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Analysis of a monophosphoryl lipid A immunostimulant preparation from *Salmonella minnesota* R595 by high-performance liquid chromatography

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Abstract

MPL® immunostimulant, a 4′-monophosphoryl lipid A (MLA) preparation obtained from the lipopolysaccharide of *Salmonella minnesota* R595, is being developed for several clinical indications. MPL comprises a mixture of MLA congeners that contain 4, 5, and 6 fatty acids. In this paper, we report a new high-performance liquid chromatography (HPLC) method for analyzing the congener composition and purity of MPL. MPL is first derivatized with dinitrobenzyloxyamine (DNBA), resulting in incorporation of the dinitrobenzyl chromophore at the reducing end of all MLA congeners. DNBA–MPL is then analyzed by reversed-phase HPLC on a Waters NovaPak C_{18}, 4 μm particle size, 300 mm×3.9 mm column. Optimal separation of DNBA–MLA species is obtained using a linear gradient of 10% to 80% isopropanol–water (95:5, v/v), 5 m*M* tetrabutylammonium dihydrogenphosphate (TBAP), in acetonitrile–water (95:5, v/v), 5 m*M* TBAP, over 45 min. A synthetic compound, corresponding to a hexaacyl MLA congener, is used for determination of the detector response factor, allowing the MLA content of MPL (i.e., purity) to be determined. Overall, this method provides better separation, higher sensitivity, and is faster and safer than previous methods used for the analysis of MPL.

Keywords: *Salmonella minnesota*; Derivatization, LC; Immunostimulants; Monophosphoryl lipid A; Disaccharides; Lipids

1. Introduction

MPL® immunostimulant is a 4′-monophosphoryl lipid A (MLA) preparation obtained by sequential acid and alkaline hydrolyses of lipopolysaccharide from *Salmonella minnesota* R595 [1]. MPL is being developed for use in several clinical indications, including prevention of septic shock [2], prevention of cardiac reperfusion injury following surgery-related ischemia [3], and as an adjuvant in therapeutic cancer vaccines and in prophylactic vaccines against several infectious diseases [1]. The successful clinical development of MPL for these indications requires the availability of validated assays to adequately characterize this product with respect to chemical composition and purity.

Chemically, MPL is a mixture of closely-related MLA species that all have the same disaccharide backbone, consisting of 4′-phospho-2′-deoxy-2′-aminoglucose-β-1′,6-2-deoxy-2-aminoglucose, but that differ in terms of their fatty acid substitutions at the 2, 2′, and 3′ positions [4,5]. The fatty acids may

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be either 3-(*R*)-hydroxytetradecanoic acid, tetradecenoic acid, or 3-(*R*)-acyloxytetradecanoic acid, in which case the acyl groups may be either dodecanoic acid, tetradecanoic acid, or hexadecanoic acid, depending on the backbone position. This heterogeneity is readily apparent by thin-layer chromatography (TLC) on silica gel, which reveals the presence of three bands of roughly equal intensity, corresponding to MLA congeners with four, five and six fatty acids (Fig. 1). All of these congener groups contribute to the biological activity of MPL [6]. Species with three and seven fatty acids may also be present at much lower levels and are regarded as impurities.

Because of the heterogeneous nature of MPL, it was necessary to develop an assay to determine both the composition with respect to the three major MLA congener groups and the total content of these congeners in MPL (i.e., purity). In the past, a combination of TLC and scanning densitometry was used to assess these parameters. However, the low resolution and poor reproducibility of the TLC technique makes validation of TLC-based assays problematic. Other methods of analyzing MPL include quantitation of amino sugars by colorimetric assay and high-performance liquid chromatography (HPLC), fatty acids by gas chromatography, and phosphorous by colorimetric assay [7–10]. These methods only quantify the constituents of MPL following degradation, and they provide no information on the congener composition of the intact MPL. It was therefore necessary to develop an alternative method of analyzing MPL.

Reversed-phase HPLC (RP-HPLC) is an attractive choice for the analysis of the congener composition in MPL, since retention on RP supports should be very sensitive to differences in fatty acid substitution. Past attempts to analyze MLA preparations with RP-HPLC have used either UV–Vis detection at 210 nm, where MLA is weakly absorbing, or radiodetection utilizing metabolically-labelled preparations

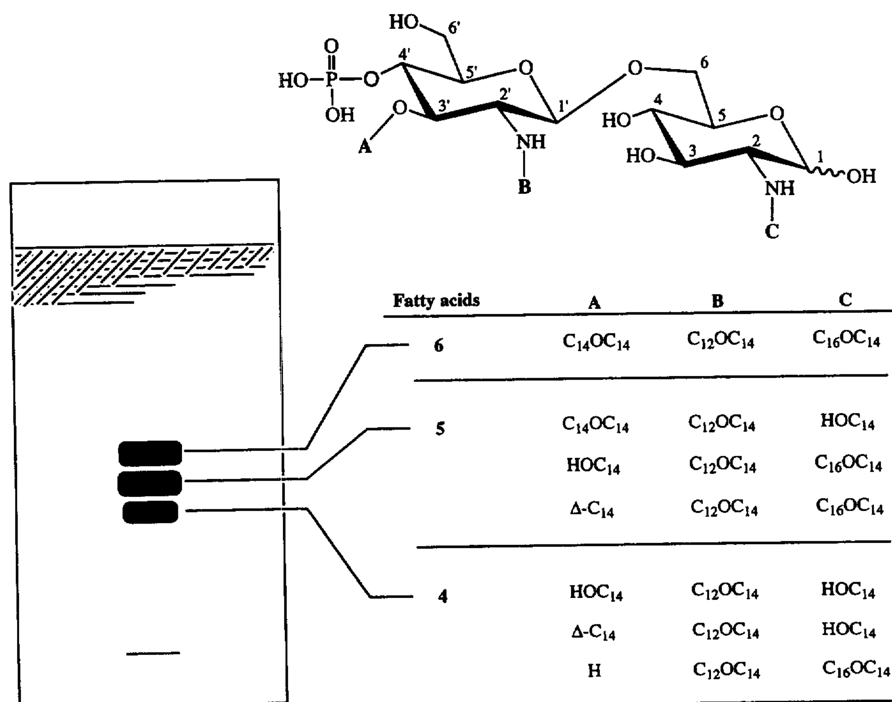

Fig. 1. Tentative structural assignments for the major MLA species present in MPL. Each TLC band contains MLA species with the same number of fatty acids. Details of the structural characterization study are given in the text. Abbreviations: HOC_{14}, (*R*)-3-hydroxytetradecanoyl; $C_{12}OC_{14}$, (*R*)-3-dodecanoyloxytetradecanoyl; $C_{14}OC_{14}$, (*R*)-3-tetradecanoyloxytetradecanoyl; $C_{16}OC_{14}$, (*R*)-3-hexadecanoyloxytetradecanoyl; Δ-C_{14}, tetradecenoyl.

[4,11]. However, UV–Vis detection of MLA is unattractive due to its low sensitivity and also because the different MLA species present in MPL may have different molar extinction coefficients. The use of radiolabelled material is unattractive for safety reasons and is obviously inappropriate for clinical materials. An additional complication in the use of RP-HPLC to analyze MPL is that the phosphomonoester group in MPL interferes with elution from RP columns. It is therefore necessary either to block the phosphate group, by treatment with diazomethane, for example, or to use an ion-pairing reagent [4,11].

In this paper, we present a method for the analysis of MPL by RP-HPLC that overcomes these difficulties by (1) derivatization of MPL with dinitrobenzyloxyamine [12], and (2) use of tetrabutylammonium dihydrogenphosphate as an ion-pairing reagent [11]. Furthermore, we report the findings from a study to validate this procedure as a USP Category I assay [13].

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile (MeCN), isopropanol (IPA), chloroform, methanol (MeOH) and tetrabutylammonium dihydrogenphosphate (TBAP) were obtained from Baker (Phillipsburg, NJ, USA). Anhydrous pyridine and 1-methyl-3-nitro-1-nitrosoguanidine were obtained from Aldrich (Milwaukee, WI, USA). Dinitrobenzyloxyamine hydrochloride (DNBA) was obtained from Regis (Morton Grove, IL, USA). MPL and synthetic MLA derivatives, designated S-X24, S-420, and S-444, were obtained from Ribi ImmunoChem Research (Hamilton, MT, USA). These derivatives contained four, five and six fatty acids, respectively.

2.2. Pre-column derivatization

Reaction of MPL with DNBA–pyridine at 60°C gave rise to the UV-active O-dinitrobenzyl oxime shown in Fig. 2 [12]. The optimum conditions for derivatization of MPL with DNBA were as follows (see Section 3): a 200 μl aliquot of a 10 mg/ml solution of DNBA in anhydrous pyridine was added to 1.0 mg MPL in a 4 ml screw-cap vial. The vial was tightly capped and then incubated at 60°C±1°C for 3 h in a heating block. Pyridine was removed by evaporation at 60°C±1°C under a stream of N_2 in a well-ventilated fume hood. The external standard was prepared using 100 μg S-444, treated in the same manner as MPL. Samples and external standard were dissolved in 200 μl $CHCl_3$–MeOH (2:1, v/v) prior to injection.

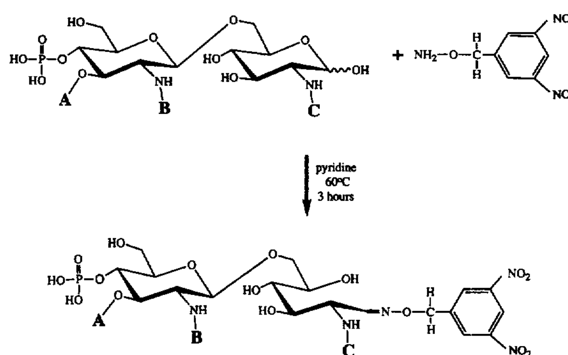

Fig. 2. Derivatization of MPL with DNBA. See Fig. 1 for details regarding the MPL structure. The stereochemistry of the O-dinitrobenzyl oxime has not been determined.

2.3. Chromatography

HPLC analyses were carried out with a Waters liquid chromatography system (Milford, MA, USA), comprising two 510 pumps, a 700 WISP autoinjector, a temperature control module (50°C), and a Waters 440 or 486 UV detector (254 nm). Waters Millennium software (v 2.0) was used for system control and data collection/processing. A NovaPak C_{18}, 4 μm particle size, 300 mm×3.9 mm stainless steel column, preceded by a Guard-Pak equipped with a C_{18} insert, was used in this study (Waters). The mobile phases were as follows: (A) 5 m*M* TBAP in MeCN–water (95:5, v/v), and (B) 5 m*M* TBAP in IPA–water (95:5, v/v). The column was equilibrated with 10% B prior to the start of each run, and the sample injection volumes were 10 μl. Sample was eluted with a linear gradient of 10% to 80% B over 45 min at a flow-rate of 1.0 ml/min. A sample prepared in the normal manner but without MPL was used as the reagent blank.

2.4. Fractionation of MPL

MPL was fractionated by normal-phase chromatography on a 30 cm×3 cm column of Bio-Sil HA silica gel (Bio-Rad, Richmond, CA, USA), using a linear gradient of 0% to 30% MeOH–water (95:5, v/v) in chloroform to elute the individual MPL TLC bands [14]. Purified TLC bands were further fractionated by first methylating the samples with diazomethane, then isolating individual components using a NovaPak 10 mm×8 cm C_{18} (4 μm particle size) radial compression cartridge (Waters) and a linear gradient of 20% to 80% IPA in MeCN over a period of 60 min at a flow-rate of 2 ml/min [15]. The runs were monitored at 210 nm, and fractions corresponding to the components of interest were pooled and evaporated.

2.5. Analysis of purified MPL components by fast atom bombardment mass spectrometry

HPLC-purified dimethyl MPL components were analyzed by positive ion fast atom bombardment (FAB) mass spectrometry [15]. Spectra were obtained on an AEI/Kratos mass spectrometer (Manchester, England), using 8 keV xenon atoms. The matrix consisted of dithiothreitol–dithioerythritol (3:1, v/v), and the instrument was calibrated by measurement of cesium iodide cluster ions over the m/z range 800–2300. Under these conditions, signals are observed for the parent ion as well as the oxonium ion fragment derived from the non-reducing end by cleavage of the glycosidic linkage.

2.6. Data analysis

The purity of MPL is defined here as the mass percent of MPL that is attributable to congener groups with four, five or six fatty acids. To calculate purity, it is first necessary to determine a theoretical weighted-average molecular mass for MPL ($M_{r,MPL}$) that is based on mol fractions and average molecular masses for congener groups with four, five and six fatty acids (see below). The theoretical mol/injection of MPL is then calculated using $M_{r,MPL}$, and the actual mol/injection is calculated using the response factor (R_F) determined with S-444. Finally, purity is calculated as the ratio of actual:theoretical mol/injection.

3. Results and discussion

3.1. Reaction of MPL with DNBA and analysis by HPLC

Analysis of DNBA-derivatized MPL by RP-HPLC on a C_{18} support, as described in Section 2, resulted in a chromatogram with three distinct clusters of peaks (Fig. 3A). The most highly retained peaks eluted between about 38 and 43 min, the intermediate cluster eluted between 27 and 35 min, and the least retained group fell between 14 and 22 min. This general pattern of peaks was observed with all production lots of MPL.

The external standard, S-444, gave a single peak that eluted at 41 min, corresponding to the most highly-retained group in the MPL pattern (Fig. 3B). Minor peaks also occurred at 31 and 20 min, presumably reflecting the presence of contaminating pentaacyl and tetraacyl species. These two minor

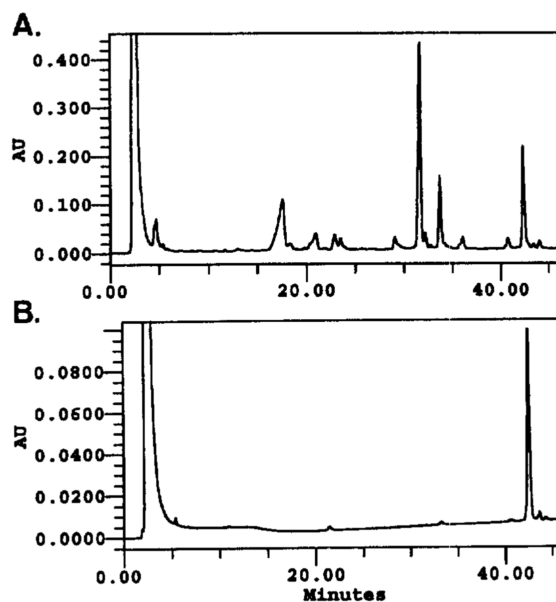

Fig. 3. HPLC chromatograms of (A) MPL and (B) the external standard, S-444. The HPLC conditions are given in Section 2.

peaks typically accounted for less than 5% of the total area eluting between 12 and 43 min.

The various MLA species present in MPL result from a combination of biosynthetic and process-related contributions [1,5]. Biosynthetic contributions arise from variations in the extent of acylation of lipid A by *S. minnesota* under different growth conditions [16], and presumably also reflect the variable substrate specificities of the acyl transferases responsible for addition of the normal chain fatty acids to the nascent lipid A structure [17]. The manufacturing process for MPL also contributes to the heterogeneity due to loss of ester-linked fatty acids during the acid and, to a lesser extent, alkaline hydrolytic steps. For example, exposure of the external standard shown in Fig. 3B to the hydrolytic conditions used in the manufacturing process resulted in generation of a pattern of peaks similar to that shown in Fig. 3A (data not shown).

It is not known if the O-dinitrobenzyl oxime formed by the reaction of MPL with DNBA has *cis*, *trans*, or mixed stereochemistry (Fig. 2). Analysis of DNBA–S-444 by HPLC revealed only one major peak, consistent with either the generation of a single stereoisomer or lack of resolution of the *cis* and *trans* isomers (Fig. 3B). The smaller peaks that occurred immediately before and after the major peak in Fig. 3B were also observed in RP-HPLC chromatograms of S-444 that had been derivatized at the 4′-phosphate by reaction with diazomethane. It is therefore unlikely that any of these peaks arose from a different stereoisomer of the oxime.

The limit of detection (LOD) of MPL under these conditions was estimated from analysis of the baseline noise present in a chromatogram for S-444, with the assumption that a signal-to-noise ratio of >3 is required for detection of signal. An injection of 5 μg S-444 gave a peak intensity of 0.089 AU, and the baseline noise in this chromatogram had an amplitude of $\leq 4 \cdot 10^{-5}$ AU. It is therefore necessary to inject 7 ng of S-444 in order to obtain a signal that is three-fold higher than baseline noise. Since the molecular mass of S-444 is 1920 g/mol, the LOD for detection of a single component by this assay is $3.6 \cdot 10^{-12}$ mol. Assuming that MPL has an effective molecular mass of 1500 g/mol (see below), it would be necessary to inject 540 ng ($3.6 \cdot 10^{-10}$ mol) in order to detect a component present at a level of 1 mol%.

3.2. Optimization of reaction conditions

The conditions for derivatization of MPL were optimized with respect to both DNBA concentration and reaction time (Fig. 4). Identical 1 mg samples of MPL were first derivatized with varying levels of DNBA for 3 h, and the resulting preparations were analyzed by HPLC as described in Section 2. The levels of DNBA incorporation were estimated from the total area counts eluting between 12.1 and 43.0 min, i.e., the region in which all MPL-derived peaks occurred. The level of DNBA incorporation reached a plateau at 10 mg/ml DNBA. Assuming an effec-

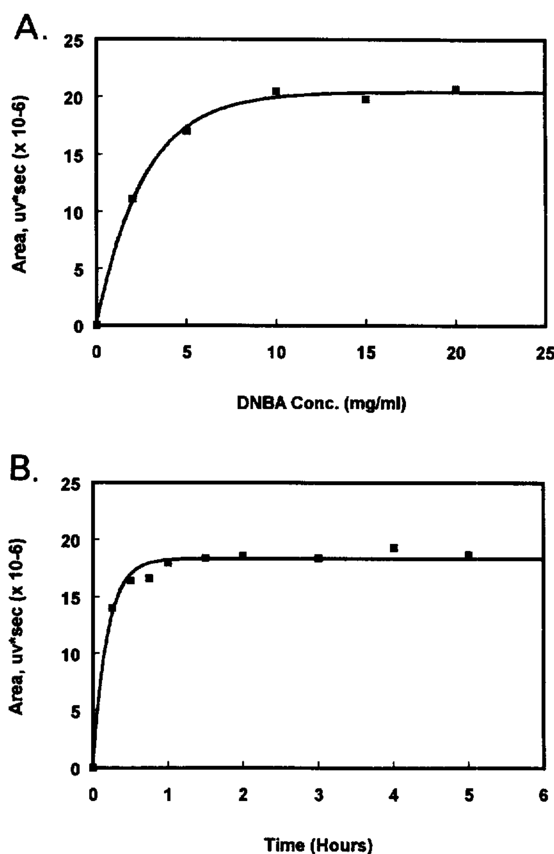

Fig. 4. Optimization of reaction conditions for DNBA derivatization of MPL. Total area counts occurring between 12.1 and 43.0 min are plotted against (A) DNBA concentration and (B) reaction time.

tive molecular mass of 1500 g/mol for MPL (see below), this concentration of DNBA provided for a fourteen-fold molar excess.

The time required for optimum derivatization was determined by incubating samples of MPL (1 mg) and DNBA (10 mg/ml) for varying times, and then analyzing the samples as described above. The reaction reached completion between 1–2 h (Fig. 4B). Based on these results, a reaction time of 3 h and a DNBA concentration of 10 mg/ml were adopted as the standard conditions for all subsequent analyses.

3.3. Correspondence between TLC and HPLC

The correspondence between TLC bands and HPLC peaks for MPL was established by HPLC analysis of isolated TLC bands. MPL was first fractionated by normal-phase chromatography on silica gel to yield pure fractions corresponding to the major TLC bands (R_F=0.25, 0.30, 0.37). These fractions were then derivatized with DNBA and analyzed as described in Section 2 (Fig. 5). The components present in the upper, middle, and lower TLC fractions eluted exclusively in the late, middle, and early peak groups, respectively. RP-HPLC and TLC therefore produce identical groupings of the MLA components present in MPL.

The small amount of overlap observed between the fractions in Fig. 5 is probably attributable to the inefficiency of the normal-phase chromatographic fractionation procedure, since a significant degree of tailing was observed with the middle and lower TLC fractions. Likewise, the differences in relative peak intensities between unfractionated MPL and the purified fractions reflects disproportionate loss of certain components in the cross-over fractions, which were not included in the main pools.

The nature of the MLA components within each peak group was established in two ways. First, the major components in each peak group were isolated as their dimethyl derivatives by RP-HPLC and analyzed by positive ion FAB mass spectrometry [15]. Three components were obtained from the early group, three from the middle group, and one from the late group. The FAB mass spectrum for the major component in the late peak group is shown in Fig. 6. In this spectrum, the peak at m/z 1781

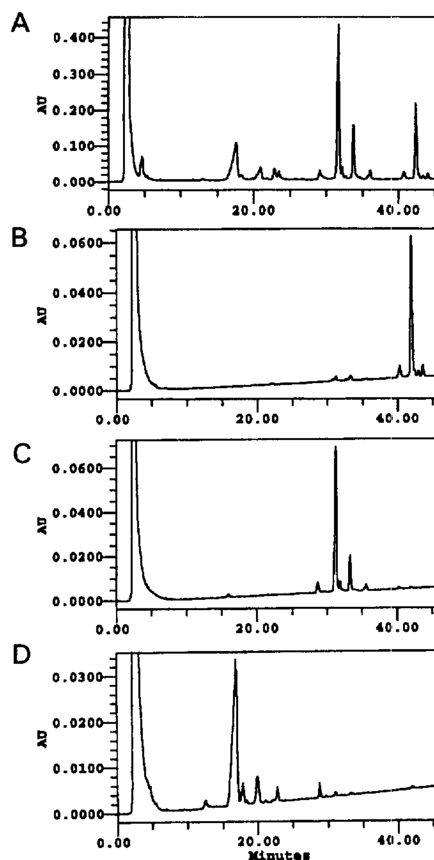

Fig. 5. HPLC analysis of the major TLC bands in MPL, using the HPLC method described in Section 2. The samples were prepared by fractionation of MPL using normal-phase chromatography on silica gel, as described in Section 2. The parent MPL is shown in (A), while the upper (R_F=0.37), middle (R_F=0.30), and lower (R_F=0.25) TLC fractions are shown in (B), (C), and (D), respectively.

corresponds to the sodium adduct of the parent ion (M_r=1758), and the signal at m/z 1115 is due to the oxonium ion fragment derived from the non-reducing end. The identity of the m/z 1781 peak as ($M+Na^+$) was confirmed by running this sample in a rubidium-spiked matrix, which resulted in a peak at m/z 1843 (data not shown). The peak at m/z 915 arises from loss of dodecanoic acid from the oxonium ion fragment. Similar spectra were obtained for the other components, and the results are summarized in Table 1. The numbers and distributions of fatty acids in each component were deduced from the masses for the parent ions and the oxonium ion fragments, taken

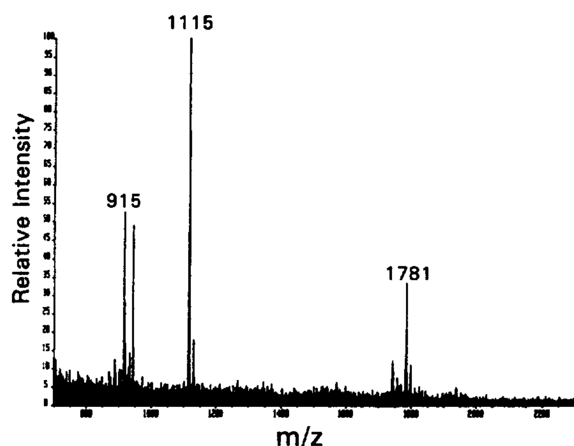

Fig. 6. Positive ion FAB mass spectrum of the dimethyl derivative of the major component isolated from the late-eluting congener group.

together with the structure determined previously for acid-hydrolyzed MLA from *S. minnesota* R595 [4]. This analysis showed that the components present in the early-, middle-, and late-eluting congener groups contained four, five and six fatty acids, respectively (Fig. 1).

The second approach involved performing a series of HPLC analyses on MPL spiked with synthetic MLA derivatives that contained four, five or six fatty acids. The derivatives designated S-426 and S-420 corresponded to the structures proposed above for components in the late- and middle-eluting congener groups, respectively, and these derivatives eluted coincidentally with the expected peaks (Fig. 7). S-X24 differed from one of the proposed tetraacyl components only in terms of having a C_{14} instead of a C_{16} fatty acid at one position, and it eluted slightly ahead of the corresponding peak in the early group (Fig. 7). Taken together, these experiments demonstrated that the early-, middle-, and late-eluting peak groups in the HPLC chromatogram for MPL contained tetraacyl, pentaacyl, and hexaacyl MLA congeners, respectively.

Table 1
Molecular masses for parent ions (i.e., [M+Na^{+}]) and non-reducing end oxonium ions for components isolated from early, middle, and late peak groups

Peak group	(M+Na^{+}) (*m*/*z*)	Oxonium ion (*m*/*z*)
Late	1781	1115
	1542	1115
Middle	1570	905
	1552	886
	1332	904
Early	1314	886
	1344	678

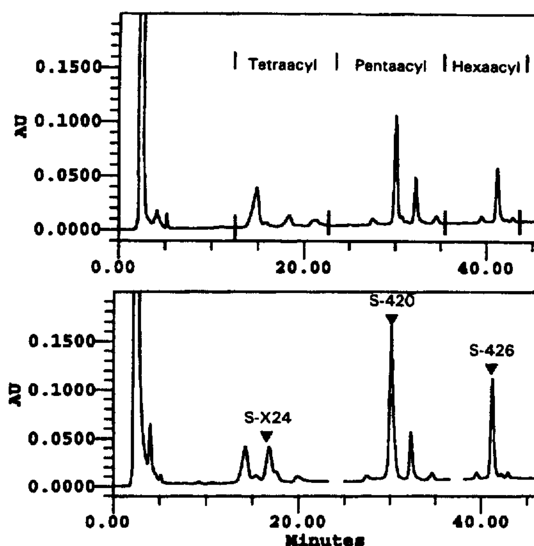

Fig. 7. Results of spiking studies with synthetic MLA derivatives. Synthetic derivatives (100 μg) were combined with MPL (1 mg), and the samples were analyzed as described in Section 2. Separate spiked samples were prepared for each synthetic derivative: (A) HPLC chromatogram of parent MPL; (B) chromatograms of spiked samples. Only the regions of the HPLC chromatograms corresponding to the relevant congener groups are shown in (B). The position of each derivative peak is indicated with an arrow. Peak groupings used for estimation of the congener composition in MPL are shown in (A).

3.4. Assessment of MPL composition and purity by HPLC

In order to determine the congener composition and purity of MPL using HPLC, it was first necessary to ascertain if the tetraacyl, pentaacyl, and hexaacyl MLA congener groups had equal molar R_F values (i.e., area counts per mole; see below). This was done using the synthetic derivatives discussed earlier. As shown in Table 2, these derivatives gave essentially equal R_F values, regardless of their fatty

Table 2
Comparison of molar response factors for synthetic MLA compounds with four, five and six fatty acids

Expt.	Response factor ($\times 10^{15}$ counts/mol)		
	Tetraacyl	Pentaacyl	Hexaacyl
1	1.55	1.40	1.70
2	2.10	2.35	1.45
3	1.95	1.35	1.45
4	1.85	1.90	1.75
Mean	1.86	1.75	1.59
S.D.	0.23	0.47	0.14
R.S.D. (%)	12.4	26.9	8.8

acid contents. The slight trend towards higher R_F values in going from hexaacyl to tetraacyl species was not significant as determined by analysis of variance (ANOVA, $\alpha=0.05$). Equal R_F values were also observed with the purified MLA congener fractions. In this case, it was first necessary to determine the effective molecular masses for each congener fraction, so that the mol/injection could be calculated. This was done by determining the phosphorous content (μmol/mg) for each congener fraction [10], with the assumption that all phosphorous was derived from MLA. By combining mol/injection and area counts/injection, the R_F values for the tetraacyl, pentaacyl, and hexaacyl congener groups were calculated to be $5.64 \cdot 10^5$, $4.98 \cdot 10^5$, and $5.47 \cdot 10^5$ area counts/nmol, respectively. These results indicated that the R_F values for the three major congener groups in MPL are not significantly different from each other. This in turn suggests that the molar extinction coefficient for the dinitrobenzyl chromophore is not influenced by structural variations in the MLA portion of the DNBA–MLA adducts or by differences in solvent composition in the IPA–MeCN elution gradient. This latter point was further supported by the observation that the absorption spectrum of DNBA–MLA did not change in solutions comprising different ratios of solvents A and B [18].

It was therefore possible to determine the relative molar amounts of the tetraacyl, pentaacyl, and hexaacyl congener groups directly from the area counts occurring in the appropriate regions in the chromatogram. This was done by first dividing the HPLC chromatogram into three regions, corresponding to tetraacyl (12.1–23.0 min), pentaacyl (23.1–36.0 min), and hexaacyl (36.1–43.0 min) congener groups (Fig. 7). The mol fraction of each congener class was then calculated by dividing the area counts for the group by the total area counts occurring between 12.1 and 43.0 min. Analyzed in this way, the MPL shown in Fig. 7 contained 18 mol% hexaacyl, 53 mol% pentaacyl, and 29 mol% tetraacyl congeners.

The purity of the MPL shown in Fig. 7 was calculated as described in Section 2. The $M_{r,MPL}$ for this MPL was calculated to be 1476 g/mol, using the mol fractions determined above and weighted-average molecular masses for the tetraacyl, pentaacyl, and hexaacyl congener groups of 1271 g/mol, 1504 g/mol, and 1724 g/mol, respectively. The theoretical moles of MPL injected in 50 μg, assuming that all mass was contributed by active MLA congeners (i.e., tetraacyl, pentaacyl, and hexaacyl), was therefore $3.38 \cdot 10^{-8}$ mol. The R_F, determined with S-444 ($M_r=1920$ g/mol), was $1.558 \cdot 10^{-15}$ mol/count. Since the total area counts occurring between 12.1–43.0 min for this MPL was 21 594 643 counts, the actual moles of active MLA congeners was $3.36 \cdot 10^{-8}$ mol. The ratio of actual:theoretical mol gave a value of 99% for the purity.

3.5. Method validation

The method was validated according to the requirements for a USP Category I assay, i.e., an assay for quantitation of a major component in a drug product [13]. Thus, the following attributes of the assay were assessed with the indicated results:

3.5.1. Precision

The assay was performed on five different days by two operators using one lot of MPL at 100% of the target sample size (i.e., 1.0 mg sample). A relative standard deviation [R.S.D., (standard deviation/mean)×100) of less than 10% was obtained for each operator.

3.5.2. Ruggedness

The results obtained for precision for the two operators were also compared using Student's t-test ($\alpha=0.05$). No difference was found between the operators.

3.5.3. Accuracy

In five assays, MPL at the 100% level was spiked with a known amount of S-444. The recoveries of both S-444 and MPL gave R.S.D.s<10%, and the amounts were not significantly different than expected, based on a Student's t-test (α=0.05).

3.5.4. Linearity and range

MPL was analyzed at the 50%, 100%, and 150% levels (0.5 mg, 1.0 mg, and 1.5 mg, respectively), and the results were compared by ANOVA (α= 0.05). No significant differences were found between the levels. A linear regression analysis of the three MPL levels gave r^2=0.999.

3.5.5. Selectivity

Injection of reagent blanks showed no integratable peaks in the MPL region of the chromatograms.

This assay therefore satisfied the requirements for validation as set forth in the USP for a Class I assay [13]. Furthermore, the derivatization and HPLC analysis procedures are simple to perform and can be set up in a short amount of time, making this assay well-suited to a quality control environment. As such, this assay will be useful in the development of MPL as an immunostimulant for use in humans.

Acknowledgments

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