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Development of an on-line preconcentration method for the analysis of pathogenic lipopolysaccharides using capillary electrophoresis–electrospray mass spectrometry Application to small colony isolates[1]

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Abstract

The present investigation describes the use of on-line chromatographic preconcentration coupled to capillary zone electrophoresis–electrospray mass spectrometry (cPC–CZE–ES-MS) for trace level analysis of negatively charged lipopolysaccharides (LPS) obtained from pathogenic strains of *Haemophilus influenzae*. The analytical performance of two different types of adsorption media [i.e., C_{18} irregular particles and poly(styrene–divinylbenzene) membrane] for anionic analytes was first evaluated using a mixture of peptide standards to determine the overall sensitivity of this approach. These chromatographic preconcentrators provided an enhancement of sample loadings of up to 5 μl with good linear response and low n*M* concentration detection limits for most peptides investigated. The application of cPC–CZE–ES-MS is further demonstrated for extracts of *O*-deacylated LPS obtained from *H. influenzae* strain Eagan. In combination with novel enzymatic releasing methods using proteinase K, this technique provides unparalleled sensitivity and enabled the identification of LPS surface antigens from as little as five bacterial colonies. Published by Elsevier Science B.V.

Keywords: *Haemophilus influenzae*; Sample preparation; Lipopolysaccharides; Saccharides; Peptides; Electrospray mass spectrometry

1. Introduction

Capillary zone electrophoresis (CZE) provides high resolution and separation efficiencies compared to conventional high-performance liquid chromatography (HPLC). This remarkable characteristic has led to the widespread application of CZE in analytical chemistry, especially for protein and peptide analyses [1]. Most applications of CZE have used UV absorbance detection as it offers simplicity, affordable price, and detection for a large number of compounds containing suitable chromophores. Concentration detection limits using UV is typically in the low μ*M* range for most peptides. Efforts to improve the sensitivity of CZE have focused on enhancement of the analytical performance of the absorbance detection system and methods providing improvement in sample loadings through preconcentration techniques. Alternate detection systems include laser induced fluorescence (LIF) [2,3], laser thermooptical absorbance (TOA) [4,5] and mass spectrometry (MS) [6–9]. However, both LIF and TOA rely on reproducible migration time for proper analyte identification in situations where standards are available. This task often becomes impractical

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for a number of complex biomolecules because of the lack of suitable standards, and such applications thus require more specific and selective detection systems. In this respect, MS using electrospray ionization (ES) has proven to be a powerful detection method for CZE, as it provides high sensitivity and structural information for a wide range of analytes. More recent advances in the fabrication of small electrospray tips (<20 μm I.D.) have enabled the reduction of the electrospray flow-rate to the sub-μl/min regime, and provided significant gains in sensitivity and increases in the signal-to-noise ratios. The coupling of conductive electrospray emitters to CZE using a sheathless interface affords significant reduction in chemical background contribution and amol mass detection limits are typically obtainable for peptides using nanoelectrospray ionization [10–14]. However, in view of the relatively small injection volume typically available with zone electrophoresis, the detection of trace level components present at sub-μ*M* concentrations in complex extracts is still a challenging issue facing this analytical technique.

To this end, a variety of sample loading techniques for improving the sensitivity of CZE have been developed, including analyte stacking [15,16], field amplification [17], transient isotachophoresis (tITP) [18,19], and on-line chromatographic preconcentration (cPC) [20–22]. Unfortunately, the electrophoretic characteristics of the analytes and the dimension constraints of a single capillary system have been limiting factors in the wider application of sample stacking and isotachophoretic preconcentration techniques. On the other hand, the use of an on-line chromatographic preconcentration–CZE cartridge that employs a membrane or small bed of packing material as the adsorptive phase enables the injection of several μl of a sample, while maintaining adequate analyte resolution during the subsequent CZE separation step. Recent reports have reviewed the analytical merits of this approach for sample extraction, clean-up, preconcentration and its further applications to CZE–ES-MS analysis of in-vivo derived metabolites, peptides and proteins [22–26]. It is noteworthy that most of these applications have been restricted to analytes which can be separated and detected as positive ions.

As an extension to earlier investigations from this laboratory on the application of CZE–ES-MS to the analysis of complex lipopolysaccharides (LPS) [27–29], we have explored the potential of on-line cPC for trace level analysis of these antigenic glycolipids. LPS are integral components of the outer cell membrane and major immunoreactive surface antigens of gram-negative bacteria. The LPS of many bacteria have been found to possess a common molecular architecture, first described for the family of *Enterobacteriaceae*, and are composed of a polysaccharide region that is linked to a hydrophobic lipid A via a core oligosaccharide [30]. In many gram-negative organisms, structural diversity within the polysaccharide region provide the molecular basis for serological typing. Certain pathogens of the genus *Haemophilus*, *Neisseria* and *Moraxella* which colonize mucosal surfaces, elaborate LPS containing shorter oligosaccharide chains referred to as lipooligosaccharides (LOS) that display extensive antigenic diversity among multiple oligosaccharide epitopes [31]. Structural variability can also be observed in the extent to which functional groups such as phosphate (P), pyrophosphate (PP), phosphoethanolamine (PE) and 3-deoxy-D-manno-2-octulosonic acid (KDO) residues are incorporated into the LPS structure which typically provided them with an overall negative charge. The analysis of these complex surface antigens can also be complicated by the relatively small amount of LPS derived from bacterial isolates. This is particularly true for the direct analysis of biological samples obtained from patients infected by these pathogens or for probing structural variation in phase variants.

In an effort to address the technical challenges facing the analysis of trace level LPS, the present report investigates the use of C_{18} stationary phase and styrene–divinylbenzene (SDB-XC) polymeric membrane for on-line cPC–CZE–ES-MS experiments. Particular emphasis was also placed on the development of an efficient extraction protocol utilizing the enzymatic release of LPS from bacterial cell membranes. The products obtained were subsequently subjected to mild hydrazinolysis in order to obtain the more hydrophilic *O*-deacylated LPS. The analytical performance of these preconcentrators was first evaluated using standard peptide mixtures and *O*-deacylated LPS from pathogenic strains of *Haemophilus influenzae* to determine the concen-

tration detection limits and the linear response. Earlier structural studies from this laboratory [32] and other groups [33,34] have led to the identification of both common and variable molecular features which are responsible for immunological specificity and virulence potential of this organism. This method was successfully applied to the characterization of *O*-deacylated LPS from *H. influenzae* strain Eagan revealing for the first time the pattern of LPS glycoforms for very small colony isolates.

2. Experimental

2.1. Chemicals and materials

Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA) and PTFE tubing from LC Parkings (San Francisco, CA, USA). Poly(styrene–divinylbenzene) membranes (SDB-XC) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Silica-based C_{18} irregular particles were taken from Sep-Pak Cartridges available from Waters (Milford, MA, USA). The peptide standards angiotensin I, Leu-enkephalin, vasoactive intestinal peptide (VIP), [Glu] fibrinopeptide B and the enzymes proteinase K, deoxyribonuclease I (DNase) and ribonuclease (RNase) were purchased from Sigma (St. Louis, MO, USA). Methanol, acetonitrile and isopropanol were from EM Science (Gibbstown, NJ, USA). The hydrazine reagent, morpholine and other buffers were obtained from Aldrich (Milwaukee, WI, USA) and formic acid from BDH (Toronto, Canada).

2.2. Bacterial strains and growth conditions

H. influenzae strain Eagan, a serotype b clinical isolate [35] from the National Research Council culture collection (NRCC 4247) was either grown at 37°C on chocolate agar plates (Quelab, Montreal, Canada) or cultivated in liquid media according to conditions described previously [32].

2.3. Preparation and extraction of lipopolysaccharides

For small scale experiments, colonies (typically 1 mm size) were scraped from the agar plate, and the proceeds of 5, 10 and 20 colonies were dispersed in 1.5-ml tubes each containing 100 μl of deionized water. The cells were freeze–dried overnight from which typical yields of 0.2 mg of dried cell mass for five colony isolates were obtained. The freeze–dried cells were dissolved in 90 μl of deionized water and a 10-μl aliquot of a 25 μg/ml solution of proteinase K was added to each vial. The suspended cell solutions were incubated at 37°C for 90 min and the digestion was stopped by raising the temperature to 65°C for 10 min. The solutions were allowed to cool at room temperature and were subsequently freeze–dried. The cells were further digested by incubating them at 37°C for 4 h in a 200 μl solution of 20 m*M* ammonium acetate buffer, pH 7.5 containing DNase (10 μg/ml) and RNase (5 μg/ml).

2.4. Preparation of O-deacylated LPS

The freeze–dried and digested cells containing the free LPS were dissolved in 200 μl of hydrazine and incubated at 37°C for 50 min with constant stirring to release *O*-linked fatty acids [36]. The reaction mixtures were cooled (0°C), the hydrazine destroyed by addition of cold acetone (600 μl), and the final product was obtained by centrifugation. The pellets were washed with 2×600 μl of acetone, centrifuged and then lyophilized from water.

2.5. Fabrication of on-line chromatographic preconcentrator

The construction of preconcentrator is similar to devices described elsewhere [23–27]. The C_{18} preconcentrator was constructed by drawing into a 2-cm piece of PTFE tubing (180 μm I.D.) a slurry solution of 40 μm irregular particles suspended in methanol (Fig. 1). A bed of 1 mm was typically used as the on-line cPC device. For the SDB-XC preconcentrator, a piece of SDB-XC membrane was inserted at the mid-point of a PTFE tubing (2 cm in length) between a fused-silica capillary transfer line (7 cm) and the CZE column (80 cm). Prior to installation, the preconcentrator was activated by rinsing it with methanol for half an hour. The entire cPC–CZE capillary was then conditioned sequentially with the elution and separation buffers.

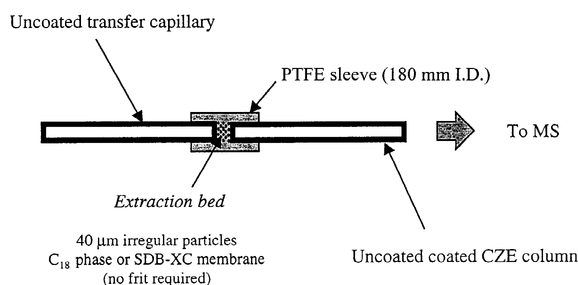

Fig. 1. Schematic description of the chromatographic preconcentration device used in the present investigation.

2.6. CZE–ES-MS and cPC–CZE–ES-MS analyses

A Crystal Model 310 CE instrument (ATI Unicam, Boston, MA, USA) was coupled to an API 300 mass spectrometer (Perkin-Elmer/Sciex, Concord, Canada) via a MicroIonspray interface. A sheath solution (isopropanol–methanol, 70:30) was delivered at a flow-rate of 2 µl/min to a low dead volume tee (250 µm I.D., Chromatographic Specialties, Brockville, Canada). All aqueous solutions were filtered through a 0.45-µm filter (Millipore, Bedford, MA, USA) before use. An electrospray stainless steel needle (27 gauge) was butted against the low dead volume tee and enabled the delivery of the sheath solution to the end of the capillary column. Unless otherwise specified, separations were obtained on 90 cm length bare fused-silica (Polymicro Technologies, Tucson, AZ, USA) using 50 m*M* morpholine/formate in deionized water pH 9, containing 2% methanol. A voltage of 30 kV was typically applied at the injection end of the capillary. The outlet of the fused-silica capillary (185 µm O.D.×50 µm I.D.) was tapered to 75 µm O.D. (20 µm I.D.). Mass spectra were acquired with dwell times of 3.5 ms per step of 1 m/z unit in full-mass scan mode, or 100 ms per channel for selected ion monitoring (SIM) experiments. A Power Macintosh PC 900 computer was used for instrument control, data acquisition and data processing.

cPC–CZE–ES-MS analyses were conducted using the same experimental set-up described above except that cPC device was inserted between the transfer line and the preconditioned CZE column. The elution buffer was composed of 10% 0.1 *M* formic acid in acetonitrile. Prior to sample injection, the capillary column was rinsed sequentially with the elution and separation buffers (five column volumes each). Sample introduction was performed at a constant pressure of either 1000 or 2000 mbar for a 3 min duration which resulted in the injection of 2.25 or 4.5 µl, respectively. After the sample loading, the capillary was rinsed with four column volumes of separation buffer. The sample was eluted from the stationary phase using a small plug of elution buffer (300 mbar for 0.1 min, 25 nl) and pushed through the stationary phase by positive pressure to prevent readsorption of the analyte [26].

3. Results and discussion

The analysis of glycolipids derived from outer cell membrane components of pathogenic bacteria presents a significant analytical challenge as these bioactive compounds have very few UV chromophores and display fatty acid side chains and modified phosphorylated groups. The preponderance of negatively charged functionalities such as phosphates and phosphoethanolamine makes them amenable to CZE separation under anionic conditions. However, their low UV extinction coefficients coupled to the limited sample loadings required to maintain adequate separation efficiencies prevent the detection of *O*-deacylated LPS at concentration below 0.1 mg/ml.

Preliminary investigations were conducted using peptides standards of different isoelectric points and hydrophobic character to optimize separation conditions under conventional CZE and when using an on-line cPC device. Such experiments were first developed using a UV detector but were subsequently extended to both CZE–ES-MS and cPC–CZE–ES-MS in order to establish the analytical performance of these techniques.

3.1. CZE–ES-MS and cPC–CZE–ES-MS analyses of peptides

Initially a mixture of four peptides, consisting of angiotensin I, Leu-enkephalin, vasoactive intestinal peptide (VIP) and [Glu] fibrinopeptide B, were chosen as model samples for evaluating cPC–CZE–ES-MS. Separations conducted using a 50 m*M*

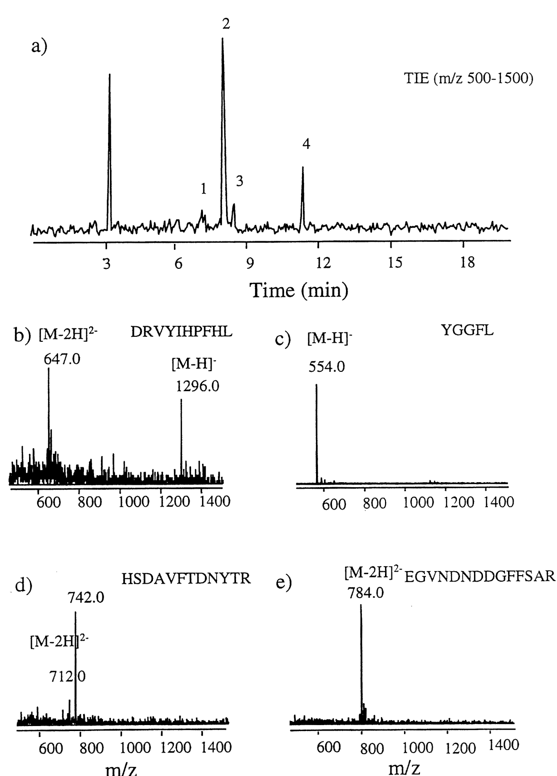

Fig. 2. CZE–ES-MS (negative ion mode) analysis of a mixture of four peptides at 10 μg/ml using full scan acquisition. (a) Total ion electropherogram (*m/z* 500–1500), extracted mass spectra for peak identified at (b) 7.2 min, (c), 8.1 min, (d) 8.5 min and (e) 11.4 min. Separation conditions: bare fused-silica (90 cm×50 μm I.D.), 50 m*M* morpholine, pH 9.0, +30 kV, 25 nl injected.

morpholine buffer, pH 9.0, were found to afford low current (8 μA) and good separation efficiencies for most peptides analyzed under these anionic conditions. The CZE–ES-MS analysis of this peptide mixture performed under full mass scan acquisition is presented in Fig. 2 for a 25 nl injection of a 10 μg/ml mixture of each peptide. Under these conditions all analytes migrated after the electroosmotic flow (EOF) observed at 3.3 min in Fig. 2. Good mass spectral response was obtained for most peptide analyzed and enabled full scan detection and meaningful identification for concentrations of approximately 10 μ*M*. Mass spectra extracted for peaks identified in the ion electropherogram of Fig. 2a are presented in Figs. 2b–e. Mass spectra are dominated by singly- and doubly-deprotonated molecules except for VIP (Fig. 2d) where a prominent ammonium formate adduct was observed instead. The mass spectral response was not identical for the different peptide candidate selected. Among the different peptides analyzed, Leu-enkephalin provided the highest sensitivity with detection limits approaching 0.9 μ*M* in full mass scan acquisition whereas such limits were nearly attained for a concentration of 7.7 μ*M* in the case of angiotensin I (Fig. 2b). A more systematic study of the sensitivity of the CZE–ES-MS techniques for these anionic peptides was conducted using SIM acquisition mode and the results obtained are summarized in Table 1. As indicated, good linear response was observed for all analytes investigated with correlation coefficients ranging from 0.978–0.999. Consistent with earlier observations, Leu-enkephalin yielded a lower limit of detection (LOSD) with a value 6 n*M* (*S/N*, 3:1) compared to other peptides analyzed that provided LOSDs typically 6–40-times higher (Table 1).

Preliminary experiments involving the cPC device

Table 1
Limits of detection for peptides analyzed by CZE–ES-MS and cPC–CZE–ES-MS

Peptides	No preconcentrator			C_{18}			SDB-XC		
	LOSD[c]		r^2	LOSD[c]		r^2	LOSD[c]		r^2
	n*M*	fmol[a]		n*M*	fmol[b]		n*M*	fmol[b]	
Angiotensin I	260	6.5	0.997	2.5	11	0.995	6.2	28	0.986
Leu-enkephalin	5.9	0.15	0.999	0.48	2.2	0.996	0.23	1.0	0.991
VIP	160	4.1	0.998	113	510	0.996	23	102	0.988
[Glu] fibrinopeptide B	17	0.77	0.979	3.2	14	0.999	1.0	4.6	0.981

[a] Based on 25-nl injection of serial dilutions of peptide standards (*S/N*, 3:1).
[b] Based on 4.5-μl injection of serial dilution of peptide standards (*S/N*, 3:1).
[c] All analyses conducted in SIM ($[M-2H]^{2+}$ ions monitored in all cases except for Leu-enkephalin where $[M-H]^-$ was selected).

were first conducted using peptide standards to establish suitable separation conditions for trace level analyses of these complex carbohydrates. The composition and acid content of the organic buffer were investigated by comparing the signal strength of the peptide peak following its release from the adsorbent abundance. cPC–CZE–UV experiments conducted using a small bed of C_{18} adsorbent indicated that an elution buffer composed of 90% acetonitrile with either 10 m*M* HCOOH or 10 m*M* HCl provided suitable desorption of the different peptides. The volume of the elution buffer was adjusted to provide quantitative release of adsorbed peptides and to minimize any adverse effect on the separation performance. For a sample loading resulting in the injection of up to 10 ng of peptides, an elution volume of approximately 25 nl yielded reproducible release of up to 60% of the adsorbed analyte.

An example of a cPC–CZE–ES-MS analysis obtained under full mass scan acquisition for a peptide mixture at concentration of 100 ng/ml is presented in Fig. 3. The identification of the different peptides is clearly visualized from the reconstructed ion electropherograms (Figs. 3b–e). The order of migration is the same as that observed previously for CZE–ES-MS (Fig. 2) except that peak resolution is partly degraded due to the concurrent application of a small inlet pressure (50 mbar) during the separation. This was found necessary in order to prevent the loss of electrical continuity imparted by the small plug of organic solvent and the presence of a bed of adsorbent which can affect the extent of the EOF. As a result of this, separation efficiencies were typically 2–10-times lower than that observed under the conventional CZE format (Fig. 2).

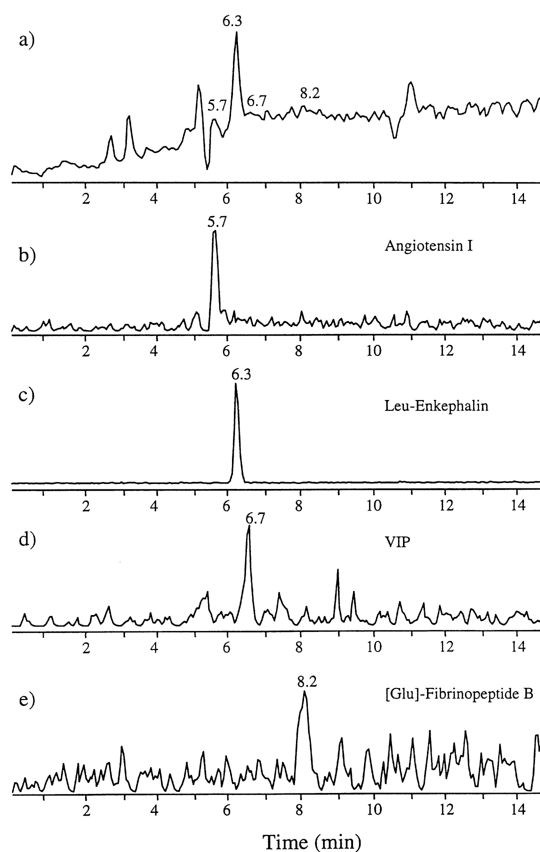

Fig. 3. cPC–CZE–ES-MS (negative ion mode) analysis of a mixture of peptide standards at 100 ng/ml using a 1-mm bed of C_{18} packing material. (a) Total ion electropherogram (*m*/*z* 500–1500), reconstructed ion electropherogram for (b) *m*/*z* 647, (c) *m*/*z* 554, (d) *m*/*z* 712 and (e) *m*/*z* 784. Separation conditions: sample loading of 3 min at 1000 mbar (2.25 μl), eluent volume 25 nl 0.01 *M* HCOOH in 90% acetonitrile, other conditions as in Fig. 2.

Serial dilutions of the same peptide mixture enabled the determination of concentration detection limits for these different analytes (Table 1). Elution conditions were optimized to provide good linear response for peptide concentrations ranging from 0.2 n*M*–10 μ*M*. Under the present conditions, a short plug of organic solvent (typically 25 nl of 0.01 *M* HCOOH in 90% acetonitrile) was necessary to efficiently desorbed the retained analyte into a minimal sample zone. Results from this study indicated that an improvement of concentration detection limits of up to 50-fold compared to CZE–ES-MS can be obtained using the on-line cPC device. As expected, such gains in sensitivity were more significant for analytes comprising a relatively high number of hydrophobic residues. The peptide VIP was not as strongly retained on the preconcentrator and yielded only modest improvements of LOSDs in spite of attempts to add ion pairing additives to the rinsing buffers. It is noteworthy that good linear correlation coefficients (r^2>0.996) were observed for all peptides investigated over the concentration range examined (Table 1).

In order to further improve the analytical performance of the preconcentration device, alternate adsorbents were investigated. Previous investigations

[22,23] have reported the use of SDB-XC membrane as efficient adsorbents for the analysis of positively-charged peptides by cPC–CZE–ES-MS. Although no data is yet available of the quantitative recovery and dynamic range of these preconcentrators, peptide and protein analyses were successfully demonstrated for target analytes present at low n*M* concentrations. In the present application, a single layer (500 μm) of this membrane was inserted in the PTFE tube by using the transfer capillary as a die cutter while the CZE column was butted against it to secure its position. cPC–CZE–ES-MS analyses were conducted using the same conditioning and extraction protocols described earlier. Separations performed using this adsorbent yield qualitatively similar electropherograms to that of the pelicular C_{18} packing material (data not shown). The most significant change noted was in the overall improvement of sensitivity for all peptides investigated (Table 1). cPC–CZE–ES-MS separations conducted in SIM mode provided at least a 12-fold improvement in LOSD values over that of CZE–ES-MS. Under the present conditions, low n*M* concentration detection limits were obtained for all analytes investigated. Again the most significant improvement in sensitivity was observed for Leu-enkephalin where an LOSD of 0.23 n*M* was obtained in SIM acquisition mode. Improvement in sensitivity over that of the C_{18} adsorbent was generally observed for all peptides analyzed. However, the only significant drawback of the SDB-XC preconcentrator was the lower linearity coefficient observed over the concentration range examined. The departure from linearity was mostly observed for concentration higher than 3 μ*M* for [Glu] fibrinopeptide B and 9 μ*M* for Leu-enkephalin, and this could possibly reflect the maximum adsorption capacity of the membrane.

3.2. Application of CZE–ES-MS and cPC–CZE–ES-MS to the analyses of O-deacylated lipopolysaccharides from H. influenzae type b strain Eagan

As part of our continuing effort to probe structural variations in glycolipids isolated from human pathogens, we have explored the possibility of integrating cPC device to enhance the sensitivity and specificity of the CZE–ES-MS method for the analysis of the carbohydrate components of bacterial LPS. The determination of the composition and structures of complex carbohydrates derived from these LPS can be very challenging due to the natural heterogeneity of glycan distribution and the presence of functional groups appended to different sites which gives large number of related LPS isoforms. It has been well-established that *H. influenzae* serotype b strains elaborate heterogeneous populations of LPS showing intra- and inter-strain variability [32–34]. On the basis of single colony studies, a number of surface exposed oligosaccharide structures, defined by their reactivities with monoclonal antibodies have been shown to be phase variable [33]. This mechanism of rapid on–off switching between certain oligosaccharide epitopes is thought to provide an important mechanism whereby *H. influenzae* can adapt to changing environmental conditions which are encountered during the pathogenic cycle of infection. This capacity to vary structural types adds to the challenge faced by the analyst.

Chemically, LPS consist of two parts having contrasting properties: a hydrophilic carbohydrate component containing acidic residues (KDO and phosphate groups) bonded to an hydrophobic lipid A component composed of a glucosamine disaccharide to which are attached *O*- and *N*-linked fatty acids. The hydrophobic character of the latter component was advantageously exploited for selective adsorption of LPS-derived molecules to reversed-phase preconcentrators. Preliminary investigations focused on the development of electrophoretic conditions conducive to the separation of *O*-deacylated LPS by CZE. Separations conducted using aqueous morpholine buffer provided adequate resolution of different LPS isoforms, but also displayed significant peak broadening possibly suggesting the adsorption of the analytes on the surface of the capillary (data not shown). Evidence of this was also noted in experiments involving the injection of serial dilution of the same LPS preparation where poor linearity and analyte response were observed at low concentration. In order to overcome this problem, an organic modifier was added to the separation buffer. Amongst the different organic solvent considered, an aqueous solution of 30 m*M* morpholine, pH 9 containing 2% (v/v) methanol was found to provide the best separation conditions. Minimization of or-

ganic content was also required to favor adsorption of the analyte in subsequent experiments involving the on-line cPC device. Under these conditions, the signal intensities for the different *O*-deacylated LPS were significantly increased and the calibration curve for the main component (*m/z* 1299) over the concentration range 10–500 μg/ml gave good correlation coefficient (r^2: 0.996) with an LOSD of 10 μg/ml (*S/N* ratio of 5:1).

The CZE–ES-MS analysis of *O*-deacylated LPS from *H. influenzae* type b strain Eagan obtained from large scale extraction of liquid cultures is shown in Fig. 4. The total ion electropherogram (TIE) corresponding to full scan analysis (*m/z* 400–1500) is presented on the top panel of Fig. 4 along with the contour representation of *m/z* vs. time in the bottom panel. The contour profile shows a series of doubly- and triply-deprotonated molecules from which molecular mass of the different analytes can be derived.

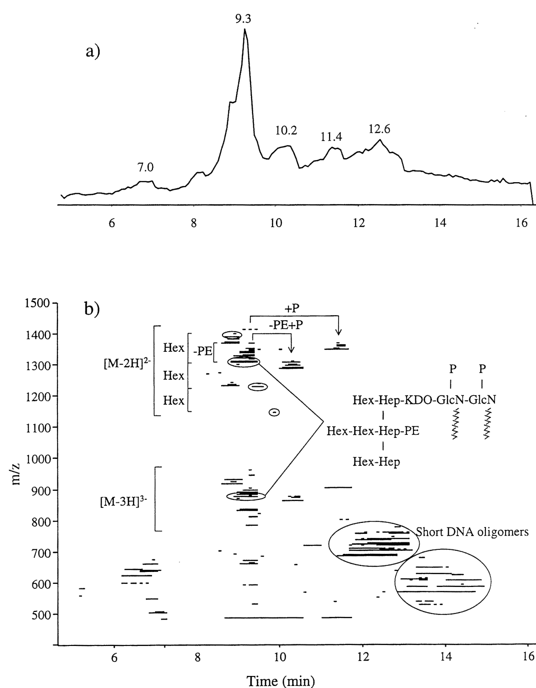

Fig. 4. CZE–ES-MS (negative ion mode) analysis of *O*-deacylated LPS from *H. influenzae* serotype b strain Eagan. (a) Total ion electropherogram (*m/z* 400–1500); (b) contour profile of *m/z* vs. time. Separation conditions: 40 nl injection of 0.5 mg/ml of *O*-deacylated LPS, bare fused-silica (90 cm×50 μm I.D.), 2% methanol in 30 m*M* morpholine, pH 9.0, +30 kV.

The most prominent *O*-deacylated LPS observed at 9.3 min corresponds to a structure composed of P_3 PE Hex_4 Hep_3 KDO $GlcN_2$ with two *N*-linked 3-OH myristic acids ($C_{14}H_{27}O_2$) where Hex, Hep and GlcN represent hexose, heptose and glucosamine residues, respectively (M_r: 2600.5). The microheterogeneity of this sample, reflected in both glycoform distribution and substitution of phosphorylated groups, is also clearly visualised in this contour profile by families of peaks with incremental changes in electrophoretic mobilities. For example, the diagonal line of negative slope indicated in Fig. 4b reflects the concurrent changes in molecular mass and electrophoretic mobilities characteristic of glycan extension. In the present case, the number of Hex residues can vary from 2 to 5 units appended to the heptose-containing core structure shown as an inset in Fig. 4b. The addition of a single phosphate group to the main component gave rise to a late migrating peak at 11.4 min (M_r: 2681.0) whereas the substitution of a phosphoethanolamine for a phosphate group is observed at 10.2 min (M_r: 2558.5). For convenience, the assignment of the major *O*-deacylated LPS observed in Fig. 4 is summarized in Table 2. It is noteworthy that the corresponding compositions are in accord with the structural model described previously for *H. influenzae* [32]. Also evident from the contour profile of Fig. 4b is the presence of small oligonucleotides migrating between 12 and 14 min. These are common contaminants obtained from large scale purification of LPS preparations using the traditional phenol–water extraction method [32,36]. Removal of these low-molecular-mass components often requires extensive fractionation using gel permeation chromatography. As part of this investigation efforts were also devoted to improve the extraction procedures for small scale isolation of LPS (see later).

In order to improve the sensitivity of the CZE–ES-MS techniques both C_{18} irregular packing and the SDB-XC membrane were evaluated as on-line preconcentrators. Serial dilutions ranging from 0.05 to 20 μg/ml of the heterogeneous population of *O*-deacylated LPS from *H. influenzae* (Fig. 4) was used to evaluate the analytical performance of the cPC devices. Preliminary cPC–CZE–ES-MS experiments indicated that both preconcentrators yield similar sensitivity with limits of detection of approxi-

Table 2
Assignment of the different *O*-deacylated LPS of *H. influenzae* strain Eagan (Fig. 4)

Time (min)	Observed ion (*m/z*)		Molecular mass		Proposed composition
	$[M-3H]^{3-}$	$[M-2H]^{2-}$	Observed	Calculated	
8.2	840	1260	2522.5	2521.3	Hex_4 Hep_3 PE KDO Lipid A[a]
8.9	920	1380	2762.5	2763.5	Hex_5 Hep_3 PE P KDO Lipid A
8.9	907	1361	2724.0	2724.4	Hex_4 Hep_3 PE_2 P KDO Lipid A
9.3	866	1299	2600.5	2601.3	Hex_4 Hep_3 PE P KDO Lipid A
9.5	812	1218	2438.5	2439.2	Hex_3 Hep_3 PE P KDO Lipid A
9.8	758	1137	2276.5	2277.1	Hex_2 Hep_3 PE P KDO Lipid A
10.2	852	1278	2558.5	2558.3	Hex_4 Hep_3 P_2 KDO Lipid A
10.8	934	1401	2804.5	2804.4	Hex_4 Hep_3 PE_2 P_2 KDO Lipid A
11.4	893	1339	2681.0	2681.3	Hex_4 Hep_3 PE_1 P_2 KDO Lipid A

[a] Lipid A is composed of $GlcN_2$, P_2 and two *N*-linked 3-OH myristic acids ($C_{14}H_{27}O_2$).

mately 100 ng/ml corresponding to the on-column injection of 450 pg of total *O*-deacylated LPS. This corresponds to a 40-fold improvement in concentration detection limits compared to that observed in previous CZE–ES-MS experiments. However, significant differences were consistently noted in the linearity of calibration curves obtained using these two adsorbents. The C_{18} irregular packing material provided a poor correlation coefficient (r^2: 0.903) compared to that observed for the SDB-XC membrane (r^2: 0.988). For this reason subsequent cPC–CZE–ES-MS experiments were conducted with the SDB-XC adsorbent.

An example of separation conducted in full mass scan acquisition (*m/z* 400–1500) using this impregnated membrane is shown in Fig. 5a Fig. 5b for a 4.5-μl injection of 4.6 and 0.98 μg/ml solutions of *O*-deacylated LPS from *H. influenzae*. As observed, good *S/N* ratios were obtained from both analyses and enabled the identification of these bacterial antigens at low μg/ml concentration. It should be reiterated that the specified concentration corresponded to the entire mixture of glycolipid and that the most prominent Hex_4 glycoform is estimated to represent approximately 25–30% of the heterogenous *O*-deacylated LPS population. Mass spectra extracted from the principal components observed in Fig. 5b at 6.2 and 6.7 min are presented in Fig. 5c Fig. 5d, respectively. Consistent with previous CZE–ES-MS analyses (Fig. 4) the major glycoform observed in Fig. 5c corresponds to the *O*-deacylated LPS composed of Hex_4 Hep_3 PE P KDO Lipid A whereas the later migrating component (Fig. 5d)

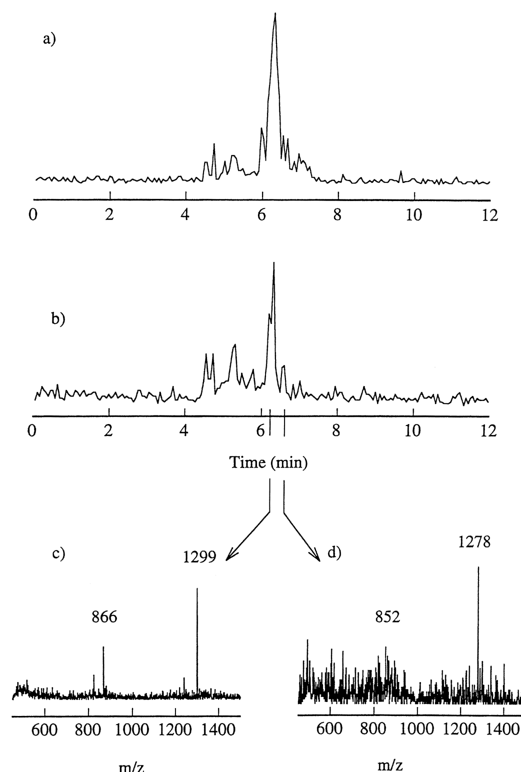

Fig. 5. cPC–CZE–ES-MS (− ion mode) analysis of *O*-deacylated LPS from *H. influenzae* serotype b strain Eagan at (a) 4.6 μg/ml and (b) 0.98 μg/ml. Extracted mass spectra at (c) 6.2 min and (d) 6.7 min. Separation conditions: SDB-XC adsorbent, 4.5 μl injected, full mass scan acquisition (*m/z* 400–1500), other conditions as for Fig. 3 except that a 2% methanol in 30 m*M* morpholine, pH 9.0 buffer was used during the zone electrophoresis.

represents a closely related analog where a PE is replaced by a phosphate group.

Application of the cPC–CZE–ES-MS technique for the analysis of trace level glycolipids was further demonstrated for bacterial isolates taken directly from Petri dish cultures of *H. influenzae* serotype b strain Eagan. However, refinement of the sample preparation protocol was required to minimize sample losses during the LPS isolation, extraction and hydrazinolysis steps. Traditionally, the isolation of LPS from large scale bacterial liquid cultures has been conducted using the phenol–water extraction method. Although this procedure enables a convenient and efficient means of processing large amount of LPS, preliminary experiments conducted using this method on single colonies (data not shown) failed to provide sufficient material for subsequent hydrazinolysis and mass spectral analyses. Alternate extraction procedures using the enzyme proteinase K to selectively release the LPS from the bacterial outer membrane were devised. This non-specific enzyme is typically used as a topological probe for membrane proteins as it effects minimal disruption of the inner membrane lipid bilayer.

The analytical protocol developed here consisted of selecting isolates in group of 5, 10 and 20 colonies. An isolate corresponding to five colonies typically gave a freeze–dried biomass of 200 μg with an expected yield of approximately 2–4 μg of crude LPS. Release of the LPS was achieved by subjecting the freeze–dried cells to consecutive treatments with proteinase K, DNase and RNase. The last two enzymatic steps were required to reduce the viscosity of the solution to minimize trapping of the LPS. Mild hydrazinolysis was then conducted on the unpurified lysate in a similar manner to that described previously [36]. A significant advantage of the present protocol is the ability to perform all of the necessary extraction and sample clean-up steps in a single vial which obviously minimizes any losses due to sample transfer.

The cPC–CZE–ES-MS analysis of an *O*-deacylated LPS sample obtained from five colonies of *H. influenzae* is shown in Fig. 6. For this analysis, the *O*-deacylated LPS extract was dissolved in a total of 40 μl from which only 4.5 μl was injected on column. The total ion electropherogram (*m/z* 400–1500) is presented in Fig. 6 together with the

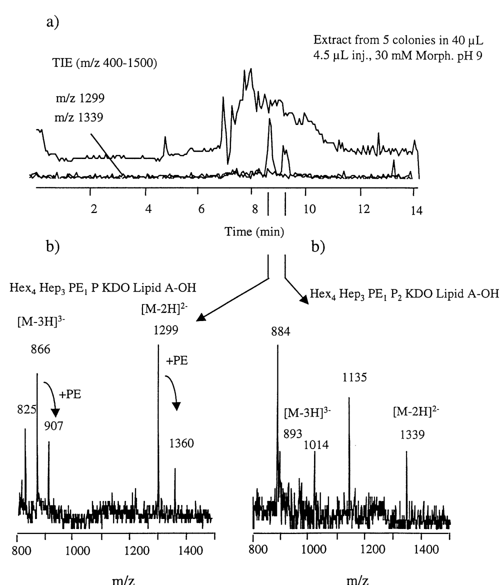

Fig. 6. cPC–CZE–ES-MS (− ion mode) analysis of *O*-deacylated LPS from *H. influenzae* serotype b strain Eagan extracted from five colonies. (a) Total ion electropherogram (*m/z* 400–1500), extracted mass spectra for peak identified at 8.5 min (b) and 9.3 min (c) in (a). Separation conditions: SDB-XC adsorbent, 4.5 μl injected (≈1 μg/40 μl), full mass scan acquisition (*m/z* 400–1500), other conditions as in Fig. 3.

extracted ion profiles for *m/z* 1299 and 1339. The mass spectra obtained from the most prominent *O*-deacylated LPS species are presented in Figs. 6b and c for peaks identified in Fig. 6a at 8.6 and 9.3 min, respectively. The principal component observed in Fig. 6b corresponds to the anticipated Hex_4 glycoform containing one P and one PE group (*m/z* 866 and 1299). Evidence of co-migrating and closely related *O*-deacylated LPS are substantiated by the observation of triply- and doubly-charged ions at *m/z* 907 and 1360 corresponding to the addition of an extra PE group to the main component and at *m/z* 825 and 1245 for the Hex_3 glycoform. The lack of resolution noted here for these different *O*-deacylated LPS compared to that observed in Fig. 4 is attributed to the concurrent application of a small inlet pressure during the separation. Other minor components baring additional ionizable functionalities such as the diphosphorylated Hex_4 glycoform (Fig. 6c) represented by the doubly- and triply-charged ions at *m/z* 893 and 1339 could easily be separated from this first peak. Also present in Fig. 6c

are ionic species at *m*/*z* 884, 1014 and 1135 which could not be readily assigned to any of the expected *O*-deacylated LOS, and the identity of these peaks is still unknown at present. It is noteworthy that in contrast to the previous CZE–ES-MS analysis shown in Fig. 4, the present extraction method does not appear to yield noticeable amounts of short oligonucleotides which is an added benefit of this experimental protocol. More importantly, this analysis revealed that the profile of *O*-deacylated LPS observed here is in close agreement with the structural model described previously [32] although different growth conditions and extraction protocols were used. The structural information coupled to the unprecedented sensitivity available from the present technique thus provides a valuable tool to probe subtle molecular changes taking place on bacterial isolates approaching a single colony.

4. Conclusions

The outer cell membrane components of gram-negative bacteria also refered to as LPS often display a remarkable structural complexity in the type and number of substituents in both the lipid A and the core structure. The high level of heterogeneity associated with these glycolipids often form the molecular basis for serological typing. The structural characterization of these glycolipids is not only important for the further understanding of the pathogenesis process but also to identify potential immunodeterminant structures that could be responsible for the virulence potential of the bacterial pathogens. The analysis of these complex carbohydrates can also be complicated by the relatively small amount of LPS derived from bacterial sources especially in situations where samples are obtained from clinical isolates or from phase variants. To this end, the present report describes the successful application of a novel LPS isolation procedure and a preconcentration method enabling the identification of trace level *O*-deacylated LPS from as little as five colonies. This chromatographic preconcentration method was first developed for peptide standards using conditions conducive to CZE–ES-MS analysis. Under optimized separation conditions, preconcentrator devices constructed from both C_{18} irregular packing material and SDB-XC impregnated membrane provided low n*M* concentration detection limits with good linear response (r^2>0.980) for serial dilutions up to 10 μ*M*. Best performances were achieved for hydrophobic peptides such as Leu-enkephalin where a detection limit of 0.23 n*M* was obtained. The application of cPC–CZE–ES-MS to the analysis of *O*-deacylated LPS from *H. influenzae* serotype b strain Eagan indicated that a 40-fold improvement in sensitivity can be obtained compared to the more traditional zone electrophoresis format. The introduction of such chromatographic preconcentration devices enabled the full mass scan acquisition of these glycolipids at a concentration of approximately 500 ng/ml corresponding to the on-column injection of 2.3 ng of total *O*-deacylated LPS. In combination with efficient LPS enzymatic extraction procedures using proteinase K, the application of cPC–CZE–ES-MS was successfully demonstrated for the analysis of five colonies of *H. influenzae*. Such advances now permits the identification of trace level glycolipids obtained from selected bacterial isolates and opens up new avenues in immunological investigations designed to probe the molecular basis of bacterial virulence.

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