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Determination of aminoglycoside antibiotics by reversed-phase ion-pair high-performance liquid chromatography coupled with pulsed amperometry and ion spray mass spectrometry☆

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ABSTRACT

This work constitutes a preliminary investigation of a high-performance liquid chromatographic (HPLC)–mass spectrometric (MS) method for confirming aminoglycoside residues in bovine tissues. A reversed-phase ion-pair HPLC method for the separation of four aminoglycosides was developed using volatile ion-pairing agents and optimized for detection with an ion spray HPLC–MS interface. The method is also compatible with a commercial pulsed amperometric detector that was used for HPLC method development and that may be useful for the screening and quantification phases of a regulatory method. Several column phases, eluent compositions, and pairing ions were evaluated for optimum HPLC–MS sensitivity. Detection limits are in the low nanogram range with the pulsed amperometric detector and with HPLC–MS in the selected ion monitoring mode. Results with bovine kidney, fortified to 20 ppm and extracted by matrix solid-phase dispersion, obtained using both detectors are presented.

INTRODUCTION

There is a great need for improved analytical methods for confirmation of aminoglycoside (AG) antibiotic residues in the edible tissues of livestock. Certain members of this class are used widely for veterinary purposes and because of their nephro- and ototoxicity, and the potential for acquired bacterial resistance, their residues in food products are of concern to the US Food and Drug Administration (FDA). For confirmation, especially in a regulatory setting, mass spectrometry has been cited as an ideal choice [1]. However, the hydrophilicity, basicity and thermal lability of these analytes preclude their direct determination by gas chromatography (GC)–mass spectrometry (MS). Therefore, high-performance liquid chromatographic (HPLC)–MS (HPLC–MS) and HPLC–MS–MS methods may have the greatest potential for accomplishing direct multi-residue identifications in complex biological matrices.

Direct determination of AGs by HPLC has been reported using refractive index [2] and ultraviolet (UV) detection [3], which lack sensitivity and selectivity. Other reports include detection by pulsed amperometry [4], including its novel combination with high-performance anion-exchange chromatography [5], and d.c. amperometry [6]. Shaikh and Allen [7] have reviewed physico-chemical methods used for AG determination in animal tissues. Traditionally, microbiological assays have been used [8], but there are an increasing number of reports of reversed-phase ion-pair HPLC methods using fluorescence detection of derivatized analytes. Such methods have been used to detect neomycin in bovine and porcine tissues [8,9] and bovine milk

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[10,11], gentamicin in bovine milk [12] and muscle tissue [13] and rabbit renal cortex [14] and streptomycin in chicken meat [15]. There are no reports of the direct spectrometric determination of AGs in animal tissue of which we are aware.

There are only a few literature reports on the on-line HPLC–MS detection of AGs. Tobramycin, kanamycin and neamine have been detected using the moving belt interface and ammonia chemical ionization [16]. Atmospheric pressure chemical ionization (APCI) mass spectra have been shown for the kanamycin components [17] with detection limits between 1 and 50 ng reported for the gentamicin C and kanamycin components by flow-injection analysis in the selected ion monitoring (SIM) mode [18]. Dihydrostreptomycin was detected by continuous-flow fast atom bombardment MS using capillary HPLC at a flow-rate of 50 nl/min [19]. Most recently, the gentamicin C components have been determined using thermospray HPLC–MS [20].

The unique features of the ion spray interface for HPLC–MS [21] make it worth investigating for the determination of these compounds. That is, for analytes that exist as ions in solution, ion desorption from the liquid to the gas phase occurs under the influence of an electric field without the need for heat or other severe conditions. Other HPLC–MS techniques such as thermospray and heated pneumatic nebulization for APCI rely on heat for either solvent or analyte vaporization, or both. There are, however, limitations to the mobile phase composition for good performance of the ion spray interface. Although the characterization of these effects is on-going, it is generally believed that use of mobile phases with low ionic strength, significant concentrations of organic modifiers such as methanol or acetonitrile and avoidance of non-volatile additives will achieve the best performance [21,22]. Therefore, it is necessary in some instances to compromise between ideal chromatographic conditions and conditions that support the ionization, desolvation and desorption processes of the ion spray HPLC–MS interface.

The AGs are difficult to retain in the reversed-phase mode even with purely aqueous eluents [2]. Therefore, most chromatographic methods are based on some form of ion chromatography, typically ion-exchange or ion-pair modes. Most of the reported ion chromatographic eluents are not well suited for use with the ion spray interface owing to the presence of non-volatile electrolytes that suppress analyte desorption and increase the background ion current in the mass spectrometer. For instance, ion-pair methods commonly employ sodium alkyl sulfonate pairing ions together with high concentrations of sodium salt buffers to improve the chromatographic peak shape [14].

Inchauspé and Samain [2] have reported the use of volatile perfluorinated carboxylic acids as ion-pairing agents to facilitate the retention of AGs in the reversed-phase mode for their preparative isolation by HPLC [2]. They later found this approach to be useful for the characterization of AGs in culture broths by off-line field desorption MS and predicted the applicability of these volatile surfactants to evolving HPLC–MS techniques [23]. Indeed, mobile phases containing trifluoroacetic acid (TFA) have been used successfully for the thermospray HPLC–MS determination of gentamicin C [20] and with ion spray HPLC–MS for the determination of peptides in the positive-ion mode [24]. TFA was not found to be effective for retaining the AGs, with the exception of the gentamicins, for which it has a unique selectivity [25]. However, Inchauspé and co-workers have successfully used the three- and four-carbon homologues, pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA), to separate mixtures of AGs [2,23,25,26].

It should be noted that, in some instances, post-column suppressors can be used on-line to remove ionic species that interfere with detection [27]. However, this adds complexity in addition to post-column dead volume and the attendant band broadening to the system. More work is needed to discover if suppressor technology can improve HPLC–MS determinations of these compounds. In this work, we explored reversed-phase ion-pair HPLC with volatile pairing ions as a means of determining AGs by ion spray HPLC–MS in the positive-ion mode without post-column suppression or make-up flows.

The feasibility of ion spray HPLC–MS for AG determinations is demonstrated here in the full-scan and SIM modes. Ultimately, confirmation will be accomplished by HPLC–MS–MS in the selected reaction monitoring (SRM) mode. With post-column addition of strong base, the method is also compatible with pulsed amperometric detection (PAD),

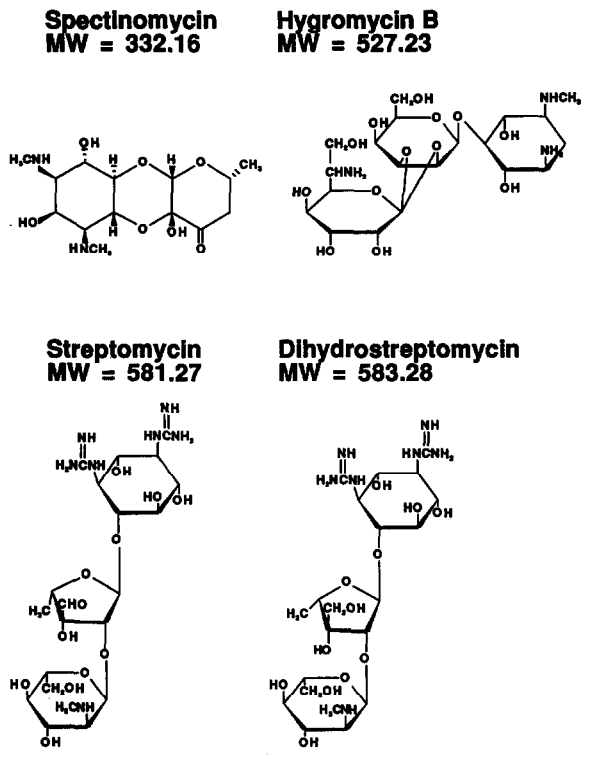

Fig. 1. Structures of the aminocyclitol and aminoglycoside antibiotics and their monoisotopic molecular weights (MW).

which could possibly be used for the screening and quantification phases of a regulatory method. Data are presented for four antibiotics of immediate interest to the US FDA (Fig. 1). Streptomycin and dihydrostreptomycin are aminoglycosides whereas spectinomycin and hygromycin B are classified as aminocyclitols [28]. They are all approved for some veterinary uses, although tolerance levels do not exist for all specific uses [29]. Current levels of regulatory interest in bovine kidney are 0.02 ppm for hygromycin B, 0.1 ppm for spectinomycin and 0.5 ppm for streptomycin and dihydrostreptomycin [30].

EXPERIMENTAL

Chemicals

Water was distilled in-house and purified with a Barnstead (Boston, MA, USA) Nanopure system. Methanol, hexane and ethyl acetate were of HPLC grade from Fisher Scientific (Rochester, NY, USA) and acetonitrile (ACN) was of Fisher Optima grade. Sodium hydroxide solution was of Fisher Certified 50% (w/w) and ammonia solution was of Fisher ACS reagent grade. Sulfuric acid, doubly-distilled from Vycor, was purchased from GFS Chemicals (Columbus, OH, USA). Spectinomycin dihydrochloride, hygromycin B, streptomycin sesquisulfate, dihydrostreptomycin sesquisulfate, pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA) were products of Sigma (St. Louis, MO, USA). The aminoglycoside standards were stored in a desiccator at 4°C and were not dried or purified further before use. Aqueous stock solutions with a free base concentration of 4 μmol/ml were found to be stable for at least 5 months at 4°C. All solutions were stored in polypropylene or polyethylene containers to avoid adsorption on glassware [31]. The amounts and concentrations specified in this paper refer to the free base form of the analytes.

Sample extraction procedure

The matrix solid-phase dispersion (MSPD) method of Barker *et al.* [32] as modified by Schenck [9] was used to extract analytes added to control bovine kidney. Control kidney was obtained at a local supermarket and homogenized in 300-g batches for 45 s at low speed in a Waring blender (VWR Scientific, Rochester, NY, USA). Tissue homogenate (0.5 g) was placed in an agate mortar and spiked with 5 μl of a 2 μg/μl aqueous stock solution of the four analytes and allowed to equilibrate for 5 min. Bondesil cyanopropyl 40-μm bulk packing material (Analytichem International, Harbor City, CA, USA) (2 g) was added to the tissue and blended manually with a pestle for 2 min. The resulting mixture was transferred through a funnel into an empty 8-ml solid-phase extraction reservoir (Analytichem International) with 20-μm frits placed at the top and bottom and compressed to a bed volume of *ca.* 4 ml. The mortar was then rinsed with 3 ml of hexane, which was poured on to the top of the column and washed with water and methanol before the next dispersion was performed. Using a Supelco (Bellefonte, PA, USA) vacuum manifold, the 3-ml hexane wash was eluted at 6.77 kPa (2 in.Hg), as were successive 5-ml aliquots of ethyl acetate, methanol and methanol–water (50:50). The analytes were eluted with 1 ml of water followed by 8 ml of

0.05 *M* sulfuric acid at 3.39 kPa (1 in.Hg). For PAD the eluate was neutralized with NaOH (50%, w/w) and 15 μl of the 9-ml eluate were injected. Sample pH was measured with a Fisher Scientific Model 815MP pH meter. For HPLC–MS an aliquot of the 9-ml eluate was concentrated by a factor of four in a SpeedVac SVC 100 (Savant Instruments, Farmingdale, NY, USA) and neutralized with ammonia solution and 10 μl were injected on to the column.

Chromatography

Separations were developed using a Dionex (Sunnyvale, CA, USA) gradient pump module equipped with a Model 4500i pulsed amperometric detector. The gradient pump module was used at a flow rate of 1 ml/min while eluents were sparged and pressurized with a Dionex eluent degas module using ultra-high-purity helium. The system included a pneumatically actuated injector with a 25-μl sample loop.

The HPLC columns used for this work were as follows: 3-μm Spherisorb ODS-2 (100 mm × 4.6 mm I.D.), packed by Keystone Scientific (Bellefonte, PA, USA), 5-μm Inertsil ODS-2 (100 mm × 4.6 mm I.D.), purchased from Keystone Scientific, and 10-μm PRP-1 (220 mm × 4.6 mm I.D.), from Hamilton (Reno, NV, USA). For HPLC–MS work, a Waters (Milford, MA, USA) Model 510 pump delivered eluent at a flow-rate of 200 μl/min to a 5-μm Spherisorb ODS-2 column, (100 mm × 2 mm I.D.), also packed by Keystone Scientific. The injector was a Rheodyne (Cotati, CA, USA) Model 7125 with a 20-μl sample loop. Spherisorb ODS-2 (3 μm) guard columns (10 mm × 4.0 mm I.D. or 10 mm × 2 mm I.D.) were used with each of the Spherisorb ODS-2 analytical columns (Keystone Scientific).

The various mobile phases tested ranged from 8 to 16% ACN and 5 to 40 m*M* PFPA or HFBA. They were prepared by diluting the acid with ACN to 50 ml in a volumetric flask and mixing this with 575 ml of water. Water and ACN were filtered through 0.2-μm nylon filter disks before use (Rainin Instrument, Woburn, MA, USA). The pH of an 8% ACN–20 m*M* PFPA eluent was measured to be 1.9. The HPLC column void volume was determined by injection of methanol. Columns were flushed overnight with ACN–water (40:60) at a flow-rate of 0.1 ml/min.

Pulsed amperometry

The pulsed amperometric detector was a Dionex Series 4500i with a solvent-compatible cell (3.5-μl volume) and a gold working electrode. The applied potential for oxidation (E_1) was 0.1 V with a pulse duration of 480 ms and a current sampling period of 200 ms. Potentials used for cleaning the working electrode (E_2 and E_3) were 0.6 and −0.8 V with pulse durations of 120 and 300 ms, respectively. The response time was set at 1 s. A Dionex post-column pneumatic controller maintained at 207 kPa (30 p.s.i.) supplied 0.3 *M* NaOH at a flow-rate of 0.8 ml/min to the HPLC column effluent, making the total flow-rate to the PAD cell 1.8 ml/min. A 122-cm reaction coil (Dionex) was used for mixing of the two streams. Full-scale output was set on the PAD to a value of 100 or 300 nA and chromatograms were recorded with a Hewlett-Packard (Avondale, PA, USA) Model 3390A integrator. The 0.3 *M* NaOH solution was prepared by sparging water with helium for 15 min and then adding the NaOH solution and swirling gently to effect mixing. The reference electrode was soaked in water for *ca.* 30 min before use and was stored in certified potassium hydrogenphthalate buffer solution (pH 4) when not in use (Fisher Scientific).

Mass spectrometry

The HPLC column effluent (200 μl/min) was connected to the ion spray HPLC–MS interface [21] via a splitting device consisting of a 1/16-in. polyether ether ketone (PEEK) tee purchased from Upchurch Scientific (Oak Harbor, WA, USA) with a 9-cm length of 0.005 in. I.D. PEEK tubing attached. The splitting ratio was estimated to be 1:5 such that 40 μl/min of effluent reached the interface. The interface was maintained at 3.4 kV and the nebulizing nitrogen pressure at 414 kPa (60 p.s.i.). The mass spectrometer was a TAGA 6000E triple quadrupole instrument, upgraded to an API III, equipped with an atmospheric pressure ion source which was used in the positive-ion mode (Sciex, Thornhill, Canada). The optimum sprayer position was determined by infusing a 100 ng/μl solution of dihydrostreptomycin dissolved in mobile phase (*i.e.*, 8% ACN in 10 or 20 m*M* PFPA) at 40 μl/min. A declustering potential of 30 V was used unless indicated otherwise. The scan rate under HPLC–MS conditions was 3 s per scan with data collected in 0.5-u steps

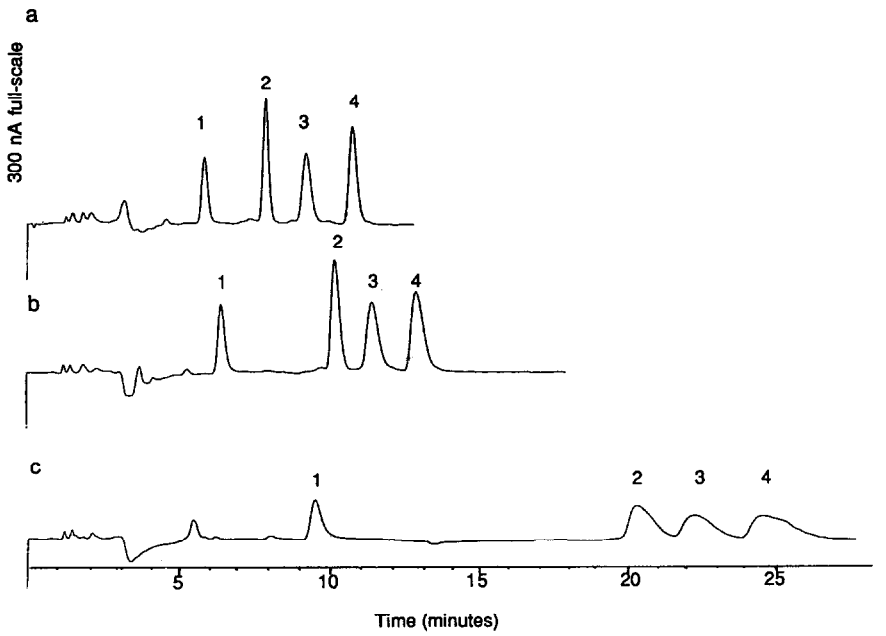

Fig. 2. HPLC–PAD elution profiles of a synthetic mixture of the four analytes using the Spherisorb ODS-2 column with various concentrations of PFPA in 8% ACN–water: (a) 20 m*M*; (b) 10 m*M*; (c) 5 m*M*. Peaks: 1 = spectinomycin (80 ng); 2 = hygromycin B (110 ng); 3 = streptomycin (145 ng); 4 = dihydrostreptomycin (145 ng).

for full-scan acquisition. Dwell periods of 750 ms were used in the SIM mode. Flow-injection analysis (FIA) was used to assess ion spray sensitivity as a function of pairing-ion concentration. For this experiment, a Brownlee micropump (Applied Biosystems, Foster City, CA, USA) delivered eluent at 40 μl/min directly to the interface with no splitting. Analytes were dissolved in the mobile phase being evaluated. The injector was a Rheodyne Model 7520 with a 1-μl internal sample loop. Data were acquired in the SIM mode with dwell periods of 75 ms. The data system was the standard Macintosh system provided by the manufacturer (Sciex).

RESULTS AND DISCUSSION

Effect of pairing-ion concentration on reversed-phase HPLC separation

For ion spray HPLC–MS it is desirable to minimize the concentration of pairing ion while using as much organic modifier as possible (see below). Also, a certain amount of chromatographic resolution

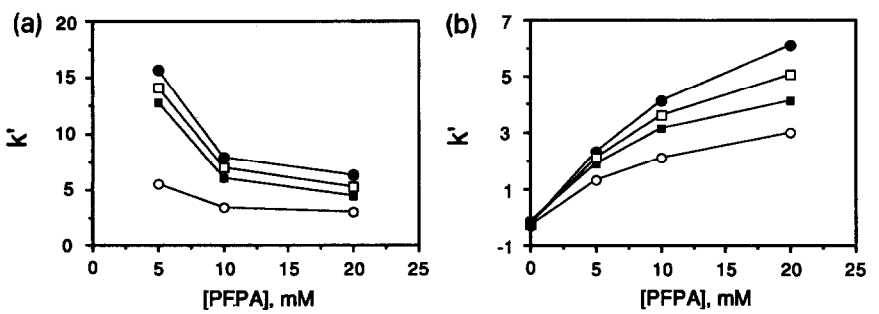

Fig. 3. Dependence of *k'* on the concentration of PFPA in 8% ACN–water for (a) Spherisorb ODS-2 column and (b) Inertsil ODS-2 ultra-end-capped column. With the Spherisorb column and no PFPA none of the analytes was observed to elute within 40 min. Data were obtained using PAD. ○ = Spectinomycin; ■ = hygromycin B; □ = streptomycin; ● = dihydrostreptomycin.

between component peaks is required in order to scan the peaks during discrete time windows with the mass spectrometer. This is necessary for monitoring ions from many analytes in one chromatographic profile in the SIM or SRM mode of operation. Of the eluents and columns tested with PAD, the highest efficiency and resolution were achieved using 8% ACN in 20 m*M* PFPA on the Spherisorb ODS-2 column. The elution profile for a synthetic mixture of the four analytes using these conditions is shown in Fig. 2a. Better resolution of streptomycin and dihydrostreptomycin is achieved with this method than with methods using alkyl sulfonate pairing ions [3,8,33].

Contrary to the predicted behavior for ion-pair systems, an increase in retention was observed with decreasing pairing-ion concentration for the Spherisorb column, as shown in Fig. 2b and c [34]. Also, increased peak asymmetry is evident in Fig. 2c. For example, the peak asymmetry for hygromycin B increases from a value of 1.07 with 20 m*M* PFPA to 1.86 with 5 m*M* PFPA. The fact that neither an ultra-end-capped polysiloxane-based Inertsil ODS-2 column nor a polystyrene (PS)–divinylbenzene (DVB) column displayed this type of behavior suggests that the effect is due to adsorption to residual silanol sites. These sites may be more effectively masked by higher pairing-ion concentrations.

The elution behaviors with the Spherisorb and Inertsil columns are compared in the plots of capacity factor (k') as a function of PFPA concentration shown in Fig. 3a and b. The Inertsil ODS-2 column shows the expected increase in capacity factor as the pairing-ion concentration is increased from 0 to 20 m*M*. Both of these columns are able to separate the four analytes at concentrations of 10 and 20 m*M* PFPA. However, resolution is too low with 10 m*M* PFPA on the Inertsil column if window acquisition is to be used with the mass spectrometer, although the analysis time is shorter. The separation efficiency on the Inertsil column is slightly lower at 20 m*M* as compared with the Spherisorb column. The Spherisorb ODS-2 packing material is the only one of the three tested that is available as 3-μm diameter particles.

A concentration of PFPA as low as 5 m*M* gave poor results with the Spherisorb ODS-2 column, as shown in Fig. 2c. This low concentration is also not effective with the Inertsil or PS–DVB columns, from which the analytes were observed to elute only partially resolved with capacity factors of less than 2.5. With the Inertsil ODS-2 column it is possible to achieve baseline separation of the four analytes by decreasing the amount of ACN to 4%, but this eluent was found not to give greater mass spectrometric sensitivity than the others tested (see below).

After 5 months of use with these acidic eluents (pH 2), the efficiency of the 100 mm × 4.6 mm I.D. Spherisorb ODS-2 column decreased by *ca.* 20%. A decrease in octadecylsilane (ODS) column life-time when using these eluents has been reported previously [26]. For that reason, the pH stability of the PS–DVB columns makes them an attractive alternative. However, at concentrations higher than 5 m*M* PFPA, spectinomycin and hygromycin were found to co-elute on the PS-DVB column. Therefore, the Spherisorb ODS-2 column and an eluent of 8% ACN in 10 or 20 m*M* PFPA was used for most of this work.

Effect of pairing-ion concentration on ion spray mass spectrometry

As mentioned previously, the general approach to ion spray HPLC–MS includes minimization of eluent ionic strength, use of as much organic modifier as practical and avoidance of buffers, unless they facilitate ionization, because of competitive

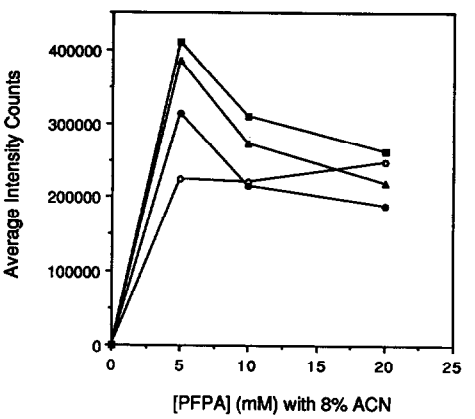

Fig. 3. Dependence of k' on the concentration of PFPA in 8% ACN–water for (a) Spherosorb ODS-2 column and (b) Inertsil ODS-2 ultra-end-capped column. With the Spherisorb column and no PFPA none of the analytes was observed to elute within 40 min. Data were obtained using PAD. ○ = Spectinomycin; ■ = hygromycin B; □ = streptomycin; ● = dihydrostreptomycin.

desorption and high chemical background caused by adducts extending to a fairly high mass-to-charge ratio (m/z). These considerations affect the choice of separation mode, especially for the aminoglycosides, which cannot be separated under typical reversed-phase conditions. We used FIA–ion spray MS experiments to assess the affect of pairing-ion concentration on sensitivity. Fig. 4 presents a plot of average MS peak intensity for three 20-ng injections of each of the four analytes at various concentrations of PFPA. Base-peak ions were monitored in the SIM mode. Related experiments in the full-scan acquisition mode showed that no significant mass spectral changes are caused by the different mobile phases tested, except that without a pairing ion present (neutral pH) no analyte signal is detected.

The observed signal is highest at 5 m*M* PFPA and decreases as the concentration is increased to 20 m*M* PFPA for each of the analytes except spectinomycin. The average response obtained with a mobile phase of 5 m*M* PFPA–4% ACN was the same as that for 10 m*M* PFPA–8% ACN. The latter eluent, however, provided better chromatographic resolution. For three of the analytes the response observed by FIA–ion spray MS is *ca.* 15–20% higher with 10 m*M* than with 20 m*M* PFPA. However, the peak heights measured on chromatograms obtained with PAD decrease by approximately the same amount when 10 m*M* is used instead of 20 m*M* PFPA owing to the longer retention. Hence concentrations of 10 and 20 m*M* PFPA may give comparable HPLC–MS results with the Spherisorb column. A concentration of 40 m*M* PFPA in the eluent resulted in a high voltage discharge between the interface and the mass spectrometer under the instrumental conditions used.

Effect of pairing-ion hydrophobicity

In reversed-phase ion-pair systems, the use of a more hydrophobic pairing ion will increase the analyte retention [35]. Therefore, HFBA was explored as a means of using a higher concentration of organic modifier in the eluent. The usefulness was tested by injecting a synthetic mixture of AGs under the two different HPLC conditions described in Fig. 5. Data were collected in both full-scan and SIM modes; only the full-scan results are shown here but the SIM results agreed well. For the same concentration of HFBA, the percentage of ACN may be doubled while still resolving the analytes. However, the longer retention times and concomitant band broadening appear to offset any increased sensitivity that might be associated with the higher concentration of organic solvent. Average peak intensity data obtained from three injections of this mixture indicate that only the spectinomycin response is significantly increased with the HFBA eluent. This is presumably because the retention time of this analyte remains approximately the same when the organic modifier concentration is increased. A more complex explanation is also possible. Inchauspé *et al.* [25] have reported variations in the number of theoretical plates with different pairing ions on an analyte-dependent basis. Ion spray phenomena may also vary by compound in the presence of different additives. In any case, HFBA did not offer a substantial improvement for the ion spray HPLC–MS analysis of this mixture of antibiotics.

In another attempt to increase the organic modifier concentration, the use of methanol was explored. Because methanol is a weaker reversed-phase eluent than acetonitrile, concentrations of *ca.* 20% (v/v) may be used to obtain similar retention. Although methanol cannot be used with PAD because it is electroactive at the potential used to oxidize the analytes, it was tested using HPLC–UV and HPLC–MS methods. In both instances broad, tailing peaks were observed for the carbonyl-containing compounds spectinomycin and streptomycin, owing to apparent hemiketal and hemiacetal formation, respectively, at this low pH. The hemiketal and hemiacetal products were identified in the ion spray mass spectra together with the ketone- and aldehyde-hydrate species normally observed so that the ion current is distributed among several ions for these analytes. However, the poor chromatographic peak shape alone precludes the use of methanol at this low pH.

Ion spray mass spectra

Full-scan mass spectra (165–600 dalton) were obtained by HPLC–MS in 8% ACN–20 m*M* PFPA from injection of 2 μg per component. Spectinomycin exists in solution as a ketone-hydrate [36] and yields a singly charged protonated molecule of this species $[M + H_2O + H]^+$ at m/z 351 as the base peak. A singly charged species corresponding to the

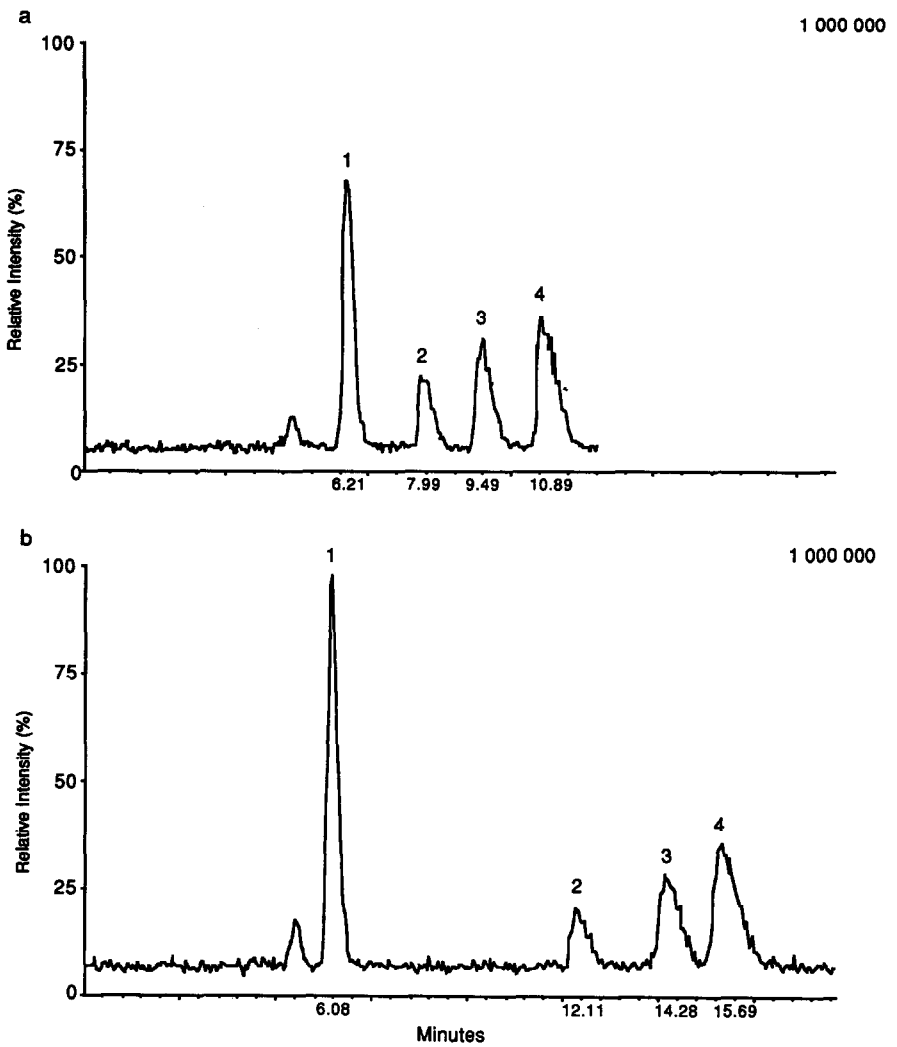

Fig. 5. Comparison of HPLC–MS ion spray elution profiles on the Spherisorb ODS-2 column using (a) 8% ACN–20 m*M* PFPA and (b) 16% ACN–20 m*M* HFBA. Data shown are reconstructed total ion current profiles from full-scan (260–400 dalton) acquisition data. Peaks: 1 = spectinomycin (400 ng); 2 = hygromycin B (550 ng); 3 = streptomycin (723 ng); 4 = dihydrostreptomycin (723 ng). Numbers at top right indicate ion counts.

non-hydrated or dehydrated molecule $[M + H]^+$ is observed at m/z 333 with a relative abundance of 8%. The predominant ions observed under these conditions for the other three analytes are doubly protonated, doubly charged species. For dihydrostreptomycin and hygromycin B the base peaks $[M + 2H]^{2+}$ are ions of m/z 293 and 265, respectively. In the mass spectrum of hygromycin B the singly charged protonated molecule $[M + H]^+$ at m/z 528 is observed with a relative abundance of 9%. Streptomycin appears to exist as an aldehyde-hydrate characterized by the base peak $[M + H_2O + 2H]^{2+}$ at m/z 301. The eluent pH is probably too low for streptomycin to exist in solution as the cyclic carbinolamine tautomer from reported [36]. Also observed is the non-hydrated or dehydrated form $[M + 2H]^{2+}$ at m/z 292 with a relative abundance of 21%. No significant fragmentation was observed at the declustering potential of 30 V used to obtain these spectra. Concentration of the ion current into one

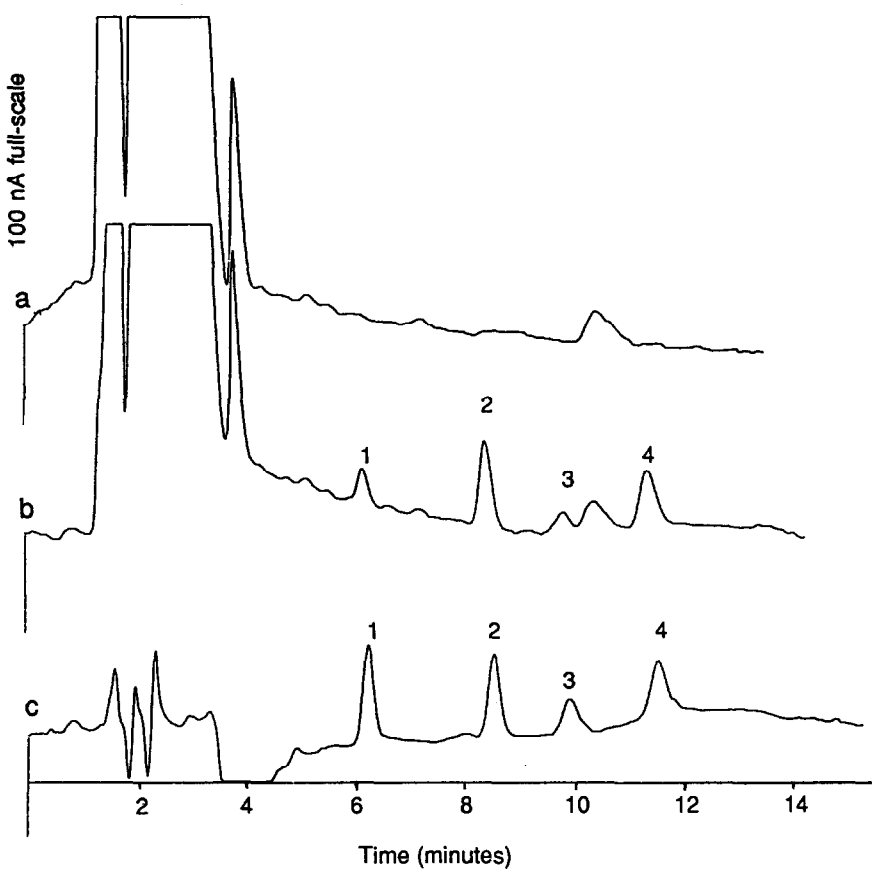

Fig. 6. HPLC–PAD of (a) MSPD extract of control bovine kidney, (b) MSPD extract of bovine kidney fortified at the 20 ppm level and (c) synthetic mixture of standards at levels of 15 ng per component injected, representative of 100% recovery. Peaks: 1 = spectinomycin; 2 = hygromycin B; 3 = streptomycin; 4 = dihydrostreptomycin.

principal species is ideal for subsequent SRM work although the lower m/z of the doubly charged species puts them in a region of higher chemical background. At a higher declustering potential of 70 V it is possible to obtain mass spectra having the corresponding singly charged species as the base peak. However, analyte signals observed for the singly charged species are much lower than those obtainable for the doubly charged species and may not be adequate for SRM work.

HPLC–PAD for aminoglycoside determination

The ion-pair system is compatible with PAD utilizing post-column addition of strong base. This HPLC–PAD system can detect *ca.* 5 ng of free base per component with signal-to-noise ratios (S/N) ranging from 4 for streptomycin to 14 for dihydrostreptomycin. These observations agree well with the detection limit of 2 ng (S/N = 3) reported for tobramycin using PAD with anion-exchange chromatography [5]. Fig. 6 shows the results for a bovine kidney sample fortified at 20 ppm of each of the analytes and extracted by MSPD together with a control kidney extract and a standard equivalent to 15 ng per component injected on-column, representative of 100% recovery. Further sample clean-up and increased recovery of spectinomycin would be necessary to reach lower concentrations with this method and to be competitive with fluorescence detection, which has been used to determine neomycin in bovine kidney at 2.5 ppm also using the MSPD technique for sample preparation [9]. It should be noted that PAD will not be limited to certain pairing-ion concentrations and non-volatile salts may

be used to improve peak shape. The only limitations we have encountered in this work are with electroactive species such as methanol and ammonium compounds, which produce an unstable baseline.

Ion spray HPLC–MS for aminoglycoside determination

Detection limits for these analytes using ion spray MS in the SIM mode are in the low nanogram range. For injection of between 4 and 7 ng of free base per component, corresponding to 12 pmol per component, S/N values range from 2 for streptomycin and hygromycin B to 6 for dihydrostreptomycin and 9 for spectinomycin. These results were obtained using the 8% ACN–20 mM PFPA eluent with the 100 mm × 2 mm I.D. Spherisorb ODS-2 column with a post-column split. The base-peak ion was monitored for each compound. Noise associated with background ion current coincident with the m/z of the hygromycin B and streptomycin ions increases the detection limits for these compounds.

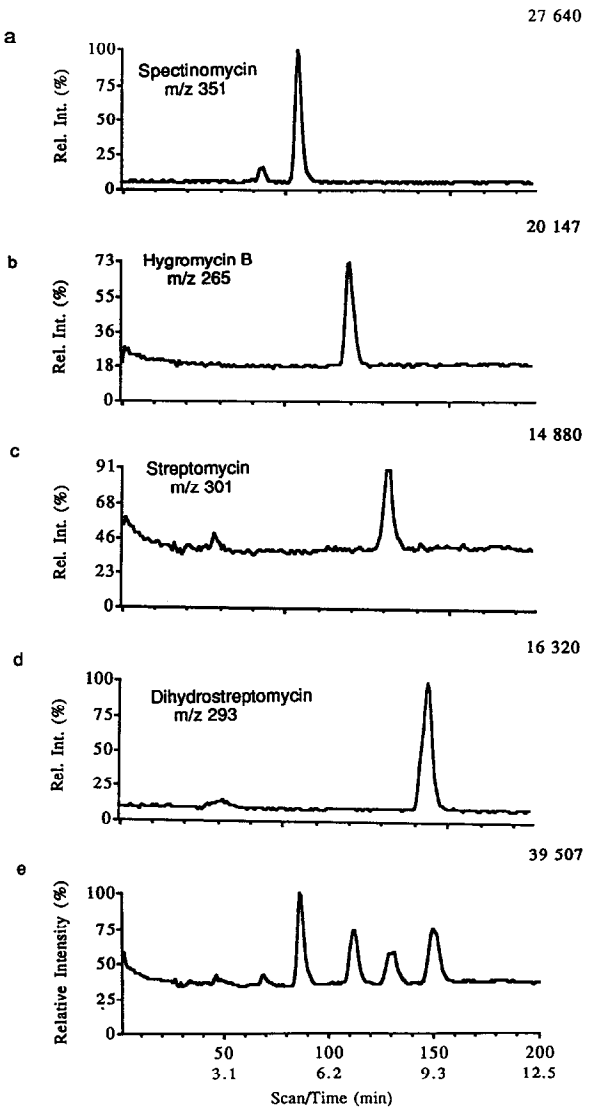

Fig. 7. (a–d) Extracted ion current profiles and (e) total selected-ion current profile of an ion spray HPLC–MS analysis of a synthetic mixture of the four analytes at the level of 40 ng per component injected, corresponding to 100% recovery from a 20 ppm sample. Numbers at top right indicate ion counts.

The determination in the SIM mode of a 40 ng per component synthetic mixture of the four analytes is shown in Fig. 7 together with an extract of bovine kidney fortified at 20 ppm of each of the analytes in Fig. 8. The decrease in ion current seen in Fig. 8c is caused by the competitive desorption of matrix components eluting prior to 3 min. The 40-ng level corresponds to 100% recovery from a 20 ppm sample. For this work, an aliquot of the 9-ml aqueous sample extract was concentrated by a factor of four and 10 μl were injected. No matrix interferences were observed in the analysis of a control kidney extract. Determination at the levels of regulatory interest using this method would require on-column detection of 44 pg of hygromycin B, 0.22 ng of spectinomycin and 1.1 ng of streptomycin and dihydrostreptomycin. These amounts are all below the instrumental detection limits discussed above. However, because only a small fraction of the sample extract was injected and no matrix interferences were observed, further concentration of the 9-ml solid phase dispersion eluate should allow the determination of all analytes, except possibly hygromycin B. If further concentration is not possible owing to solubility limitations or overloading of the HPLC column, an additional clean-up step will be necessary. Determination of hygromycin B may require extraction of a larger tissue sample.

Also, because the sensitivity of this method is adversely affected by the high water content of the eluent (92%) and the 10–20 mM concentration of PFPA necessary to effect separation, we shall continue to evaluate columns that would allow use of more compatible eluents. Use of microbore HPLC columns (1 mm I.D.) would increase the method sensitivity owing to peak-volume concentration and

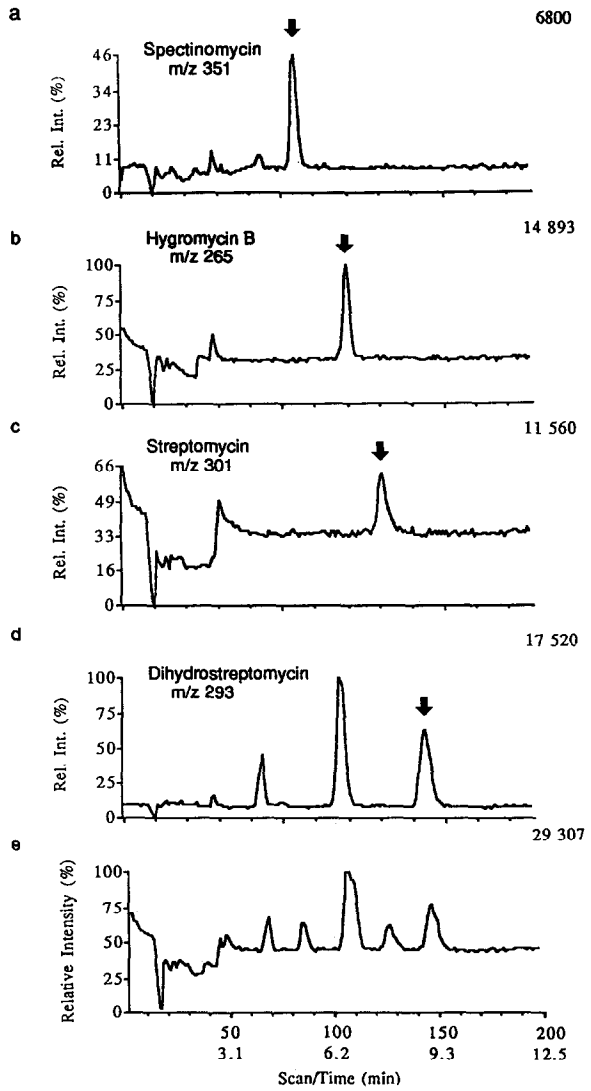

Fig. 8. (a–d) Extracted ion current profiles and (e) total selected-ion current profile of an ion spray HPLC–MS analysis of an MSPD extract of bovine kidney fortified at 20 ppm with each of the four analytes. Numbers at top right indicate ion counts.

the fact that at the lower flow-rates used with these columns no post-column split is required. However, because of their reduced loading capacity their applicability to these complex sample matrices remains to be demonstrated.

CONCLUSIONS

Although increasing concentrations of PFPA in the eluent were found to decrease the ion spray sensitivity for most of the compounds studied, a concentration of at least 10 m*M* was necessary for adequate chromatographic resolution on the columns tested. A concentration of 20 m*M* PFPA in 8% ACN provided the best separation efficiency and resolution on the Spherisorb ODS-2 column. PAD was useful for method development as it can detect as little as a few nanograms of underivatized aminoglycosides. Further clean-up will be needed if PAD is to be used for screening and quantification in the sub-ppm range in bovine kidney extracts. The detection limits of ion spray HPLC–MS in the SIM mode are also in the low nanogram range. Further concentration and possibly further clean-up of the bovine kidney extracts will be necessary to achieve determination at target levels. The even greater selectivity with respect to matrix interferences of the SRM mode to be used for eventual confirmation should allow injection of more concentrated extracts. Although sensitivity in the SRM mode remains to be optimized for confirmatory work, these results demonstrate the feasibility of this approach for determining aminoglycoside and aminocyclitol residues in bovine kidney. Future work will focus on the optimization of SRM parameters, improvement of analyte isolation and recovery from bovine kidney, evaluation of other chromatographic modes and application of the method to other aminoglycoside antibiotics and other biological matrices.

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