $[F_2]^+$); 111 (62, $[F_3 + H]^+$); 79 (33, $[F_4 + H]^+$); 52 (100[a])
Cephapirin (423.4)		

[a] The indicated mass is outside the instrument's (Finnigan MAT 4500) calibration range and is probably inaccurate.

Administration (FDA) has recently indentified approximately 60 drugs which are likely residues in such animal-derived human foods and for which current analytical methodology is deficient in some way [7]. Analytical methods for β-lactam antibiotic residues provide a means of monitoring, controlling and measuring residues in animal food products.

Screening methods are often the most cost effective techniques because they use rapid sample analysis and are usually amenable to multiresidue analysis in field laboratory environments. For these reasons the detection of antibiotics in milk continues to be carried out by bioassay techniques such as microbiological tests [8], immunoassay [9,10], competitive binding [8,11,12] and enzyme inhibition [13]. With the possible exception of immunoassay, none of the screening procedures can distinguish β-lactam antibiotics from one another. Methods involving fluorescence [14] and thin-layer chromatography [15] were reported for analysis of penicillin G in milk. However, these procedures are not acceptable for current regulatory purposes. Therefore, specific chemical cleanup, separation and confirmation procedures for β-lactam antibiotics are needed for accurate identification and quantitation of suspect trace level residues in biological matrices.

Several methods using various extraction and deproteinization procedures [16] followed by high-performance liquid chromatography (HPLC) employing a variety of stationary and mobile phases for the separation have been investigated [17–23]. Ultraviolet and fluorescence detectors have been most routinely used but, where interferences have arisen, confirmation of analyte identity has been demonstrated through gas chromatography (GC)–mass spectrometry (MS) thus introducing problems associated with GC analysis [24,25]. Recently, Meetschen and Petz [26] described a method using GC for analysis of β-lactams with neutral side chains which required a time-consuming partitioning cleanup and derivatization. Confirmatory methods, whether they are a second independent quantitative analytical method based on a different methodology or highly definitive MS, ideally provide unequivocal proof of the suspected drug residue. Thermospray HPLC–MS has been successfully used to analyze non-volatile and thermally labile compounds [27–29] including several β-lactams [30–34]. Structurally different underivatived cephalosporins have been under extensive investigation by laser-induced vaporization, desorption chemical ionization, and fast atom bombardment mass spectrometry [35].

Another promising technique is atmospheric pressure ionization. Atmospheric pressure chemical ionization (APCI) forms ions in a discharge by means of chemical ionization [36–39] and electrospray (ESP) forms ions through ion evaporation in highly charged micron-size droplets [40,41]. The ESP ionization techniques is very mild and the obtained sensitivities are extremely good, although some restrictions are placed upon the conductivity and the flow rate of the mobile phase. Electrospray ion currents usually are maximized with solvents of low conductivities at flow-rates from 1 to 10 μl/min. These low flow-rates require liquid chromatography on the microbore scale to avoid post-column splitting. Recently, nanoscale capillary liquid chromatography and capillary zone electrophoresis have been successfully combined with quadrupole mass spectrometry via an ESP interface. These two microbore-scale methodologies have been applied to the separation and determination of sulfonamides commonly administered in subtherapeutic doses to promote growth in animals [42]. Very recently, the related "ion spray" technique was used for the investigation of veterinary drugs residues in bovine kidney. McLaughlin and Henion [43] applied reversed-phase ion-pair HPLC coupled with pulsed amperometry and ion spray MS to determine trace levels of four aminoglycosides.

Continuing our interest in developing new HPLC–MS methods for accurate determination of drug residues in biological matrices, we have studied the influence of different HPLC mobile phase and buffered solutions on the ionization process for β-lactam antibiotics. Both positive and negative ionization operational modes were applied to form characteristic ions. It was anticipated that several drugs requiring confirmatory procedures would exhibit only a single ion (most

commonly $[M + H]^+$ in positive and $[M - H]^-$ in negative ion operational mode) making identification difficult. Therefore, work enhancing the fragmentation through use of higher collisional activated dissociation (CAD) was used to aid in structural elucidation. For HPLC–ESP-MS analysis of a mixture of β-lactam antibiotics in milk, a post-column split ratio for 70:1 of the eluent from a 150 × 2 mm I.D. column was used to gain the required lower flow-rate for a successful ESP-MS operation.

EXPERIMENTAL

Material

Water was bidistilled and purified with a Milli-Q water system (Millipore, Bedford, MA, USA) prior to use. Methanol (MeOH), acetonitrile (MeCN) and 2-propanol (IPA) were of "HPLC/GC grade" quality (Baxter Healthcare, Muskegon, MI, USA). For mobile phase additives trifluoroacetic acid (TFA) (J. T. Baker, Marietta, GA, USA), formic acid (FOA), acetic acid (HOAc), ammonium acetate (NH_4OAc), heptafluorobutyric acid (HFBA), tetrabutylammonium hydroxide (TBAH) (Aldrich, Milwaukee, WI, USA) and ammonium hydroxide (NH_4OH) (Fisher Scientific, Rochester, NY, USA) were used. Arginine and gramicidin S from bacillus brevis (Sigma, St. Louis, MO, USA) were employed as tuning compounds in positive ion ESP-MS operation. The β-lactam antibiotic penicillin G (potassium salt), ampicillin (anhydrous), amoxicillin, cloxacillin (sodium salt) and cephapirin (sodium salt) were from Sigma. The drug standards were stored in a dry atmosphere at 4°C and were not dried or purified further before use.

Instrumentation

For flow infusion (FI) experiments a Sage 341B micro syringe pump (Sage Instruments, Division of Orion Research, Boston, MA, USA) and a 50-μl PS C-160 FN syringe for a Waters U6K injector (Dynatech Precision Sampling, Baton Rouge, LA, USA) were utilized.

The eluents used in the flow injection or separation of the drug mixture were delivered by an Isco (Lincoln, NE, USA) 100D syringe pump/pump controller Model 174262. The samples were injected with an Actuator, Model 732 with a 5-μl sample loop (Alcott Chromatography, Norcross, GA, USA) or with a Valco C14W.5 injector with a 0.5-μl sample loop (Isco) and separated on a 150 × 2 mm Ultremex 3, C_{18} column (Phenomenex, Torrance, CA, USA.) A Waters Model 484 MS tunable absorbance detector (Waters Assoc., Milford, MA, USA) was linked to the higher flow-rate port of an Acu-Rate IC-70 splitter (LC Packings International, San Francisco, CA, USA) and set to 230 nm. A Hewlett-Packard (Avondale, PA, USA) HP 3396A integrator was used to record the ultraviolet (UV) absorption chromatograms.

For small sample injections in micro-HPLC–ESP-MS determination a Valco C14W.5 injector with an internal 0.5-μl sample loop (Isco) and for larger injections a pheodyne 8125 injector with variable sample loop (Rheodyne, Cotati, CA, USA) was tested. The Rheodyne injector contributed to a larger void volume but was able to handle 1-, 5- and 30-μl injections of drug mixtures. A 15 cm × 320 μm I.D. Fusica C_{18} capillary column with a particle size of 3 μm protected by a μ-guard precolumn (LC Packings International) was connected through a 5-cm inlet capillary transfer line to the injector. The 35-cm column outlet capillary transfer line was linked to the Analytica electrospray needle. It is not recommended to directly interface the capillary column to the electrospray needle by removing the outlet capillary. This would damage the column and also increase the void volume.

An Analytica HPLC–ESP-MS interface Model QBV 25-AL (Analytica of Branford, Branford, CT, USA) was installed on a Finnigan MAT 4500 single quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) and used for the initial FI-CAD voltage experiments. For the FI-ESP-MS buffer studies and the negative ion ESP experiments a Analytica HPLC–ESP-MS interface, Model 100547-3, was connected to a Hewlett-Packard Engine, Model HP 5989A, single quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA). Two types of ESP needles were used with comparable results: The three layered original Analytica needle used a 50 μm I.D. stainless-steel tube to deliver

solvent, a 125 μm I.D. second layer tube to deliver an additional solvent to enhance ESP operation, especially when using high percentages of water. The third tube was used to add a gas to aid in nebulization of highly conducting or aqueous solvents. The 33-gauge Hamilton needle use a 110 μm I.D. SS-304 stainless-steel tube with a 210 μm O.D. and no second tube for additional solvent delivery (Hamilton, Reno, NV, USA). This needle was used in a cut length of 25 cm and with a 90° bevel at the tip. The same nebulization gas addition capability was maintained as with the original needle type. For all HPLC–ESP-MS determinations of the drugs a new computer controlled and more sensitive Analytica HPLC–ESP-MS interface, Model 101737 with autotune capability, was applied. For instrument control and data acquisition a Hewlett-Packard HP Vectra 80486-33 EISA personal computer/MS-DOS Chemstation software version M2.43 was utilized. Sample pH was measured with a Cole Parmer (Chicago, IL, USA) pH meter and an Orion Ross combination pH 81-15 electrode (Orion Research, Boston, MA, USA) as 20°C.

FI-ESP-MS Conditions

Mobile phases such as MeOH–water (8:2), (1:1) and (2:8) were tested for positive ion MS operation. A concentration of 100 ng/μl of the drug was dissolved in the appropriate mobile phase. The mobile phase additives were dissolved in MeOH–water (1:1) in the following concentration: 0.1% (v/v) TFA (pH 2.2), 0.1% (v/v) FOA (pH 3.1), 1% (v/v) HOAc (pH 3.4), 50 m*M* NH_4OH (pH 10.5), 10 m*M* HFBA (pH 2.2) and 10 m*M* TBAH (pH 12.1). For negative ion ESP operation the mobile phases IPA–water (8:2) and (1:1) were applied. The analytes were infused at a flow-rate of 1.2 μl/min and 2.4 μl/min. At both flow-rates similar signal responses were observed and other flow rates did not improve the signal intensity. To avoid a severe corona discharge in the ESP interface oxygen was coaxially mixed with the nebulized liquid.

For the flow injection-ESP-MS experiments on the Finnigan MAT 4500 instrument the ESP interface, Model QBV 25-AL, was operated at a needle voltage of 3.4 to 3.8 kV. Best results for positive ion operation was obtained with skimmer (S) and lens (L) voltages of $S_1 = 24$ V, $S_2 = 17$ V, $L_1 = -22$ V, $L_2 = -4$ V and $L_3 = -58$ V. The potential difference between the skimmer and the end of the capillary controlled the extend of fragmentation through CAD. The potential difference was varied between 40 and 240 V. The mass spectrometer scanned from a mass-to-charge ratio (m/z) 50–500 at a step size of 0.1 units and a rate of 0.33 scans/s. In the ESP interface, Model 100547-3, for the HP 5989A instrument, the needle is grounded and charging occurs by keeping the cylindrical electrode (V_1) at about 2.7 kV, the end plate (V_2) at 2.7 kV and the capillary (V_3) at 2.8 kV (positive ion operation required reverse polarity, and V_{1-3} operated about 1 kV higher than in negative ion operation). Best results for negative ions were obtained with $S_1 = -40$ V, $S_2 = -17$ V, $L_1 = 38$ V, $L_2 = 83$ V and $L_3 = 37$ V. Positive ion operation required reversed polarity and only slight adjustments of L_1, L_2 and L_3. The CAD voltage was varied between −40 to −400 V (40 to 400 V). The mass spectrometer scanned from m/z 40–500 at a step size of 0.1 units and a rate of 0.5 scans/s. The threshold value was set to 250. The system was checked by analyzing adenosine-5′-monophosphate in IPA–water (1:1) for negative ion and arginine/gramicidin S in MeOH–water (1:1) for positive ion operation.

Sample preparation procedure

Blank milk was collected from β-lactam free cows at North Carolina State University (Raleigh, NC, USA). A 0.5-ml aliquot of milk was diluted with a equal volume of a solution consisting of MeCN–water (1:1). The sample was vortex-mixed for 10–15 s, placed in a microseparation system with an M_r 10 000 cutoff filter and centrifuged for approximately 30 min at 3000 *g* with a 40° fixed-angle rotor. The five tested β-lactam antibiotics were spiked at different lens and skimmer concentration levels into the milk blank. A 0.5–5-μl aliquot of the colorless blank or spiked ultrafiltrate was injected into the HPLC system equipped with a UV and a MS detector. A synthetic mixture of the drugs was

made with the same solvent combination as used for the milk sample.

HPLC–ESP-MS conditions

HPLC separation was performed using a mobile phase consisting of 40% (v/v) MeCN and 1% (v/v) HOAC in water (pH 3.0). The mobile phase flow-rate into the 70:1 splitter was 300 μl/min. The splitting reduced the flow-rate of the eluent entering the ESP interface to 4.3 μl/min. Higher flow-rates decreased the sensitivity of the ESP-MS system and produced instable signals. To estimate the dead time (t_0) of the HPLC system, pure MeCN as an non-retained standard was injected. The measured values were in the range of $1.475 \leq t_0 \leq 1.480$ min. The resulting low flow-rate and the relative long transfer line to the MS detector gave a delay of about 10–15% in the total ion current (TIC) or extracted ion chromatogram compared to the retention time t_R measured from the UV chromatogram.

The Analytica HPLC–ESP-MS interface, Model 101737 has a different lens and skimmer arrangement compared to the former interface type used for flow injection or FI-ESP-MS experiments and was autotuned with a synthetic mixture of the five β-lactam antibiotics (100 ng/μl each). The mass spectrometer scanned from m/z 200–450 at a step size of 0.1 units and a rate of 0.33 scans/s. The threshold was 500 and the abundance of each mass was sampled six times during a scan. In single ion monitoring (SIM) data acquisition a dwell time of 200 ms resulted in 4.33 cycles/s.

Microbore HPLC–ESP-MS Conditions

Isocratic microbore HPLC separation was performed using a mobile phase consisting of 30% (v/v) MeCN and 1% (v/v) HOAc in water (pH 2.9). The mobile phase flow-rate through the column and into the ESP interface was 4 μl/min. For injection volumes over 0.5 μl the sample was concentrated on-column by using pure water as a weak eluent. The elution time was dependent from the injected sample volume and varied from 1 to 8 min.

The Analytica HPLC–ESP-MS interface, Model 101737, was equipped with a new fire polished gold plated glass capillary and autotuned with a synthetic mixture of the five β-lactam antibiotics (100 ng/μl each). In SIM data acquisition a dwell time of 200 ms resulted in 4.33 cycles s.

RESULTS AND DISCUSSION

Effect of CAD voltage of positive ion MS spectra

The initial work for β-lactam antibiotics evaluated the CAD spectra that could be generated between the capillary and skimmer in the ESP interface. The increase in potential different between the capillary and the skimmer transferred of more internal energy into the molecule through collisional activation, resulting in the formation of structurally relevant product ions.

Most of the CAD fragment ions detected for each antibiotic could be identified based on sample cleavages of various groups from the molecule. The mass spectra at two different CAD voltages are listed in Table I for all five tested antibiotics. Low CAD voltages (<+100V) generally form molecular adducts and only very few characteristic fragment ions. With CAD energies in the range of +100 V to +200 V more fragmentations occurred. Usually, the fragmentation followed a pattern with all tested penicillins showing a characteristic cleavage product of the β-lactam ring $[C_6H_9HSO_2 + H]^+$ at m/z 160 and a further loss of COOH at m/z 114. Fragments formed by the cleavage of the amide moiety are more specific for the different penicillins. Penicillin G formed a $[C_6H_5CH_2]^+$ ion at m/z 91, ampicillin exhibited a $[C_6H_5CHNH_2]^+$ ion at m/z 106 as the base peak, amoxicillin showed a loss of NH_3 at m/z 349, cloxacillin, that contains one chlorine, showed two fragments at m/z 321 and 178 assignable to its amide moiety. The ESP ionization of the cephalosporin, cephapirin, produced no fragments from a cleavage inside of the β-lactam ring. Instead, a loss of the carboxylic group (–COOH) was tentatively identified at m/z 220. Several ions with lower m/z ratios had their origin from a successive cleavage of the amide groups.

Electrospray response in various mobile phase combinations

Sometimes, ESP intensity in a given solvent can vary drastically. The signal intensity is usually dependent on the hydration of the analyte in a given solvent as well as if the analyte is charged in solution. Fig. 1 shows the trend of the relative ESP intensity of $[M + H]^+$ ion of penicillin G, amoxicillin and cephapirin in different MeOH–water mobile phase combinations at a capillary-skimmer potential of 80 V. To compensate for slightly different instrumental tuning parameters, an internal standard of 100 ng/μl arginine was used. Usually, the more organic in the solvent, the higher the ESP response. Acetonitrile also resulted in slightly better ion currents compared to methanol when equal percentages of either organic component in water were used. Different solvent ratios changed not only the intensity of the detected fragments but also affected adduct ions. Higher methanol content increased the intensity of the $[M + H + MeOH]^+$ ion. Higher water concentrations in the mixture increased the intensity of the $[M + Na + H_2O]^+$, $[M + K + H_2O]^+$ or $[M + H + H_2O]^+$ ions in the ESP mass spectrum. The mass spectrometer did not scan high enough to detect a dimer ion. Therefore, no valid evidence could be obtained to confirm dimer ion formations at a low capillary-skimmer potential depending on the mobile phase combination.

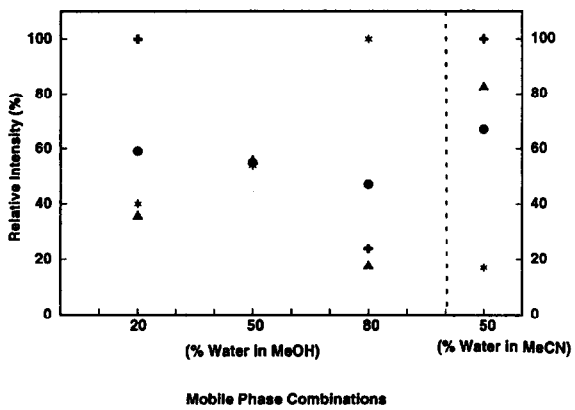


Fig. 1. Electrospray intensity for penicillin G (★) amoxicillin (+) and cephapirin (▲) in different mobile phase combinations at a capillary-skimmer potential of +160 V. ● = Average response.

Electrospray response using various mobile phase additives

A general approach to HPLC–ESP-MS includes minimization of eluent ion strength, use of as much organic modifier, such as methanol or acetonitrile, as practical and avoidance of buffer or other non-volatile mobile phase additives, unless they facilitate ionization. Mobile phase additives compete with sample ionization and result in a higher chemical background caused by adducts extending to a fairly high m/z ratio [44,45]. Therefore, it is necessary to compromise between ideal chromatographic conditions and conditions that support the ionization, desolvation and desorption processes of the HPLC–ESP-MS interface.

We used FI-ESP-MS experiments to assess the effect of various acids, bases and salts which change the pH of the mobile phase, and ion-pairing compounds on sensitivity. A lower pH of the eluent should favor the free acid form of the drugs, and thus increases the ESP response in positive ion mode. Suitable reversed-phase (RP) modifiers are volatile acids such as TFA, FAO, HOAc, volatile salts such as NH_4OAc, normally used in thermospray ionization to enhance ionization, and basic NH_4OH. There are an increasing number of reports using RP ion-pair HPLC methods to determine drug residues. Therefore, the effect of HFBA and TBAH addition to the eluent on ESP response of the five β-lactam antibiotics was tested. For this study, four different CAD voltages were applied for each drug infusion and the resulting mass spectra and the corresponding signal intensities were recorded. Fig. 2 presents bar graph plots of the relative ESP response of the $[M + H]^+$ ion (left column) and a characteristic $[fragment]^+$ ion (right column) of 100 ng/μl of each β-lactam antibiotic. The results obtained with the basic additive TBAH are not shown in Table II because the signals of all tested drugs were strongly suppressed in this eluent system. The only signal observed was the $[TBAH]^+$ ion at m/z 242.

In general, all five β-lactam antibiotics showed higher $[M + H]^+$ intensity at CAD voltages of +80 V and +160 V. An increase of the CAD voltage to +240 V or +320 V caused a severe drop in signal intensity. Therefore, the results at

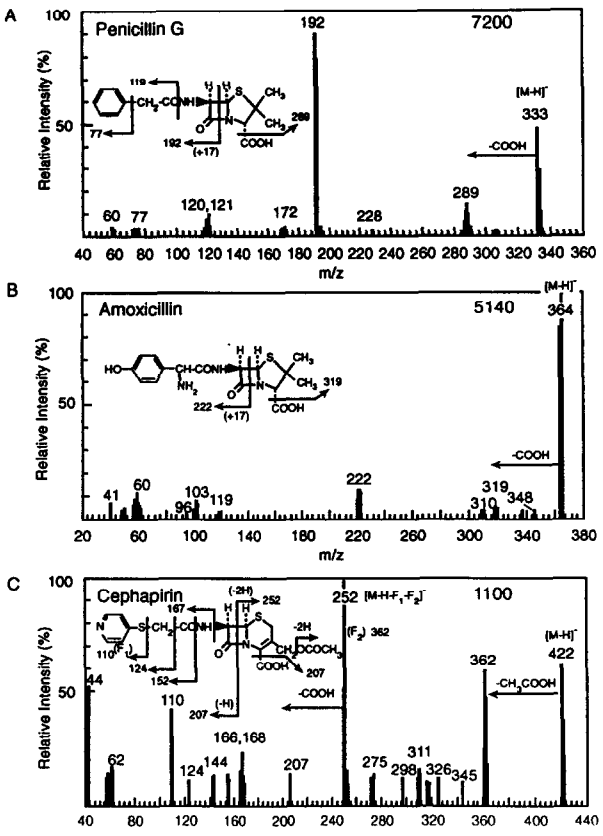


Fig. 2. Negative ion spectra of (A) penicillin G, (B) amoxicillin and (C) cephapirin at a CAD voltage of −120 V. Numbers at top right indicate ion counts.

higher CAD voltages were not listed in Table II. Comparing the intensity of characteristic fragment ions, a similar trend was observed throughout the whole palette of testing mobile phase systems. Using the mobile phase MeOH–water (1:1) without further additives gave poor ESP response. Addition of the strong acid TFA increased the ESP response of penicillin G and cloxacillin. For the other three drugs no significant increase or even distinct lower ESP responses are found. Addition of weaker acids such as FOA and HOAc drastically increased the ESP response of the $[M + H]^+$ ion as well as the fragment ions of all five drugs. A reasonable explanation for this increase may be a better droplet formation and nebulization capability due to the higher volatility and lower viscosity of these two acids. The near neutral pH of the eluent, achieved by the addition of NH_4OAc, weakened the ESP response of amoxicillin, ampicillin and cephapirin. Penicillin G and cloxacillin showed reasonably high ESP responses at lower CAD voltages. The use of the base NH_4OH as a mobile phase additive was well suited for penicillin G and cloxacillin. The addition of HFBA showed a reasonably good signal intensity for these two drugs. For the other three drugs no real advantage of using the two additives was found.

Table III shows the average ESP respond of all five tested compounds in the seven mobile phase system at four different CAD voltages. Over all, best ESP responses for drugs were achieved with 0.1% (v/v) FOA and 1% (v/v) HOAc in MeOH–water (1:1). The stronger acids TFA and HFBA did not facilitate ionization and are therefore not an ideal choice for future HPLC–ESP-MS work. The use of the volatile salts NH_4OAc and NH_4OH increased the ESP response in comparison to the unmodified eluent but the gain of signal intensity was rather limited. The use of FOA, HOAc or NH_4OAc to modify the mobile phase may be an interesting compromise between required chromatographic and electrospray conditions for a successful RP chromatographic separation and ESP-MS detection of β-lactam antibiotics.

Negative ion electrospray MS spectra

Penicillin contains a 6-aminopenicillanic acid nucleus and cephalosporins possess a 7-aminocephalosporinic acid nucleus. Due to their carboxylate group, the drugs are also suited to negative ion MS detection. A concentration of 100 ng/μl of each drug in IPA–water (8:2) was infused into the ESP interface at a flow-rate of 1.2 μl/min. In general, the negative ion spectra showed lower signal intensities, exhibiting all a $[M - H]^-$ ion and producing less fragmentations at higher capillary-skimmer voltages compared to positive ion spectra. The mass spectra at lower CAD voltages normally formed only the molecular ion and higher CAD voltages increased the background noise. The fragmentations seen in the mass spectra in Fig. 2 resulted mainly from a loss of COOH or HCO_2CH_3 and opening of the

TABLE II

COMPARISON BETWEEN DIFFERENT CAD VOLTAGES AND MOBILE PHASE ADDITIVES

CAD voltage = voltage measured at the end of the capillary (Analytica, Model 101737); relatvie intensity = relative signal intensity of the $[M+H]^+$ ion or $[fragment]^+$ ion; penicillin G, *m/z* 335 and *m/z* 160; ampicillin, *m/z* 350 and *m/z* 106; amoxicillin, *m/z* 366 and *m/z* 349; cloxacillin *m/z* 436 and *m/z* 277; cephapirin *m/z* 424 and *m/z* 111.

Drug	Mobile phase additive in MeOH–water (1:1)	Relative intensity (%) of $[M+H]^+$ ion at CAD = +80 V	Relative intensity (%) of $[fragment]^+$ ion at CAD = +160 V
Penicilin G	None, pH 8.4	30	5
	+0.1% (v/v) TFA, pH 2.2	44	21
	+0.1% (v/v) FOA, pH 3.1	44	15
	+1% (v/v) HOAC, pH 3.4	91	100
	+50 m*M* NH_4OAc, pH 7.1	55	61
	+50 m*M* NH_4OH, pH 10.5	100	50
	+10 m*M* HFBA, pH 2.2	68	41
Ampicillin	None, pH 8.4	4	16
	+0.1% (v/v) TFA, pH 2.2	5	6
	+0.1% (v/v) FOA, pH 3.1	100	100
	+1% (v/v) HOAc, pH 3.4	44	90
	+50 m*M* NH_4OAC, pH 7.1	9	10
	+50 m*M* NH_4OH, pH 10.5	14	10
	+10 m*M* HFBA, pH 2.2	2	0
Amoxicillin	None, pH 8.4	31	29
	+0.1% (v/v) TFA, pH 2.2	3	3
	+0.1% (v/v) FOA, pH 3.1	100	96
	+1% (v/v) HOAC, pH 3.4	73	100
	+50 m*M* NH_4OAC, pH 7.1	3	3
	+50 m*M* NH_4OH, pH 10.5	16	11
	+10 m*M* HFBA, pH 2.2	6	43
Cloxacillin	None, pH 8.4	3	0
	+0.1% (v/v) TFA, pH 2.2	63	67
	+0.1% (v/v) FOA, pH 3.1	31	100
	+1% (v/v) HOAC, pH 3.4	31	100
	+50 m*M* NH_4OAC, pH 7.1	56	93
	+50 m*M* NH_4OH, pH 10.5	100	93
	+10 m*M* HFBA, pH 2.2	34	63
Cephapirin	None, pH 8.4	2	4
	+0.1% (v/v) TFA, pH 2.2	9	5
	+0.1% (v/v) FOA, pH 3.1	100	100
	+1% (v/v) HOAC, pH 3.4	89	43
	+50 m*M* NH_4OH, pH 7.1	0.4	2
	+50 m*M* NH_4OH, pH 10.5	24	0.2
	+10 m*M* HFBA, pH 2.2	14	12

β-lactam ring, forming ions at *m/z* 289 and 192 for penicillin G, *m/z* 319 and 222 for amoxicillin, and *m/z* 207, 167, and 110 for cephapirin, respectively. It was found that at higher solvent pH, better ion currents were obtained from selected antibiotics such as β-lactams, aminoglycosides, tetracyclines, and sulfonamides [46]. Typically, pH 10 achieved through the addition of 50 m*M* NH_4OH resulted in highest ESP responses for $[M-H]^-$ ion for the three β-lactam antibiotics compared to the non-buffered solution (pH 7). This observation is consistent

TABLE III

COMPARISON BETWEEN DIFFERENT CAD VOLTAGES AND MOBILE PHASE ADDITIVES

Averaged data from all five drugs. CAD voltage = voltage measured at the end of the capillary (Analytica, Model 101737); relative intensity = relative signal intensity of the $[M + H]^+$ ion or $[fragment]^+$ ion; penicillin G, m/z 335 and m/z 160; ampillicin, m/z 350 and m/z 106; amoxicillin, m/z 366 and m/z 349; cloxacillin, m/z 436 and m/z 277; cephapirin m/z 424 and m/z 111.

Mobile phase additive in MeOH–Water (1:1)	Relative intensity (%) of $[M + H]^+$ ion at CAD =				Relative intensity (%) of $[fragment]^+$ ion at CAD =			
	+80 V	+160 V	+240 V	+320 V	+80 V	+160 V	+240 V	+320 V
None, pH 8.4	20	10	5	5	15	12	10	8
+0.1% (v/v) TFA, pH 2.2	35	28	5	2	30	28	3	1
+0.1% (v/v) FOA, pH 3.1	100	50	25	7	40	81	60	3
+1% (v/v) HOAc, pH 3.4	75	57	38	8	48	100	47	18
+50 m*M* NH_4OAc, pH 7.1	35	12	5	2	20	42	6	2
+50 m*M* NH_4OH, pH 10.5	70	35	10	5	30	40	21	14
+10 m*M* HFBA, pH 2.2	35	20	2	1	28	38	15	3

with the formation of anions in solution for the samples analyzed but these negative ion ESP signals were only one-fourth as sensitive as the $[M + H]^+$ peak in position ion ESP operation.

HPLC–UV chromatograms

Methodology was developed for the detection of the five β-lactam antibiotics directly from milk ultrafiltrate by RP chromatography using UV detection. Various elution systems consisting of MeCN, water, 1% (v/v) HOAc and 0.01 *M* NH_4OAc were investigated for separation of the individual drugs from milk ultrafiltrate. The addition of NH_4OAc to MeCN–water–HOAc mobile phase systems reduced the k' values of all five drugs to under 6 and no baseline separation could be achieved. Removing the salt increased the k' values and improved the peak symmetry. From the four tested mobile phase systems with 20%, 30%, 40% and 50% (v/v) MeCN and 1% (v/v) HOAC in water an addition of 40% (v/v) organic resulted in a baseline separation of all five drugs in a reasonable run time (about 15 min). Under these HPLC conditions the β-lactam antibiotics were retained, permitting their separation from most bovine milk extract interferences and providing a clean analytical window for their detection. Fig. 3A shows the UV chromatogram generated at 230 nm of a blank milk ultrafiltrate. Chromatograms of bovine milk ultrafiltrate spiked with a concentration of 80 ppm of each drug are given in Fig. 3B. The UV chromatogram of a synthetic mixture of 20 ppm of each drug reported in Fig. 3C showed a similar elution profile but a less intense solvent front at the beginning of the run. The average capacity factors of antibiotics in this mobile phase system were $k'_1 = 1.50$, $k'_2 = 3.53$, $k'_3 = 4.38$, $k'_4 = 5.26$ and $k'_5 = 8.17$.

HPLC–ESP-MS

Full-scan mass spectra (m/z 200–450) were obtained by HPLC–ESP-MS in 40% MeCN with 1% HOAc in water from injection of 500 ng per component. Fig. 4 displays the extracted ion current profiles of the five separated drugs (peaks 1–5). Due to the 70:1 post-column split ratio, a longer elution time and a slight tailing of the peaks occurred. The determination in the selected-ion monitoring (SIM) acquisition mode of a blank milk ultrafiltrate (A), 143 pg per

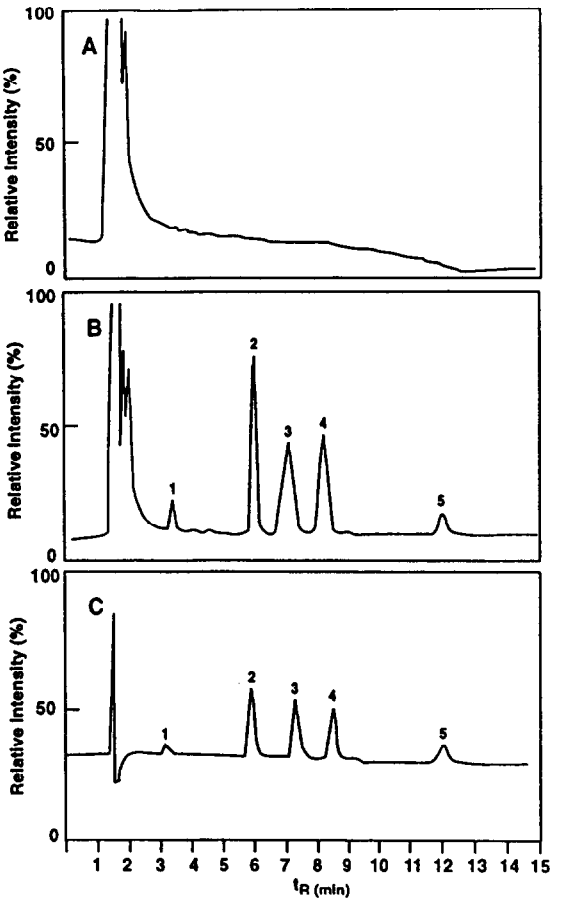


Fig. 3. HPLC–UV chromatograms of (A) blank milk ultrafiltrate, (B) 40 ng of each drug spiked into milk ultrafiltrate, (C) synthetic mixture of 10 ng of each drug. Peaks: 1 = penicillin G; 2 = cloxacillin; 3 = cephapirin; 4 = amoxicillin; 5 = ampicillin. Injection column 0.5 μl; flow-rate 0.3 ml/min; λ = 230 nm; AUFS = 1.0.

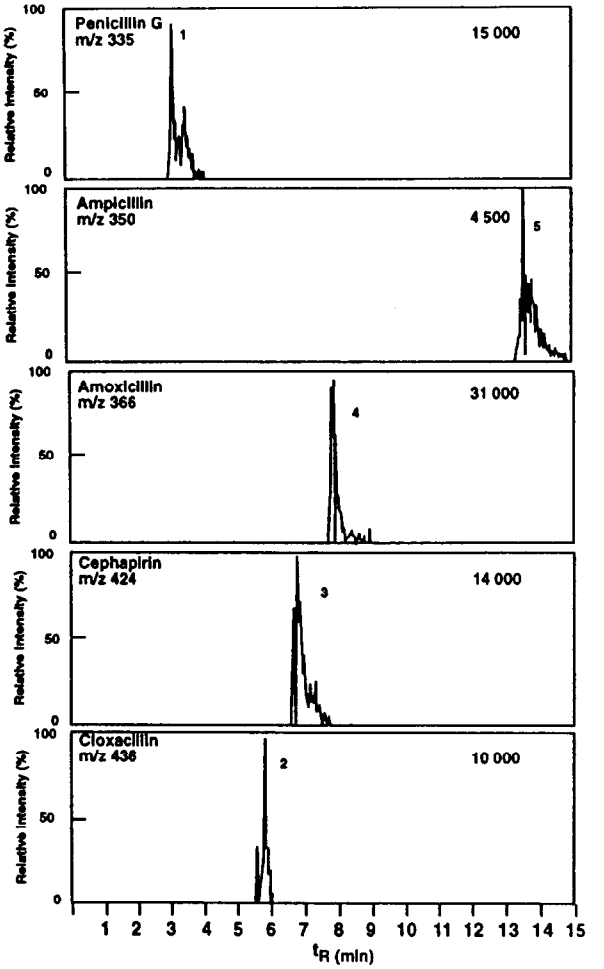


Fig. 4. Extracted ion current profiles of an HPLC–ESP-MS analysis of a synthetic mixture of the five analytes at a level of 7.1 ng per component corresponding to an on-column injection of 500 ng of each drug. Number at top right indicates ion counts. Peaks: 1 = penicillin G; 2 = cloxacillin; 3 = cephapirin; 4 = amoxicillin; 5 = ampicillin.

component spiked milk ultrafiltrate (B) and synthetic mixture (C) of the five analytes is shown in Fig. 5. The 143 pg level corresponds to 100% recovery from an injection of 0.5 μl of a 200 ppm sample. For this amount injected, signal-to-noise ratios ranged from 2 for penicillin G, 2 for cloxacillin, 4 for cephapirin, 6 for amoxicillin, to 10 for ampicillin. In the SIM acquisition mode the $[M + H]^+$ ion of each free drug was monitored and no interference from milk ultrafiltrate appeared.

Linearity of the HPLC–ESP-MS method was determined by spiking drug standards in a concentration of 20, 60, 80 and 200 ppm into blank milk ultrafiltrate corresponding to 143, 428, 571 and 1428 pg of each drug after splitting of the eluent from the HPLC system. Within this range r ($n = 4$) for penicillin G was 0.9997, for cloxacillin 0.9966, for cephapirin 0.9994, for amoxicillin 0.9979, and for ampicillin 0.9961 (Fig. 6).

Approaches to determine ppb levels in complex matrices —preliminary experiments

Determination at the levels of regulatory interest using this method would require on-column

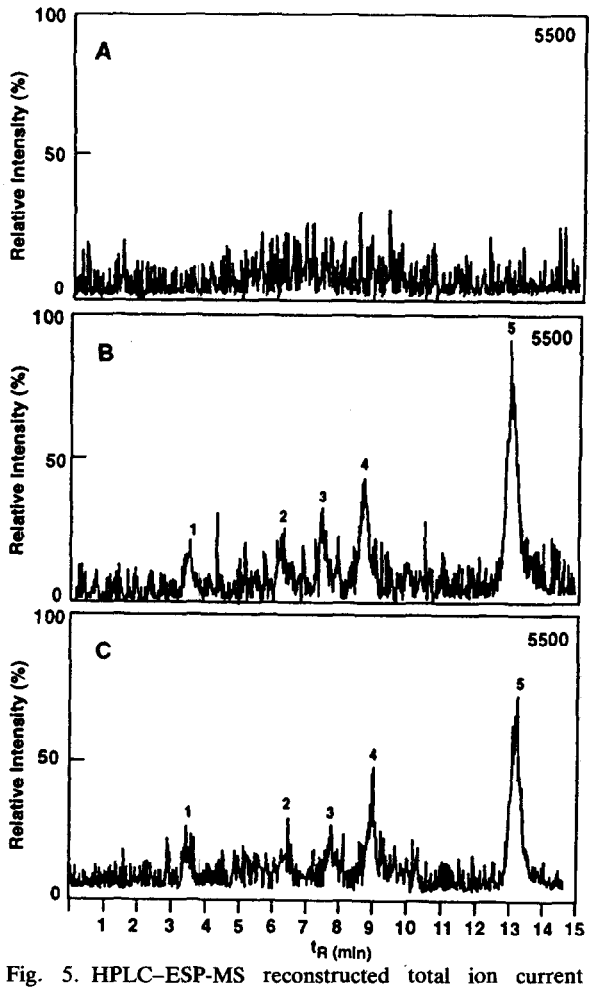


Fig. 5. HPLC–ESP-MS reconstructed total ion current profiles of (A) blank milk ultrafiltrate, (B) 143 pg of each drug in milk ultrafiltrate, and (C) synthetic mixture of 143 pg of each drug in the SIM data acquisition mode. This corresponds to an on-column injection of 10 ng of each drug. Number at top right indicates ion counts. Peaks: 1 = penicillin G; 2 = cloxacillin; 3 = cephapirin; 4 = amoxicillin; 5 = ampicillin.

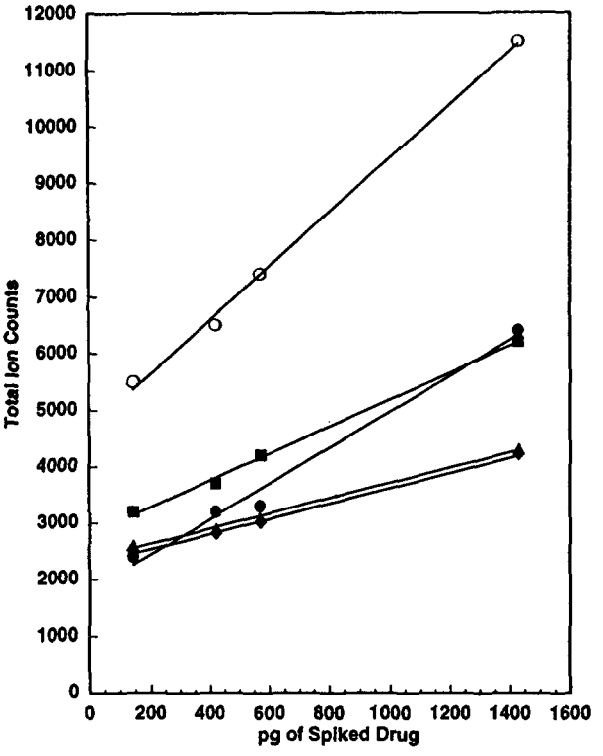


Fig. 6. Heights of peaks in total ion current profile *versus* amount for β-lactam antibiotics introduced into the mass spectrometer. ● = Penicillin G; ◆ = cloxacillin; ▲ = cephapirin; ■ = amoxicillin; ○ = ampicillin.

detection of 5 pg/μl penicillin G, 10 pg/μl of cloxacillin, amoxicillin, ampicillin, and 20 pg/μl of cephapirin according to the known tolerance levels for the particular drug used on dairy cows [47]. All of these amounts are currently below the instrument detection limits discussed above. To detect 5 ppb levels of these drugs, an injection of about 30 μl is required to achieve a mass loading suitable for ESP-MS detection. Usually, an on-column concentration step is needed prior to elution at this higher injection volume [48].

Preliminary studies have demonstrated that β-lactam antibiotics can be concentrated directly on an analytical column from filtrates with a weak solvent such as water and eluted under isocratic conditions or with a solvent gradient. Similar approaches were already used successfully for determination of novobiocin [49], virginiamycin [50] and tetracyclines [51]. Samples with high matrix interference can be treated under a procedure sometimes termed "heart-cutting" [17], and rechromatographed under different conditions. This approach has been successfully used for determination of penicillin G and lincomycin resides in milk and tissue [19].

Another possibility would be the utilization of a recently introduced mechanical vibration-assisted ESP source [52]. This ultrasonic mechani-

cal vibration nebulization-assisted electrospray (ultraspray) allows consistent charged droplet production less affected by solution flow rate, conductivity and solution chemistry than with a conventional ESP interface. The initial charged droplet size produced is primarily a function of the frequency of mechanical vibration. This new interface type has the advantage that it can directly operate at flow-rates up to 400 μl/min and the disadvantages stemming from a post-column splitting of the eluent would be omitted without loosing the ability to work with well suited and efficient 150 × 2 mm columns. The signal intensity of the $[M+H]^+$ peak of 100 ng/μl penicillin G in MeOH–water (1:1) with addition of 1% (v/v) HOAc within the flow-rate range of 2–50 μl/min was nearly constant.

To further investigate the feasibility of a direct HPLC–ESP-MS separation at flow-rates under 10 μl/min, we also tested a packed RP capillary column system operating at a flow-rate of 2–10 μl/min [53] and, therefore, more favorable operation without splitting of the solvent flows. A synthetic mixture of 5 pg/μl of each drug was eluted with the mobile phase containing 30% (v/v) MeCN and 1% (v/v) HOAc in water. The separation was performed on a 15 cm × 320 μm I.D. Fusica C_{18} capillary column. The void volumes of various parts (*e.g.*, injector, transfer lines, connections, conventional stainless-steel electrospray needle and large injection volume) caused band broadening which reduced resolution. Fig. 7 presents the ion trace of all five β-lactam drugs acquired under the SIM mode. Also, remarkable is the change in elution order of penicillin G and cloxacillin on the microbore column compared to the 2.1 mm I.D. column. To effectively pursue this promising small scale separation technique we are currently working on the reduction of void volumes which will improve spray formation and ESP ionization efficiency.

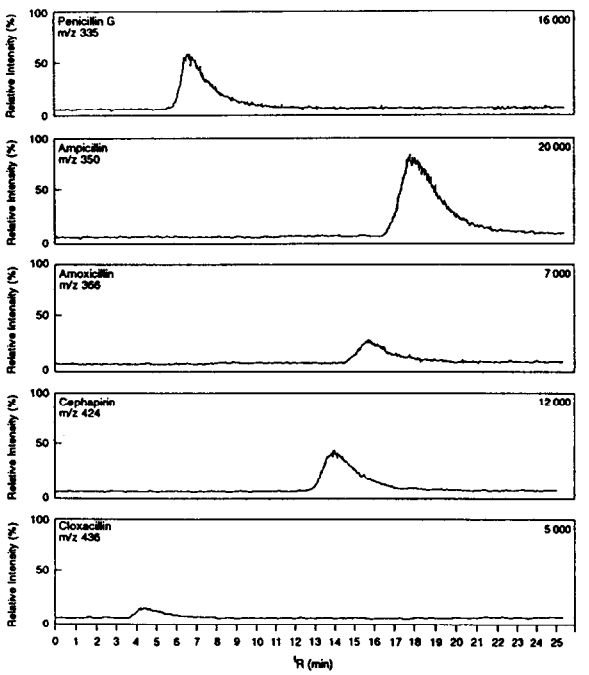


Fig. 7. Extracted ion current profiles of an microbore HPLC–ESP-MS analysis of a synthetic mixture of the five analytes at a level of 5 pg/μl per component. Number at top right indicates ion counts of the particular mass peak seen in the ion trace. Peaks: 1 = cloxacillin; 2 = penicillin G; 3 = cephapirin; 4 = amoxicillin; 5 = ampicillin.

CONCLUSIONS

The HPLC–ESP-MS method described for a multiresidue analysis of β-lactam antibiotics was developed based on preliminary flow injection and infusion experiments clarifying CAD and solvent dependence of the analysis. Due to the low flow-rate limitation required for successful ESP ionization, a post-column splitting of the eluent from the column was necessary. The lower amount of analytes entering the ESP interface also produced a necessary sacrifice of overall detection sensitivity. Therefore, the 5 ppb level of regulatory interest was not reached with this method despite good chromatographic resolution of the analytes.

Further improvements are suggested by discussing on-column concentration techniques and possibilities for an elimination of the eluent splitting prior to ESP ionization and MS detection. So far, the use of a RP microbore HPLC column shows promising results if the chromatographic separation can be improved and void volumes reduced to reasonable levels.

Future work will focus on the optimization of

instrumental parameters, improvement of apparatus performance, and evaluation of other chromatographic modes to multiresidue analysis.

ACKNOWLEDGEMENTS

We thank Mr. D. Heller from FDA, Center for Veterinary Medicine, Beltsville MD, USA, for helpful discussions concerning the project, Dr. K.L. Tyczkowska of the College of Veterinary, North Carolina State University, Raleigh, NC, USA, for the blank milk ultrafiltrate, and Analytica of Branford Inc., Branford, CT, USA, for the flow injection experiment with the mechanical vibration-assisted ESP interface.

This research was supported by US Food and Drug Administration Cooperative Agreement No. FD-U-000581.

REFERENCES

1 E.H. Flynn, *Cephalosporins and Penicillins, Chemistry and Biology*, Academic Press, New York, 1872.
2 D.F. Wishart, *J. Am. Vet. Med. Assoc.*, 185 (1984) 1106.
3 K.L. Tyczkowska, R.D. Voyksner and A.L. Aronson, *J. Chromatogr.*, 594 (1991) 195, and references cited therein.
4 M.M. Siegel, R.K. Isensee and D.J. Beck, *Anal. Chem.*, 59 (1987) 989.
5 A. Goodman-Gilman, L.S. Goodman, T.W. Rau and F. Murad (Editors), *The Pharmacological Basis of Therapeutics*, MacMillan, New York, 7th ed., 1979.
6 M.E. Wolff, *Medical Chemistry*, Wiley, New York, 4th ed., 1979.
7 *Fed. Reg.*, 52 (1987) 165, 196; 54 (1989) 196; US Government Printing Office Superintendent of Documents, Washington, DC.
8 D.M. Mac Cauley and V.S. Packard, *J. Food Prot.*, 44 (1981) 696.
9 J.J. Ryan, E.E. Wildman, A.H. Duthie, H.V. Atherton and J.J. Aleong, *J. Dairy Sci.*, 69 (1986) 1510.
10 P. Rohrer, M. Schaellibaum and J. Nicolet, *J. Assoc. Off. Anal. Chem.*, 48 (1985) 59.
11 S.E. Charm and R. Chi, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 304.
12 D.L. Collins-Thompson, D.S. Wood and I.Q. Thompson, *J. Food Prot.*, 51 (1988) 632.,
13 S.A. Thorogood and A. Ray, *J. Soc. Dairy Technol.*, 37 (1984) 38.
14 R.D. Munns, W. Shimoda, J.E. Roybal and C. Vieira, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 968.
15 W.A. Moats, *J. Agric. Food Chem.*, 31 (1983) 1348.
16 W.A. Moats, *J. Assoc. Off. Anal. Chem.*, 3 (1990) 343; and references cited therein.
17 J. Carlquist and D. Westerlund, *J. Chromatogr.*, 344 (1985) 285.
18 K. Tyczkowska and A.L. Aronson, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 773.
19 W.A. Moats, *J. Chromatogr.*, 507 (1990) 177.
20 A.I. MacIntosh, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 880.
21 W.A. Moats and R. Malisch, *J. Assoc. Off. Anal. Chem. Int.*, 75 (1992) 257.
22 W.A. Moats, *J. Chromatogr.*, 593 (1992) 15.
23 K.L. Tyczkowska, R.D. Voyksner, R. Straub and A.L. Aronson, *J. Assoc. Off. Anal. Chem.*, submitted for publication.
24 R.M. Simpson, F.B. Suhre and J.W. Shafter, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 23.
25 M.A. Hayes, *J. Chromatogr. Sci.*, 26 (1988) 146.
26 U. Meetschen and M. Petz, *J. Assoc. Off. Chem.*, 73 (1990) 373.
27 M.L. Vestal, *Science*, 226 (1984) 275.
28 C.R. Blackey, J.J. Carmody and M.L. Vestal, *J. Am. Chem. Soc.*, 102 (1980) 5931.
29 D. Pilosof, Kim H. Yong, D.F. Dyckes and M.L. Vestal, *Anal. Chem.*, 56 (1984) 1236.
30 S.E. Unger and B.M. Wanach, *Spectroscopy*, 1 (1986) 33.
31 S. Suwnrumpha and R.B. Freas, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 983.
32 K. Tyczkowska, R.D. Voyksner and A.L. Aronson, *J. Chromatogr.*, 490 (1989) 101.
33 R.D. Voyksner, K. Tyczkowska and A.L. Aronson, *J. Chromatogr.*, 567 (1991) 389.
34 K.L. Tyczkowska, R.D. Voyksner and A.L. Aronson, *J. Vet. Pharmacol. Therap.*, 14 (1991) 51.
35 M. Scandola, G. Tarzia, G. Gaviraghi, D. Chiarello and P. Traldi, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 851.
36 G.W. Griffin, I. Dzidic, D.I. Caroll, R.N. Stillwell and E.C. Horning, *Anal. Chem.*, 45 (1973) 1204.
37 E.C. Horning, M.G. Horning, D.I. Caroll, I. Dzidic and R.N. Stillwell, *Anal. Chem.*, 45 (1973) 936.
38 E.C. Horning, M.G. Horning, D.I. Caroll, I. Dzidic and R.N. Stillwell, *Adv. Biochem. Psychopharmacol.*, 7 (1973) 15.
39 M.W. Siegel and W.L. Fite, *J. Phys. Chem.*, 80 (1976) 2871.
40 C.M. Whitehouse, R.N. Dreyer, M. Yamashita and J.B. Fenn, *Anal. Chem.*, 57 (1985) 675.
41 J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong and C.M. Whitehouse, *Mass Spectrom. Rev.*, 9 (1990) 37.
42 J.R. Perkins, C.E. Parker and K.B. Tomer, *J. Am. Soc. Mass Spectrom.*, 3 (1992) 139; and references cited therein.
43 L.G. McLaughlin and J.D. Henion, *J. Chromatogr.*, 591 (1992) 195.
44 A.P. Bruins, T.R. Covey and J.D. Henion, *Anal. Chem.*, 59 (1987) 2642.
45 M.G. Ikonomou, A.T. Blades and P. Kebarle, *Anal. Chem.*, 62 (1990) 957.

46 R. Straub and R.D. Voyksner, *Proceedings of the 40th Annual Conference on Mass Spectrometry and Allied Topics, Washington, DC, May 31–June 5, 1992*, p. 1879.
47 J.W. Harman, *Food Safety and Quality –FDA Strategy Needed to Address Animal Drug Residues in Milk, Report to the Chairman*, Human Resources and Intergovernmental Relations Subcommittee, Committee on Government Operations, House of Representatives, Washington, DC, 1992, pp. 60–62.
48 W.A. Moats, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 343.
49 W.A. Moats and L. Leskinen, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 776.
50 W.A. Moats and L. Leskinen, *J. Agric. Food Chem.*, 36 (1988) 1297.
51 W.A. Moats, *J. Chromatogr.*, 358 (1986) 253.
52 C.M. Whitehouse, S. Shen, and J.B. Fenn, presented at the *40th Annual Conference on Mass Spectrometry and Allied Topics, Washington, DC, May 31–June 5, 1992*; paper TOA 1:15.
53 M.T. Davis and T.D. Lee, *Protein Sci.*, 1 (1992) 935.