Application of Nanoscale Packed Capillary Liquid Chromatography (75 μ m id) and Capillary Zone Electrophoresis/Electrospray Ionization Mass Spectrometry to the Analysis of Macrolide Antibiotics

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Nanoscale separation techniques, nanoscale packed capillary columns (75 μ m id), and capillary zone electrophoresis (CZE), on-line with electrospray mass spectrometry (ESI/MS), were applied to the separation of a series of ten macrolide antibiotics. Both techniques use sub-microliter-per-minute flow rates through the analytical column and therefore require an electrospray probe that incorporates coaxial sheath flow. Positive ion electrospray mass spectra of these compounds yielded mainly protonated molecules. Fragmentation to yield structurally significant fragment ions was achieved by collision-induced dissociation (CID) at increased skimmer voltages. Separations were achieved using both techniques, with CZE/ESI/MS showing improved peak shapes and detection limits combined with faster analysis times. Nanoscale packed capillary columns provided better chromatographic resolution and was less susceptible to peak broadening caused by overloading of the analytes. (*J Am Soc Mass Spectrom* 1992, 3, 563-574)

Macrolides are a class of antibiotics produced by *Streptomyces* soil bacteria. Macrolides are widely used broad-spectrum antimicrobial agents, which are useful against gram-positive bacteria [1-4]. Erythromycin is perhaps the most commonly prescribed macrolide in the United States, but other members of this class have been developed and are commonly used in other countries [5-9].

Research in our Tokyo laboratory has involved the mechanisms of bacterial macrolide resistance. Our work, and that of other researchers, has indicated several mechanisms involving macrolide esterases and rRNA methylases [7, 10-14] and 2'-phosphotransferase [15-18].

The residual potency of the antibiotic in the reaction mixture with macrolide-inactivating enzyme has usually been determined by microbioassay. During the course of these studies, however, the need has become apparent for a physicochemical method capa-

ble of determining drug purity, degradation products, and enzymatically induced structural changes in the macrolide. Because of the possibility of the formation of several products, a combination of a mass-spectrometric technique with a separation technique would be desirable. These compounds cannot be analyzed by direct electron or chemical ionization probe or by gas chromatography mass spectrometry (GC/MS). A liquid chromatography mass spectrometry (LC/MS) approach, however, is a possibility.

Historically, the difficulty in coupling conventional high performance liquid chromatography (HPLC) and mass spectrometry has been the basic incompatibility of the vacuum system with the large volume of mobile phase produced when operating at a flow rate of 1 mL/min. This first led to the development of a variety of LC/MS interfaces designed either to remove the solvent external to the ion source (moving belt) or to reduce the effective mobile-phase flow rate by means of a postcolumn splitter (direct liquid introduction, DLI). A step toward improving the performance of both of these interfaces was the reduction of column effluent volume through the use of microbore

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or minibore HPLC columns, with flow rates of 20–200 $\mu\text{L}/\text{min}$. Neither interface was ideal, however, because thermal degradation and memory effects could be encountered with the moving-belt interface, and plugging of the pinhole often occurred with the DLI interface. The thermospray interface, with its ease of operation and its ability to handle 1-mL/min flow rates, overshadowed the other LC/MS techniques soon after its introduction. We have been involved in a research program aimed at the development of nanoscale separation techniques—nanoscale packed capillary liquid chromatography (nCLC) [19–22] and capillary zone electrophoresis (CZE) [23–27]—characterized by sub-microliter-per-minute flow rates, in conjunction with mass spectrometry. We have interfaced these techniques to both continuous flow fast atom bombardment (CF-FAB) and electrospray mass spectrometry, both of which can efficiently handle these mobile-phase flow rates.

Capillary zone electrophoresis, developed by Jorgenson and Lukacs [28], employs capillary tubes, usually made of fused silica, with inner diameters of 10–100 μm . The column is filled with a buffer solution, a voltage potential is applied across the capillary tube, and separation of analytes with different partial charges occurs as a result of their differential migration in the electric field. The separation is primarily a function of charge with secondary contributions from molecular size and shape [29]. The voltage drop across the column is typically 30 kV (300 V/cm). CZE has a pistonlike flow profile rather than a parabolic flow profile; hence separation efficiencies are extremely high [29]. Relative electrophoretic migration times (the operational equivalent of HPLC retention times) are a function of both electromigration of the analyte in the electric field, and the electroosmotic flow (i.e., the liquid flow resulting from the movement of highly solvated ions). This liquid flow is usually 50–350 nL/min. Electrophoretic migration times can be altered by adjusting the pH, by changing the buffer composition (restricted to volatile buffers when coupled to mass spectrometry), or by adding an organic modifier to the buffer to reduce the electroosmotic flow. In addition, chemical modification of the capillary walls has allowed improved separations of positively charged analytes, particularly basic compounds, by preventing adsorption [30].

Treatment of the fused silica column with 3-aminopropyltrimethoxysilane (APS) changes the charge on the column wall from the negative charge found in bare fused silica to a positive charge, due to the presence of the amino propyl groups. This change in the charge on the walls leads to a change in the direction of electroosmotic flow because, under these conditions, the electroosmotic flow is driven by an excess of negative ions in solution. Thus the direction of the electroosmotic flow is from high negative potential to ground. The electroosmotic flow in CZE is typically strong enough to sweep charged analytes

and neutral species to “ground” regardless of the sign of the charge. A review of CZE/MS, including a more detailed description of CZE, has recently been published [27].

The second sub-microliter-per-minute technique is nCLC. Standard HPLC column packings are used, either reversed-phase or normal-phase, and standard HPLC techniques such as gradient operation are available. Instead of a 4.6-mm id column, 75- μm id fused silica capillary columns with mobile-phase flow rates of 50–800 nL/min have been used. This LC column id eliminates many of the problems associated with the smaller open-tubular LC columns, which had very low loading capacities [31], and with microbore columns, which suffered from band broadening due to differences between the column packing bed in the interior of the column and that near the wall [32]. For column id-to-particle diameter ratios of less than 10, the entire packing bed will be uniform, resulting in increased separation efficiencies [32, 33].

In electrospray ionization (ESI), a recently exploited mass-spectrometric technique, small, highly charged droplets are generated from a solution by passing the solvent stream through a needle at high voltage [34, 35]. No pinhole is used to generate the spray; instead, a plume is formed by the charge repulsion of the droplets. Sample ions are formed from the droplets, possibly by an ion evaporation mechanism [36]. Flow rates for the generation of the spray are ~ 5 –10 $\mu\text{L}/\text{min}$. Unlike conventional HPLC, the flow rates generated by both CZE and nCLC are actually lower than ideal for this interface but can easily be augmented by the addition of a makeup solution. CZE/ESI/MS, with the makeup flow introduced either coaxially [26, 37, 38] or via a liquid junction [39], has been reported.

Electrospray (ion spray or nebulization-assisted electrospray [40]) is a very gentle ionization technique; resulting spectra contain mainly molecular ions with limited fragmentation. Electrospray has been successfully used for the analysis of a wide variety of compounds, including proteins and peptides [21, 26, 39, 41–45], azo dyes [40, 46], and marine neurotoxins [47, 48]. Electrospray mass spectra of drugs representing diverse classes have been reported, including gramicidin S [35]; morphine, codeine, and cocaine [49]; insulin [50]; and quaternary ammonium drugs [51].

We now report the results of our study of the application of CZE and nCLC in conjunction with ESI for the analysis of macrolide antibiotics.

Experimental

Instrumentation

The mass spectrometer data system used was a VG 12-250 quadrupole mass spectrometer and data system (VG/Fisons, Altrincham, UK). Reference spectra

of the standards were acquired over the mass range 100–1200 *u* at a scan rate of 10 s/scan. The mass range employed was 400–1200 *u* at 5 s/scan for the nCLC/ESI/MS, acquired in the peak centroid mode. Selected ion monitoring (SIM) on the eight masses of the protonated molecules was used for the CZE/ESI/MS.

A Vestec electrospray source, model 611B (Vestec Corp., Houston, TX), was used throughout the experiments. This source differs from other electrospray source designs [35] in that it does not rely on the use of a nitrogen curtain gas but instead uses a heated block for desolvation [52]. The block was held at 266 °C, and the chamber temperature was kept at 55 °C. The needle voltage was held at ~3 kV, giving a spray current of approximately 0.2–0.25 μ A, and the skimmer voltage was varied between 4 and 55 V.

The probe used was of a coaxial flow design. The concept of coaxial flow for use with capillary columns was originally developed for the postcolumn derivatization of analytes following capillary column chromatography where the introduction of additional solution could be delivered to the capillary tip without loss of chromatographic resolution [53]. The principle of coaxial flow has been used by Smith and co-workers for electrospray [37, 38] and has previously been used in our laboratory for the combination of nanoscale separations with continuous-flow fast atom bombardment (FAB) [19–27, 54]. In our CZE/nCLC probe, the analytical capillary passes through a Valco "tee" union, through a 1/16-in. stainless steel tube, and through the union holding the electrospray needle (Figure 1). The analytical capillary protrudes slightly from the end of the needle but is still within the Taylor cone [55]. Sheath flow is introduced through one side of the tee and flows through the interstitial space between the analytical capillary and the stainless steel tubing and electrospray needle. The electrical connection to the needle is made at the

Valco tee. The original metal sleeve around the probe handle has been replaced with plexiglass to prevent electrical discharge, and both metal endplates of the probe handle must be grounded. This electrospray probe design allows separations to be performed at the sub-microliter flow rates while total flow at the needle tip can be kept at the ~5–10 μ L/min required for stable electrospray operation. In addition, this design permits independent optimization of the separation column conditions and of the sheath flow conditions.

The electrospray needle should be as small as possible, with an inner diameter just large enough for the column to pass through. For nCLC work, where 75- μ m id $\times$ 150- μ m od fused-silica capillary tubing is used, the needle is made from 0.007-in. id $\times$ 0.013-in. od tubing. For CZE work, it was found that thicker-walled tubing led to more reliable operation (fewer problems of electrodrilling holes through the capillary wall), so 75- μ m id $\times$ 360- μ m od capillary is used. The needle size used for CZE operation is cut from 0.017-in. id $\times$ 0.028-in. od stainless steel tubing.

CZE System

The CZE apparatus was constructed in house and has been described elsewhere [23–25, 27]. It uses a Glassman HV power supply (Glassman High Voltage, Whitehouse Station, NJ), which was operated at +30 kV or –30 kV in these experiments. The high voltage end of the CZE capillary is located in a plexiglass box, and the CZE "ground" is the electrospray needle itself (at ~+3 kV) so that the effective voltage drop was 27 or 33 kV.

Injectons were made by raising the end of the electrophoresis capillary above the level of the electrospray needle, placing the end of the electrophoresis capillary in the sample solution, and allowing siphoning of the sample into the capillary tube for a timed interval. The end of the capillary is then placed in the

Figure 1. Schematic of CZE and nCLC/ESI/MS probe. The probe is shown with the needle used for nCLC work. For CZE analyses, where a larger od fused silica capillary is used, the needle size was 0.017 in. id and 0.028 in. od.

CZE buffer, and the voltage is applied to initiate the electrophoretic separation. Electroosmotic flow rates through the 75- μ m id capillary are 300-400 nL/min. Because ESI requires 5-10 μ L/min total flow to generate a stable spray, makeup sheath flow is required and was generated in these experiments by a Harvard Apparatus model 909 syringe pump (Harvard Apparatus, South Natick, MA). For separations of the macrolides with neutral or basic mobile phases (pH 7, 8.5, and 9), untreated silica capillary was used. For the separations of the macrolide antibiotics with acidic mobile phases (pH 2.35, 2.6, 3.4, 5, and 7), the CZE capillary was treated with APS [25]. The buffers employed for the separations were 0.01-M acetic acid or ammonium acetate; pH adjustments were made with ammonium hydroxide or acetic acid down to pH 3.4. For pH below 3.4, formic acid and trifluoroacetic acid were used to achieve the desired pH values.

nCLC System

The nCLC column used was a 2-m $\times$ 75- μ m id, 150- μ m od fused-silica capillary packed to a depth of 25 cm with 10- μ m AQ-C18 (YMC Inc., Morris Plains, NJ). The column preparation procedure has been described in detail elsewhere [22]. Mobile-phase flow through the analytical column was generated using a Gilson Gradient HPLC system and controller, operated at 0.8 mL/min. Flow through the capillary column was reduced by a factor of 1000 with a splitting tee. A 34-cm length of 75- μ m id capillary was used to provide the back-pressure required to force approximately 800 nL/min of mobile phase through the capillary column.

A water-acetonitrile gradient (25% to 100% over 30 min, with both mobile-phase solvents containing 0.1% TFA) was employed for the separation. As with the CZE/ESI/MS, a sheath flow consisting of 50-50 methanol-3% aqueous acetic acid was used. The sheath flow was delivered using a Harvard syringe pump, model 909 (Harvard Apparatus, South Natick, MA).

Injections onto the analytical column were performed by placing the end of the column into the sample vial in a stainless steel pressure vessel [54, 56]. Samples were forced onto the column by pressurizing with helium for a timed interval.

Chemicals

Structures and names of the macrolide antibiotics studied are shown in Figure 2. Standard samples of erythromycin A (EMA), oleandomycin (OL), spiramycin (SPM), and tylosin (TYL) were purchased from Sigma Chemical Co. (St. Louis, MO). Troleandomycin (TAO), formerly known as triacetyloleandomycin, was a gift from Sankyo Co., Ltd. (Tokyo), and josamycin (JM) was a gift from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo). Midecamycin (MDM)

and miokamycin (MOM) were a gift from Meiji Seika Kaisha, Ltd. (Tokyo). Leucomycin-A5 (LM-A5) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Rokitamycin (RKM) was purchased from Toyo Jozo (Tokyo). Compounds were used without further purification.

Mobile phases consisted of HPLC-grade acetonitrile and methanol (Fisher Scientific, Fairlawn, NJ). Water was obtained from a Milli-Rho/Milli-Q system (Millipore Corp., Bedford, MA). Trifluoroacetic acid was from Fairfield Chemical Co., Blythewood, SC.

Bacterial Digests

For study of the OL inactivation products, 0.5 mg OL was combined with 800 mL bacterial extract (containing the inactivating enzyme) and 200 mL 80-mM ATP in pH 7.8 TMK buffer (0.06-M KCl, 0.01-M magnesium acetate, and 0.006-M 2-mercaptoethanol in 0.1-M Tris-HCl). An aliquot was taken at the beginning of the reaction and was designated as OL-0h. A second aliquot was obtained after reaction at 37 °C for 24 h and was designated as OL-24h. Both aliquots were heated at 100 °C for 3 min to stop the enzymatic reaction. The solutions were centrifuged at 5000 g for 15 min, and the supernatant was taken and lyophilized. A 2-mg portion of each sample was reconstituted in 200 μ L water and analyzed by nCLC. The gradient used for these samples was the same as that employed for the standards, except for the incorporation of a 15-min wash at 100% H₂O (0.1% TFA) at the start of the analysis to assist in the removal of salts.

Results and Discussion

Fragmentation Patterns and Skimmer Voltage Effects

Macrolides are comprised of an amino sugar (B), a nitrogen-free sugar (S), and a 14- or 15-membered ring aglycone portion (A) (see Schemes I and II). The nitrogen-free sugar (S) and the amino sugar (B) can either be both attached directly to the aglycone (A) to give a structure with the shorthand notation SOAOB, or the sugar (S) can be bound to the amino sugar (B), which is then bound to the aglycone (A) to give a structure with the shorthand notation AOBOS.

Electrospray spectra of the individual macrolide antibiotics were obtained by continuous infusion of solutions (1 mg/mL in 50-50 water-methanol) through a 75- μ m id fused-silica capillary. Tabulated spectra for the macrolides studied are given in Table 1, and ESI mass spectra for JM, at skimmer potentials of 11, 25, and 40 V, are shown in Figure 3. At low skimmer voltages, most of the macrolide antibiotics yielded abundant protonated molecules. At higher skimmer voltages, fragment ion relative abundances increased, with a concomitant loss of protonated molecule

STRUCTURES OF COMPOUNDS STUDIED

16-membered ring macrolides

Midecamycin (MDM)

Compound	C-3	C-4	C-9	C-14	C-15	C-3"	C-4"
Midecamycin (MDM)	OCOCH ₂ CH ₃	OCH ₃	OH	H	CH ₃	CH ₃ , OH	OCOCH ₂ CH ₃
Miokamycin (MOM)	OCOCH ₂ CH ₃	OCH ₃	OCOCH ₃	H	CH ₃	CH ₃ , OCOCH ₃	OCOCH ₂ CH ₃
Leucomycin-A5 (LM)	OH	OCH ₃	OH	H	CH ₃	CH ₃ , OH	OCOCH ₂ CH ₂ CH ₃
Rokitamycin (RKM)	OH	OCH ₃	OH	H	CH ₃	CH ₃ , OCOCH ₂ CH ₃	OCOCH ₂ CH ₂ CH ₃
Josamycin (JM)	OCOCH ₃	OCH ₃	OH	H	CH ₃	CH ₃ , OH	OCOCH ₂ CH(CH ₃) ₂
Spiramycin (SPM)	OH	OCH ₃		H	CH ₃	CH ₃ , OH	OH
Tylosin (TYL)	OH	CH ₃		CH ₂ CH ₃	CH ₃ , OH	CH ₃ , OH	OH

14-membered ring macrolides

Erythromycin A (EMA)

Compound	C-6	C-8	C-11	C-12	C-13	C-2'	C-3"	C-4"
Erythromycin A (EMA)	CH ₃ , OH	CH ₃	OH	CH ₃ , OH	CH ₂ CH ₃	OH	OCH ₃ , CH ₃	OH
Oleandomycin (OL)	CH ₃ , H	CH ₂ O	OH	CH ₃ , H	CH ₃	OH	OCH ₃ , H	OH
Troleandomycin (TAO)	CH ₃ , H	CH ₂ O	OCOCH ₃	CH ₃ , H	CH ₃	OCOCH ₃	OCH ₃ , H	OCOCH ₃

Figure 2. Structures of compounds studied (LM = LM - A5).

abundance. Skimmer voltage-induced CID effects in atmospheric pressurized ionization (API) sources have been reported by several research groups [57-59] and have been described for the Fenn-Whitehouse API-electrospray source [35] by Loo et al. [60] and as occurring in the skimmer-collimator region in the

Vestec source [52]. The changes in ion abundance are shown in Figure 4 for MDM, a 16-membered ring macrolide, and TAO, a 14-membered ring macrolide. For these compounds, a skimmer setting of 11 V gave the most abundant protonated molecule signal, and a skimmer voltage setting of 25 V was the best choice if

structural information, as well as molecular weight information, was desired. The ion abundance of the $(M + Na)^+$ showed less of a decrease with increasing skimmer voltage than the $(M + H)^+$ ion. This phenomenon has also been observed for sulfonamides [61] and may prove useful in distinguishing between protonated and sodiated species.

Fragmentations of these protonated molecules are quite predictable (Schemes I and II) and typically

produce an ion from the sugar ring (S^+), an ion from the B ring plus oxygen (BOH^+), and an ion arising from loss of the sugar from the protonated molecule $[M + H - (S - H)]^+$. In macrolides where the S ring is attached directly to the macrolide ring (A), the ion B^+ is observed in lieu of the $(BOH)^+$ ion. For LM-A5, which has an esterified hydroxyl group (O_2CR) on the sugar, the OCR portion of the ester is lost as a substituted ketene from the protonated molecule to give $[M + H - (OCR - H)]^+$. An ion formed by loss of carboxylic acid from the tertiary carbon, $(S - HO_2CR)^+$, is observed for MDM and RKM. Similar fragmentations have been observed in the FAB mass spectrum of OL [15], and similar reaction pathways have been described for the FAB mass spectra of steroid and flavonoid glycosides [62].

In general, more abundant fragment ions are observed for the 16-membered macrolides than for the 14-membered ring macrolides. These same macrolides also have both the sugar rings (S) and the aminosugar rings (B) attached directly to the macrolide ring rather than in series, and we are therefore unable to distinguish which structural feature may account for this difference.

One macrolide, RKM, has two B rings. Under electrospray ionization, this compound shows a doubly protonated molecule, resulting from protonation of both $N(CH_3)_2$ groups, as well as a singly protonated molecule. The abundance of the doubly charged ions decreases with increasing skimmer voltage.

CZE Separations

Our first approach to combining a separation step with ESI for the analysis of mixtures of macrolides was to try CZE/ESI/MS. CZE separations were done at various pH levels ranging from pH 2.35 to pH 9. When basic mobile phases were used, the separations were carried out in an untreated silica capillary at 30 kV CZE voltage. At acidic pH levels, separations were done in an APS-treated silica capillary at -30 kV CZE voltage. An initial problem encountered was column overloading. High sample loadings in CZE (ng levels) resulted in broad unresolved peaks. This is one of the limitations of the CZE approach—overloading of the capillary can readily occur. Using our present CZE/ESI/MS system, the upper limit of loading onto the column is often very close to the lower limit of detection of our quadrupole mass spectrometer under full scan conditions, leading to a very narrow dynamic range. For this reason, data were acquired in the selected ion monitoring (SIM) mode for the CZE analyses.

To avoid overloading and to be able to distinguish between components of the same molecular weight, two separate mixtures of the macrolide antibiotics were used in the CZE/MS experiments—one mixture of six macrolides (TYL, RKM, MOM, MDM, EMA,

Table 1. Tabulated ESI mass spectra at skimmer voltages 11, 25, and 40 V

Compound	Structure	A ring	Skimmer voltage	S ⁺	B ⁺	BOH ⁺	BOSH ⁺	[M + H - (S - H)] ⁺	[M + H] ⁺	[M + Na] ⁺	[M + H + CH ₃ OH]	[M + CH ₃ OH + Na] ⁺	Additional ions
MDM	AOBOS	16	11	9 (201) ^a		6 (174)	5 (375)	10 (814)	100 (814)	14 (836)			
			25	41		36	9	18	100	13			
			40	40		100	10	10	25	12			
MOM	AOBOS	16	11	8 (243)			7 (417)	7 (656)	100 (898)	50 (920)			(S - HOCCCH ₃) ⁺
			25	20			8	7	100	41			15 (183)
			40	10			8	8	26	26			40
													32
LM-A5	AOBOS	16	11	39 (215)		20 (174)	12 (388)	22 (558)	80 (772)		100 (804)		(M - H + 2Na) ⁺
			25	100		72	20	36	63		62		(M + H - COCH ₂ CH ₂ CH ₂) ⁺
			40	36		100	27	18	12		15		73 (816)
													8 (702)
													31
													13
													4
RKM	AOBOS	16	11				5 (445)	5 (558)	100 (828)	24 (850)	48 (860)	26 (882)	(S - HO ₂ CH ₂ CH ₃) ⁺
			25				15	10	87	31	44	16	17 (197)
			40				8	7	30	12	22	10	32
													11
JM	AOBOS	16	11	19 (229)		10 (174)	4 (403)	11 (800)	100 (828)	15 (850)	31 (880)		(M + H + H ₂ O) ⁺
			25	61		53	12	20	100	47	26		24 (846)
			40	51		94	8	8	17	23	10		31
													12
SPM	B'AOBOS	16	11	33 (145)		100 (174)	8 (319)	5 (699)	25 (843)		27 (875)	17 (897)	(M + 2H) ²⁺
			25	20		100	17	3	12		11	10	(M + 2H + CH ₃ OH) ²⁺
			40	49		76	15	4	11		20	10	25 (438)
													3
													3
													0
													0
TYL	S'AOBOS	16	11			20 (174)	13 (319)	19 (772)	100 (916)	29 (926)	52 (948)		(M + H - (S - H) - (S' - H)) ⁺
			25			100	20	41	81	41	81		5 (588)
			40			100	32	50	52	25	42		17
													15
EMA	SOAOB	14	11	7 (159)	27 (158)		9 (317) ^b	100 (576)	59 (734)	29 (757)			(M + H - (S - H) - H ₂ O) ⁺
			25	7	100		4	64	14	14			30 (559)
			40	8	100		2	7	5	13			23
													4
OL	SOAOB	14	11	10 (145)	35 (158)		6 (303) ^b	100 (544)	31 (688)	10 (710)			
			25	4	100		2	28	9	8			
			40	10	100		0	5	2	1			
TAO	SOAOB	14	11	18 (187)	100 (200)			13 (628)	88 (814)	29 (836)	13 (846)		
			25	18	100			5	19	12	5		
			40	11	100			1	3	9	3		

^aRelative abundance (mass).

^bPossible rearrangement.

Figure 3. ESI mass spectra of JM at skimmer voltages 11, 25, and 40 V.

and SPM) and a second mixture of four antibiotics (JM, LM-A5, OL, and TAO). Separations were investigated at a series of pH levels: pH 7, 8.5, and 9 using a plain fused-silica capillary and pH 2.35, 2.6, 3.4, 5, and 7 using an APS-treated capillary. During the study at acidic pH levels, it was noted that the addition of TFA, needed to reach the lowest pH value, led to a reduction in sensitivity. Addition of an organic modifier, 2-propanol, was investigated at pH 3.4 and pH 8.5 in an attempt to improve the separation [63]. As expected, changing the CZE buffer to one containing 10% 2-propanol decreased the electroosmotic flow

rates, leading to longer analysis times. Unfortunately, separations were not improved.

Optimal separations by CZE were obtained at either pH 8.5 on untreated silica or pH 3.4 on an APS-treated column. The pH 3.4 separations (Figure 5a and b) were the best obtained overall because peak widths were narrower than at pH 8.5 (approximately 190,000 theoretical plates for OL in Figure 5a). Analysis time was rapid, with all of the components eluting within 7 min at pH 3.4 and 10 min at pH 8.5.

Approximately 44 fmol of each component was injected on-column in Figure 5a, whereas 300 fmol of each macrolide was used to produce the electropherograms in Figure 5b. Figure 5b illustrates the variation in sensitivity among the macrolides, EMA being the most sensitive and MOM being the least sensitive (and the least stable) of these compounds; it also demonstrates the effect of column overloading. To detect the MOM peak, approximately seven times as much material was injected as in Figure 5a. As can be

Figure 4. Relative ion abundances as a function of skimmer voltage for midcamycin (MDM) and troleandomycin (TAO).

Figure 5. (a) Selected ion electropherograms for the protonated molecules from a mixture of four macrolide antibiotics separated by CZE/ESI/MS. Buffer: 0.01-M acetic acid, pH 3.4. Column: $1.1 \text{ m} \times 75 \text{ }\mu\text{m}$ id APS. Amount injected: ~ 44 fmol. (b) Selected ion electropherograms for the protonated molecules from a mixture of six macrolide antibiotics separated by CZE/ESI/MS. Buffer: 0.1-M acetic acid, pH 3.4. Column: $1.1 \text{ m} \times 75\text{-}\mu\text{m}$ id APS. Amount injected: ~ 300 fmol.

seen by comparing the peak widths in Figure 5a and b, the effect of overloading the CZE column led to peak broadening and a reduction in the number of theoretical plates—EMA shows only 30,000 theoretical plates in this analysis.

Unfortunately, we were unable to obtain baseline separations of the macrolides by CZE. Because CZE separations are based primarily on the partial charge on the molecule with secondary contributions from molecular shape and weight, it is not surprising that these structurally similar compounds do not readily separate in CZE. The separations obtained at these two pH levels were, in effect, "opposites" of each other, with the later-eluting peaks on silica being the earlier-eluting peaks on APS, and vice versa. In both APS and silica separations, differences in electrophoretic migration times among the other macrolides are small but reproducible.

As expected for a group of compounds with the same functional groups, CZE separations are based only on the molecular weights of the compounds. CZE can separate OL (mol wt 687) and EMA (mol wt 733) from TYL (mol wt 915) but cannot resolve the compounds that differ by only 14 u in molecular weight, such as MDM (mol wt 813), RKM (mol wt 827), and JM (mol wt 827). Identification of these compounds based solely on their electrophoretic migration times by CZE/UV, for example, would be impossible. The advantage of doing the analysis by CZE/ESI/MS is that the compounds, even if overlapping, can be distinguished on the basis of their molecular weights and fragment ions.

SPM showed two peaks at pH 8.5 and at pH 3.4 when run in the mixture and separately. The peak migrating differently from the other macrolides may be due to the formation of a doubly charged analyte, as SPM is the only one of these macrolides with two amine groups. The other peak that appears in the same m/z 843 window may be due to a different isomer or an impurity, whose identity could not be determined from these SIM/MS experiments.

nCLC Separations

Because the separations achieved by CZE were limited (although sufficient for some applications when combined with mass-spectral differentiation), we explored combined nCLC/ESI/MS for these compounds. One of the main advantages of the nCLC approach, when used in conjunction with the quadrupole mass spectrometer, is its increased sample loading capacity. CZE column overloading occurred when higher levels of analyte (necessary for the acquisition of full scan data) were injected. Furthermore, the ability to perform gradient analyses with nCLC greatly aids method development, and it also allows on-column sample preconcentration from dilute solutions.

For ease of comparison, the same mixtures were

used for the nCLC separation as were used for the CZE separations. A mixture of 15 pmol of each of the four antibiotics shown in the CZE experiments (Figure 5a) was chromatographed using a simple linear gradient, and all four components were readily separated (Figure 6a). The peak occurring at scan 307 in the reconstructed ion chromatogram for LM-A5 was due to a degradation product of the TAO sample solution, which will be discussed later.

An nCLC separation on the same mixture of six macrolide antibiotics used in the CZE separations shown in Figure 5b was performed with 30 pmol of each antibiotic injected onto the column. All six components were readily resolved through application of the same linear gradient (Figure 6b), with SPM being very close to its detection limit. (SPM was below the detection limit by CZE when data were acquired in

Figure 6. (a) Reconstructed ion chromatograms from a mixture of four antibiotics separated by nCLC/ESI/MS. Packed capillary LC column: 2 m $\times$ 75- μ m id, 25 cm packed with 10- μ m AQ C-18. Gradient 25–100% (30 min) $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (both 0.1% TFA). Amount injected: $\sim$ 15 pmol. (b) Reconstructed ion chromatograms from a mixture of six antibiotics separated by nCLC/ESI/MS. Packed capillary LC column: 2 m $\times$ 75 μ m id, 25 cm packed with 10- μ m AQ C-18. Gradient 25–100% (30 min) $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (both 0.1% TFA). Amount injected: $\sim$ 30 pmol.

the full scan mode.) The multiple peaks in the mass window corresponding to TYL may result from isomers of the drug.

Spectra obtained for JM and MOM during the analyses illustrated in Figure 6a and b are shown in Figure 7. These demonstrate the simplicity of the spectra obtained using the electrospray interface at low skimmer voltage.

nCLC/ESI Applications

Two applications of the nCLC/ESI technique to the analysis of macrolide antibiotics are reported here. The first involves the structural determination of a degradation product from TAO, and the second involves the analysis of inactivated OL from a bacterial digest.

TAO degradation product. The reconstructed ion chromatogram for m/z 772, the mass of the protonated LM-A5 molecule, showed two peaks. The chromatographic peak occurring at scan 307 in the reconstructed ion chromatogram in the nCLC separation shown in Figure 6b was traced to a degradation product of the TAO sample solution.

This TAO degradation product, which has the same molecular weight as LM-A5, could be differentiated from LM-A5 by its retention time and by the presence of a fragment ion at m/z 586. Based on its molecular weight and its presence in TAO, this degradation product is probably a diacetylleandomycin resulting from hydrolysis of one acetate ester. On the basis of its ESI mass spectrum, it is possible to deduce which acetyl group is "missing". The ion of m/z 586, noted in its spectrum, arises via loss of a monoacetylated S ring, $[M + H - (S - H)]^+$. Thus the S ring still retains an acetyl group. Another fragment ion can be observed (at 25 V skimmer voltage) at m/z 158, which can be assigned as a B^+ fragment. This ion is 42 u lower in mass than the B^+ ion from TAO, indicating

that the acetyl group has been lost from the B ring. Hence, based on its ESI mass spectrum, the degradation product in the sample solution is a diacetylleandomycin with acetyl groups on the A and S rings.

Bacterial digest. To determine if the nCLC method could be applied to the analysis of products resulting from bacterial inactivation of macrolide antibiotics, a digest of OL and bacterial extract was prepared. A sample containing all of the reagents but in which the inactivation reaction was stopped immediately was designated "OL-0h"; the sample in which the enzymatic reaction was allowed to proceed for 24 hours was designated "OL-24h". Earlier experiments involving bioassays had indicated the formation of an inactivated OL in the reaction mixture, designated "IOL". Off-line FAB/MS experiments on purified IOL had indicated the presence of phosphorylated OL as the enzymatic reaction product, with protonated molecular weight of 768 u [25].

nCLC/ESI analysis of the OL-0h sample showed only unreacted OL, whereas the OL-24h sample showed almost no OL but instead an earlier-eluting product of m/z 889. A mixture of these two samples was used to produce the chromatograms in Figure 8a and b, showing the separation. The on-line mass spectrum of the m/z 889 product (Figure 8c) also showed an ion corresponding to the loss of 144 u, which was attributed to an $[M + Na - (S - H)]^+$ ion. This indicates that the sugar ring is unchanged from that of OL. In addition to this product, a trace of the

Figure 7. On-line ESI mass spectra obtained for JM and MOM from the nCLC analyses shown in Figure 6.

Figure 8. Reconstructed ion chromatograms of the OL reaction product (a) and unreacted OL from an nCLC/ESI analysis of the mixture (b), and ESI mass spectrum of the reaction product (c).

expected phosphorylated OL (IOL) was also observed at m/z 790 (corresponding to $[M + Na]^+$). The difference in mass between IOL and the new product is 121 u, so the new product appears to be a condensation product, complex, or adduct of IOL with a Tris molecule ($IOL + Tris + H^+$). This m/z 889 compound is not likely to be a physiological product of OL but is most likely an artifact resulting from lyophilization of the IOL in the Tris solution, which led to a highly concentrated solution before evaporation. Some $(OL + Tris + H)^+$ and $(OL + Tris + Na)^+$ adduct was detected by nCLC/ESI as well. The extraction procedure previously used for generation of IOL involved extraction into BuOH prior to lyophilization, and thus the IOL was not exposed to highly concentrated Tris buffer. Further studies involving FAB MS/MS analysis of the adduct are in progress, but nCLC has been shown to be valuable in the identification of macrolide inactivation products as well as for extraction procedure evaluation.

Comparisons of CZE/ESI/MS and nCLC/ESI/MS

From a practical point of view, nCLC is advantageous in terms of ease of operation for those versed in the use of larger id columns. Use of these small packed capillary columns is as easy as conventional HPLC, and column preparation is not difficult or time-consuming. A further advantage is the ability to transfer previously developed conventional HPLC methodologies directly to the nCLC columns. Of the two techniques, analysis using CZE/ESI/MS is significantly more rapid while providing increased separation efficiency (i.e., narrower peaks) and thus enhanced detection limits (by approximately an order of magnitude for full scan data). Chromatographic resolution by CZE, however, was much poorer than by nCLC, due to similarities between the molecules in size, shape, and charge. A major disadvantage of CZE/ESI/MS using this system was the ready occurrence of column overloading.

nCLC separations of these particular compounds were better than by CZE but were also slower and were rendered even more time-consuming by the column reequilibration time required between gradient runs. Reequilibration of nCLC columns is no more time-consuming than for conventional HPLC, however, and reequilibration, of course, would not be needed for isocratic operation.

Comparison of the separating capabilities of nCLC and CZE is more difficult because the techniques exploit different physical properties of the analytes. Essentially, they must be viewed as complementary and not competitive analytical techniques. Both techniques are easily interfaced with the electrospray ionization source. The same probe and sheath flow control can be used for both CZE and nCLC. Switching between an open tubular CZE column and a

packed nCLC column is easy and rapid, taking only about 10 min.

Conclusion

Positive-ion electrospray ionization of macrolide antibiotics yields spectra with abundant protonated molecular ions. Fragmentations useful for structure determination can be enhanced by increasing the skimmer voltage.

Separation of synthetic mixtures of macrolide antibiotics can be achieved using either CZE or nCLC, but the higher loading capacity of the nCLC column proves distinctly advantageous where mass-spectrometric sensitivities are limiting. However, CZE peak widths are narrower, detection limits are lower, and the analysis times are shorter, but for this group of closely related compounds, chromatographic separations were better by nCLC. Both techniques are readily interfaced with the mass spectrometer using the electrospray probe where the sub-microliter-per-minute flow rate of the analytical techniques is supplemented by coaxial delivery of additional solution required for stable electrospray operation.

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