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High-performance liquid chromatographic method to resolve and determine lipopolysaccharide sub-groups of *Escherichia coli* endotoxin in isolated perfused rat liver perfusate

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ABSTRACT

A high-performance liquid chromatographic method was developed for resolving heterogeneous preparations of fluorescently labelled endotoxin derived from *Escherichia coli* (Serotype 0111:B4) into separate lipopolysaccharide sub-groups. The endotoxin was chromatographed on an analytical gel permeation column using a mobile phase of acetonitrile (20%, v/v) and 100 m*M* phosphate buffer (pH 7.75). Four fluorescent peaks were resolved, representing sub-groups of markedly different molecular sizes. Three of the four sub-groups contained the core polysaccharide 2-keto-3-deoxyoctonate, confirming that they contained lipopolysaccharide. Fluorescein isothiocyanate (FITC)-labelled endotoxins derived from *Vibrio cholerae* and *Salmonella minnesota* chromatographed using the same system eluted with distinctly different patterns of peaks from each other and from *E. coli*. Extraction of *E. coli* FITC–endotoxin from a buffer solution using a phenol–diethyl ether method and subsequent chromatography allowed the determination of three of the four fluorescent sub-groups over the concentration range 1–15 μg/ml.

INTRODUCTION

Endotoxin is a general term describing a class of lipopolysaccharide molecules located in the outer cell membrane of Gram negative enteric bacteria. The presence of endotoxin in the systemic circulation can cause a number of pathological effects, via either direct toxicity to cells or the induction of secondary effector secretion.

The lipopolysaccharide molecules consist of a polysaccharide moiety which provides the toxin with antigenic specificity [1] and a lipid moiety which is thought to be responsible for the pathological effects of endotoxin. Endotoxin isolated from bacterial cultures by any of the commonly used methods [2,3] usually contains several sub-

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forms of lipopolysaccharide, owing to the presence of mutant bacteria which are deficient in certain biochemical pathways. These so-called rough form (R-form) bacteria cannot, to various degrees, synthesize the complete polysaccharide moiety produced by normal or so-called smooth (S-form) bacteria. Moreover, it has been suggested that smaller lipopolysaccharide molecules produced by R-form bacteria are cleared from the plasma *in vivo* more rapidly than the larger molecules produced by the S-form bacteria [4]. Studies on the disposition of endotoxin in the body and in specific organs should therefore deal with the individual components of this substance rather than treating it as a single entity.

Methods currently available for assay of endotoxin in biological fluids include the *Limulus* amoebocyte lysate (LAL) assay [5], gas chromatography–mass spectrometry [6] and gamma/beta counting of endotoxin radiolabelled with ^{14}C, ^{3}H, ^{125}I or ^{51}Cr [7–10]. None of these methods allows the separate determination of different lipopolysaccharide components which may be present in a sample. Although size-exclusion chromatography using Sepharose and similar gels has been used by a number of workers to study the molecular form of endotoxin preparations [11–13], these methods are not suitable for analytical studies as they require large amounts of sample and the detection methods used (radioactivity or assay for the 3-deoxy sugar 2-keto-3-deoxyoctonate) are time consuming.

The aim of this study was to develop a method utilizing high-performance liquid chromatography (HPLC) to resolve and separately determine R- and S-form components of endotoxin for studies in perfusate samples from isolated perfused rat livers. Although lipopolysaccharide molecules possess no useful absorbance or fluorescence properties, endotoxins labelled with the fluorochrome fluorescein isothiocyanate (FITC) [14] are commercially available, and have been used in studies of the binding of endotoxin to hepatocyte membranes [15]. Such fluorescent labelling could potentially prove suitable for the direct detection of small amounts of endotoxin following chromatography. HPLC assay would ideally provide resolution, and separate determination, of S- and R-form lipopolysaccharide based on the different physico-chemical properties of the lipopolysaccharide molecules. Additionally, HPLC might allow the detection of modifications occurring to the lipopolysaccharide molecules, and would require only small volumes of sample.

EXPERIMENTAL

Chemicals

The FITC–endotoxin was purchased from Sigma (St. Louis, MO, USA). It was prepared from *E. coli*, (Serotype 0111:B4) using phenol extraction, and labelled with FITC by conjugation to amine groups on the lipopolysaccharide (LPS) molecule [14]. The acetonitrile used in the mobile phase was of HPLC grade (Mallinckrodt Australia, Melbourne, Australia). All other chemicals were of analytical-reagent grade and obtained from either Sigma or Merck (BDH Chemicals, Melbourne, Australia).

Fluorescence spectrum of FITC–endotoxin

Excitation and emission scans were done on FITC–endotoxin dissolved in distilled, deionized water in a quartz cuvette (Tintometer, Salisbury, UK) using a Hitachi (Tokyo, Japan) Model 2000 spectrofluorimeter. To determine the effect of pH on fluorescence, the intensity of a 1 μg/ml solution of FITC–endotoxin was measured (excitation at 496 nm, emission at 517 nm) at pH 3.90, 5.05, 5.50, 6.55, 7.00, 7.29, 7.50, 8.50 and 9.47.

HPLC conditions and apparatus

Chromatography was carried out on a constant-flow high-pressure liquid chromatograph consisting of a Model 6000A solvent-delivery system, Model U6K injector, an RCSS Guard-Pak, a Model 481 variable-wavelength UV absorbance detector operating at 280 nm (all from Millipore–Waters, Milford, MA, USA) and a Model LS4 spectrofluorimeter (Perkin-Elmer, Beaconsfield, UK) operating with excitation at 496 nm and emission at 517 nm (excitation and emission slit widths 10 nm). The column used was a Waters Ultrahydrogel Linear (300 × 7.8 mm I.D.), packed with a gel of cross-linked hydroxylated polymethacrylate in a blend of pore sizes. The mobile phase used was 100 m*M* phosphate buffer

(pH 7.75) containing 20% (v/v) acetonitrile, at a flow-rate of either 0.2 or 0.4 ml/min. The signals from the detectors were collected and processed using Maxima Version 3.1 software (Dynamic Solutions, Millipore, Milford, MA, USA).

Column calibration

The Ultrahydrogel column was calibrated using a series of protein standards of defined molecular size (Pharmacia, Piscataway, NJ, USA). The following proteins were dissolved in 100 m*M* phosphate buffer at the indicated concentrations: thyroglobulin 18 mg/ml (Stokes radius = 85.0 Å), ferritin 1.5 mg/ml (Stokes radius = 61.0 Å), catalase 15.4 mg/ml (Stokes radius = 52.2 Å), aldolase 20 mg/ml (Stokes radius = 48.1 Å), albumin 24.4 mg/ml (Stokes radius = 35.5 Å), ovalbumin 18.4 mg/ml (Stokes radius = 30.5 Å), chymotrypsinogen A 19.7 mg/ml (Stokes radius = 20.9 Å) and ribonuclease A 19.8 mg/ml (Stokes radius = 16.4 Å). A 25-μl volume of each protein solution was injected on to the column and eluted at a flow-rate of 0.2 ml/min. Peaks were detected using the UV detector operating at 280 nm.

HPLC of FITC–endotoxin and sub-unit size determination

E. coli FITC–endotoxin dissolved in distilled, deionized water at a concentration of 53 μg/ml was spiked with a trace amount of free FITC dissolved in methanol. The solution was chromatographed under the same conditions as the standard proteins and the eluate was monitored for both fluorescence and absorbance activities. The retention times of the resulting fluorescence peaks were used to determine the relative molecular mass range of the resolved endotoxin components by comparison with the retention times of the protein standards. FITC–endotoxin from *Vibrio cholerae* and *Salmonella minnesota* bacteria (Sigma) were also chromatographed to compare endotoxins from different genera of bacteria.

Validation of HPLC method: LPS content of peaks determined by assay for 2-keto-deoxyoctonate

The *E. coli* LPS molecules comprising the endotoxin contain several molecules of the 3-deoxy sugar 2-keto-3-deoxyoctonate (KDO) in its core polysaccharide region [12]. To confirm that the fluorescence peaks detected following HPLC of the endotoxin contained LPS, the column eluate was collected and assayed for the presence of KDO. FITC–endotoxin (1.2 mg/ml in distilled, deionized water) was chromatographed at 0.2 ml/min and fractions of the column eluate were collected in siliconized glass tubes using a Frac-100 fraction collector (Pharmacia). Column fractions were assayed for KDO according to the method of Waravdekar and Saslaw [16], as modified by Cynkin and Ashwell [17]. Samples of the column eluate (1 ml) and a blank (mobile phase, 1 ml) were hydrolysed by the addition of 400 μl of 5 *M* H_2SO_4 and heating at 80–90°C for 30 min. After cooling to room temperature and addition of periodic acid (BDH Chemicals, Kilsyth, Victoria, Australia) (0.025 *M* in 0.06 *M* H_2SO_4, 500 μl), the solutions were mixed and heated at 55°C for exactly 25 min. After cooling to room temperature, sodium arsenite (Ajax Chemicals, Auburn, NSW, Australia) [2% (w/v) in 0.5 *M* HCl, 1 ml] was added. The samples were vortex mixed to discharge the transient iodine colour and 2-thiobarbituric acid (Sigma) [0.6% (w/v), pH 2.0, 3 ml] was added. The tubes were heated at 95°C for exactly 12 min for colour development. Extraction reagent consisting of amyl alcohol (BDH Chemicals)–12 *M* HCl (1:1, v/v) (3 ml) was added, the samples were vortex mixed vigorously and the two phases separated by centrifugation at 150 *g* for 15 min at 4°C. The absorbance of the top layer was measured at 534 nm using a Hitachi U 2000 double-beam spectrophotometer, zeroed against the blank.

Application of HPLC method: assay of FITC–endotoxin in isolated perfused rat liver perfusate

We assessed the usefulness of the form of chromatography described above in determining *E. coli* FITC–endotoxin using the isolated perfused rat liver (IPRL) preparation. In this preparation, samples are taken from a reservoir of perfusate which is recirculating through the isolated liver. The perfusate consists of 1% (w/v) bovine serum albumin and 0.1% (w/v) glucose in Krebs Henseleit hydrogencarbonate buffer. Standards of

FITC–endotoxin were prepared in such a perfusate at concentrations of 0, 1, 5, 10 and 15 μg/ml, together with replicates (10 μg/ml, $n = 6$) and controls (10 μg/ml in distilled, deionized water, $n = 3$). To separate the lipopolysaccharides from perfusate protein, the standards (1 ml) and replicates (1 ml) were extracted using the phenol–water method of Westphal and Jann [2], with a subsequent diethyl ether wash to remove all traces of phenol. The extracted samples were concentrated by evaporation to dryness under nitrogen at 50°C followed by reconstitution in 300 μl of distilled, deionized water.

Calibration graphs and assay validation

Chromatography was performed as described above using 100 μl of the standards, replicates and controls at a flow-rate of 0.4 ml/min, and the areas of each resolved LPS peak were calculated using Maxima software, Version 3.1. For each peak, a calibration graph was constructed of peak area *versus* standard concentration, using least-squares regression. The within-day accuracy of the assay was defined as the closeness of the assayed concentration of each LPS to its known concentration. This was calculated from the mean difference (absolute value) between the apparent (from the calibration graph) and nominal (10 μg/ml) concentration of each LPS in the six replicates, and is expressed as a percentage of the nominal concentration. Precision was defined as the standard deviation of the apparent concentrations about the mean, and was expressed as a percentage of the mean. The recovery of FITC–endotoxin following extraction was calculated by comparing the peak areas of the replicate samples of extracted FITC–endotoxin with those of the non-extracted controls.

RESULTS AND DISCUSSION

Fluorescence spectra of FITC–endotoxin

The FITC–endotoxin had an excitation maximum at 258 nm. However, excitation at this wavelength involved the production of significant Raman peak interference, overlapping the emission maximum of 517 nm. Excitation of the solution at 496 nm eliminated this interference while still producing significant excitation of the FITC molecules. A calibration graph of fluorescence intensity *versus* FITC–endotoxin concentration at an excitation wavelength of 496 nm and an emission wavelength of 517 nm was linear ($r^2 = 1.00$) at concentrations of FITC–endotoxin between 0.1 and 10 μg/ml (data not shown). These wavelengths were used for detection in subsequent chromatography.

A sigmoidal relationship existed between pH and fluorescence intensity of FITC–endotoxin (Fig. 1), an effect similar to that noted previously for FITC–asialoorosomucoid [18]. To maximize the emission intensity and thus increase the assay sensitivity, and also to minimize intensity fluctuations which could occur with only small changes in pH in the range 5.5–7.5, 100 m*M* phosphate buffer (pH 7.75) was used in the mobile phase for HPLC.

HPLC: calibration of column, size estimation and KDO content of resolved LPS peaks

Retention by the Ultrahydrogel column of the standard globular proteins best approached linearity when compared with the Stokes radius of the protein molecules ($r^2 = 0.98$) (Fig. 2) rather than relative molecular mass ($r^2 = 0.87$) or log (relative molecular mass) ($r^2 = 0.96$).

Chromatography of *E. coli* FITC–endotoxin separated four fluorescently active groups which are arbitrarily defined as LPS1, LPS2, LPS3 and LPS4 in order of increasing retention time and decreasing molecular size (Fig. 3). The results of the KDO assay on the column eluate were positive for LPS1, LPS2 and LPS4, confirming that the fluorescence peaks contained LPS. KDO of LPS2 appears as a prominent shouler on the peak

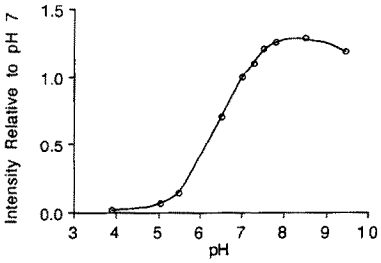

Fig. 1. Sigmoidal relationship found between fluorescence of FITC–endotoxin and solvent pH. Excitation wavelength = 496 nm; emission wavelength = 517 nm.

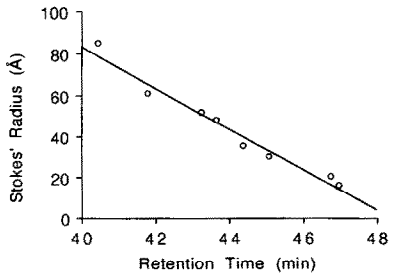

Fig. 2. Calibration graph for the Ultrahydrogel column, produced by chromatographing globular proteins with a range of accurately defined molecular sizes. A linear increase in retention time occurred with decreasing molecular size. Flow-rate = 0.2 ml/min. r^2 = 0.98.

of LPS1. LPS3 did not show KDO activity; however, as LPS3 represented only 0.6% of the total fluorescence area, the amount of KDO may have been below the detection limit of the KDO assay. The KDO assay further showed the absence of any significant amounts of non-fluorescently labelled LPS, and continuing the chromatography to 120 min (data not shown) demonstrated that there was no free FITC (with a retention time of 110.8 min) in the FITC–endotoxin preparation.

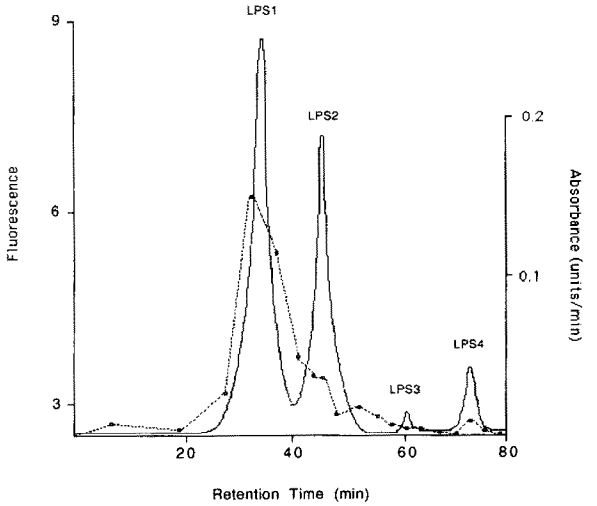

Fig. 3. Chromatogram of *E. coli* FITC–endotoxin run on an Ultrahydrogel column at a flow-rate of 0.2 ml/min. Fluorescence peaks are denoted LPS1, LPS2, LPS3, and LPS4 in order of increasing retention time and molecular size. The column eluate was assayed spectrophotometrically for the presence of KDO, indicated by absorbance at 534 nm. Solid line = fluorescence; broken line = absorbance.

Only LPS2 had a retention time within the range of the protein calibration graph, with an apparent Stokes radius of 24.6 Å (Table I). Estimation of the absolute size of the LPS sub-groups from the calibration graph should be considered approximate as evidence suggests that the endotoxin molecules from *E. coli* 0111:B4 are rod-like in shape [12], and thus may behave differently to globular proteins during passage through the column packing. However the retention data do show the LPS sub-groups comprising the endotoxin preparation have a wide range of molecular sizes.

Of the total fluorescence activity in the FITC–endotoxin preparation, LPS1 was the major component (67.1%) (Table I), followed by LPS2 (26.7%). These possibly correspond to the two physically and chemically distinct LPS components found by Morrison and Leive [12] following Sepharose 4B fractionation of phenol extracted *E coli* (0111:B4) endotoxin. Morrison and Leive calculated the range of relative molecular masses of the two components from sedimentation and diffusion coefficients to be 1 300 000–1 500 000 and 700 000–900 000. LPS3 and LPS4 were minor components of the preparation, together making up 7.1% of the total fluorescence activity (Table I). The small molecular size components are presumably R-forms of the endotoxin.

Fig. 4 shows that endotoxins isolated from *S. minnesota* and *V. cholerae* bacteria are, like that from *E. coli,* heterogeneous in composition, containing sub-groups of various relative molecular mass. The endotoxins isolated from the different genera of bacteria produce peaks with different retention times.

Extraction of endotoxin from IPRL perfusate

Separation of (lipo)polysaccharide from protein using phenol extraction is effected by the high partition coefficient and dielectric constant of the homogenous solution of perfusate and phenol at 68°C. On cooling to room temperature, the solution separates into a water phase containing the (lipo)polysaccharide and a phenol phase containing the protein [2]. Chromatography of phenol–diethyl ether-extracted perfusate spiked with FITC–endotoxin resulted in four fluorescent

TABLE I

RETENTION TIME, STOKES RADIUS AND AREA PERCENTAGE OF FITC-ENDOTOXIN COMPONENTS FOLLOWING ULTRAHYDROGEL CHROMATOGRAPHY AT A FLOW-RATE OF 0.2 ml/min

Parameter	Component				
	LPS1	LPS2	LPS3	LPS4	FITC
Retention time (min)	36.0	45.9	60.3	72.3	110.8
Stokes radius (Å)	>85	24.6	<16.4	<16.4	–
Area (% of total area)	67.1	26.7	0.6	5.5	0.0

peaks with retention times identical with those of the four peaks obtained after chromatography of non-extracted FITC–endotoxin, except for the LPS4 peak, which was absent following extraction. A small peak eluted just after LPS2 in extracted but not non-extracted FITC–endotoxin, and may be LPS4 modified in some way to decrease its retention time during the extraction procedure, or a component of LPS1, LPS2 or LPS4 which is resolved from its parent peak. An absorbance peak eluting coincident with the LPS1 fluorescence peak suggests that some material, absorbing light at 280 nm, may have been associated with LPS1 following extraction. Such an association did not alter the retention time of LPS1.

Assay validation

LPS1, LPS2 and LPS3 could be determined by measuring peak areas in extracted perfusate standards containing FITC–endotoxin over a concentration range of 1–15 μg/ml. The calibration graphs constructed by linear regression of peak area and concentration data were LPS1 (μg/ml) = 0.165 + 0.133(peak area), r^2 = 0.98; LPS2 (μg/ml) = 0.256 + 0.061(peak area), r^2 = 1.00; LPS3 (μg/ml) = 0.063 + 0.376(peak area), r^2 = 0.99. The accuracy and precison of the assay, calculated from replicate samples containing FITC–endotoxin at a concentration of 10 μg/ml, are shown in Table II. Despite the absence of an internal standard, the accuracy and precision of the assay are within acceptable limits. FITC–endotoxin of various relative molecular masses were tested as potential internal standards for the assay owing to the similarity of their structure to the polysaccharide moiety of the LPS molecule. However, none were suitable as they eluted coincident with or overlapping the LPS peaks.

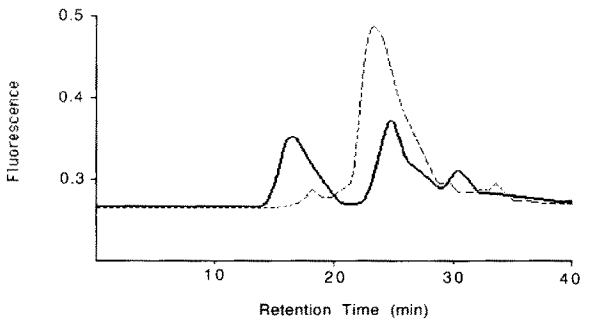

Fig. 4. Comparison of chromatograms of FITC–endotoxin from *V. cholerae* (solid line) and *S. minnesota* (broken line) run on the Ultrahydrogel column at a flow-rate of 0.4 ml/min.

TABLE II

ACCURACY AND PRECISION OF THE ASSAY AND RECOVERY OF FITC–ENDOTOXIN FOLLOWING EXTRACTION FROM IPRL PERFUSATE

Parameter	Component			
	LPS1	LPS2	LPS3	LPS4
Accuracy (%)	22.8	10.8	24.2	ND[b]
Precision (%)	12.7	15.9	25.0	ND
Recovery (%)	43.1	52.0	138.6[a]	ND

[a] The areas of the control and replicate peaks were not significantly different (Student's *t*-test, $p > 0.05$).

[b] ND = not detected.

CONCLUSION

The methods developed in this study allowed the HPLC resolution of a fluorescently labelled endotoxin preparation from *E. coli* Serotype 0111:B4 into four fluorescent sub-groups of varying molecular sizes. Following extraction from IPRL perfusate, three of the four sub-groups could be determined over the concentration range 1–15 μg/ml. The use of the Ultrahydrogel column and the fluorescently labelled endotoxin allowed microgram amounts of LPS to be detected in small samples (50–100 μl) using standard HPLC instrumentation.

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