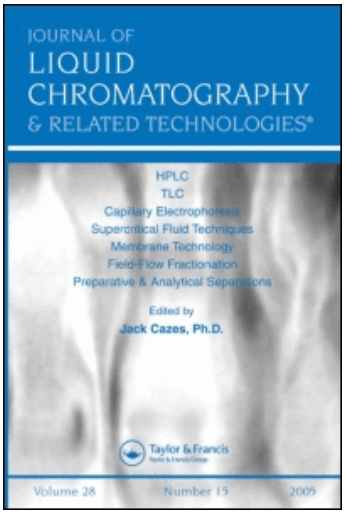

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REMOVAL OF THE NONIONIC DETERGENT EMULGEN 911 FROM SOLUBILIZED MICROSOMES BY HPLC

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ABSTRACT

The removal of a nonionic detergent, Emulgen 911, by HPLC from solubilized microsomes is described. Microsomal samples were dissolved in phosphate buffer containing sodium cholate and tritiated Emulgen 911. The HPLC column was eluted with phosphate buffer containing 20% (v/v) glycerol, sodium cholate and, for one set of conditions with Lubrol WX. The detergent, Emulgen 911, when eluted with sodium cholate, binds tightly to the column eluting after the inclusion volume. Elution in the presence of Lubrol WX resulted in decreased retention, with Emulgen 911 eluting within the inclusion volume. Microsomal samples chromatographed with sodium cholate are resolved into two peaks. The first eluting peak is associated with the cytochrome P-450 fraction. Effective detergent removal under these conditions was 88% of the eluted radioactivity. For purified cytochrome $P\text{-}450_1$ detergent removal was 99% efficient and provided a sample with a ratio of 2.5 microgram detergent per nanomole of P-450. Elution with Lubrol WX did not provide a full quantitative picture because of overlapping protein and detergent peaks but it provided evidence for strong hydrophobic interactions between the bonded phase and Emulgen 911, and between the two nonionic detergents.

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INTRODUCTION

Proteins which are an integral parts of membranes require solubilization by use of detergents prior to any purification step (1). Protein constituents of the endoplasmic reticulum are solubilized by means of nonionic/or ionic detergents. A combination of non-ionic and ionic detergents has been successfully used for the solubilization of microsomal preparations prior to purification (2). One of the most frequently used combinations in the purification of cytochrome P-450 is sodium cholate and Emulgen 911. The latter is a nonionic surfactant consisting of a mixture of polyoxyethylene nonylphenyl ethers (3). The presence of detergents in the purified protein samples represents a serious problem due to potential inhibition of enzymatic activity and slow denaturation of the enzyme (2,4). These effects are associated with both types of detergents but removal of nonionic detergents from solubilized proteins is comparatively more difficult than removal of ionic detergents (1,2). Consequently, it is worthwhile to devise efficient methods for removal of nonionic detergents from protein samples. A frequently used method for removal of Emulgen 911 involves the use of XAD-2 resin; by sequential batch treatments the concentration of detergent is lowered to acceptable levels. The use of Sephadex LH-20 has been reported for removal of Lubrol WX (5). Application of this latter procedure to Emulgen 911 is not as efficient, with an optimal 60% effective removal in a slow process (2). A recently

published procedure involving chromatography in hydroxylapatite (6) has become the most widely used method for removal of nonionic detergents. In the present communication we explore the removal of Emulgen 911 from rat liver microsomes by HPLC. This technique has been recently used to fractionate cytochrome P-450 samples either on ion exchange (7) or size exclusion columns (8).

MATERIALS AND METHODS

Charles River CD strain male rats were used in the present study. A sample of [^{3}H]-Emulgen 911 was generously provided by Mr. Wayne Levin, Hoffman LaRoche, Nutley, NJ. No specific activity was given for the tritiated detergent, a neat aliquot of 1 µl represented ca. 10^6 cpm and appropriate dilutions were made from this stock. A sample of highly purified cytochrome P-450$_1$ from rabbit lung was provided by Dr. Richard M. Philpot, Laboratory of Pharmacology, NIEHS, Research Triangle Park, N.C.

SOLUBILIZATION OF MICROSOMES

Rat liver microsomes were solubilized at room temperature in 100 mM potassium phosphate buffer, pH 7.5, containing 0.5% sodium cholate and 0.1% Emulgen 911 according to literature procedures (9). For the experiments with [^{3}H]-Emulgen 911, 2 ml of protein solution (10 mg protein/ml) was diluted with 2 ml of radiolabeled buffer and equilibrated at room temperature for 1 hr. The final activity of the solubilized microsomes was 102 408 cpm/10 µl at a protein concentration of 5 mg/ml. From this solu-

tion, aliquots of 250 µl (250 µg Emulgen, 1.25 mg protein) were injected into the HPLC column.

CHROMATOGRAPHIC CONDITIONS

The eluents used were: buffer A, 100 mM potassium phosphate (pH 7.5) buffer solution containing 0.5% sodium cholate, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 20% (v/v) glycerol; buffer B, as above with the addition of 0.2% Lubrol WX. A control (blank) sample of [^{3}H]-Emulgen 911 buffer (20 µl, ca. 95 547 dpm) was run under each set conditions. The equipment consisted of a M6000A pump, U6K injector, model 440 dual-channel UV absorbance detector, and a model 730 data module (all from Waters Associates, Inc., Milford, MA 01757). Peak absorbance was monitored simultaneously at 280 nm and 405 nm. The column used was a Spherogel TSK-3000SW (0.75 x 60 cm, Altex-Beckman). Samples were eluted isocratically at a flow rate of 1 ml/min. In all cases fractions were collected between 8 and 60 minutes. Instagel (6 ml) was mixed with the fractions and counting was done in a Packard Tricarb 460 CD liquid scintillation counter.

HPLC of [^{3}H]-Emulgen 911. a) Elution with 0.5% sodium cholate/Lubrol WX. An aliquot of a 0.1% solution of tritiated Emulgen 911 buffer (20 µl, 123 261 dpm) was chromatographed as described above. Fractions (1 ml) were collected from 8 to 60 min. The column was subsequently eluted with water (2 x 30 ml) and methanol (2 x 30 ml); aliquots (1 ml) of these fractions were taken for scintillation counting. Total recovery of radioac-

tivity from the column was 94%. The distribution of label among the fractions was: sodium cholate/Lubrol WX buffer (91.2%), water (6.4%), and methanol (2.3%). b) Elution with 0.5% sodium cholate. As described above, radiolabeled Emulgen 911 (20 µl, 95 547 dpm) was chromatographed using sodium cholate as the only detergent component in the buffer. Total recovery of radioactivity was 94%. The distribution of label was: sodium cholate buffer (51%), water (23%), and methanol (26%).

HPLC of cytochrome $P\text{-}450_1$ Solubilized cytochrome $P\text{-}450_1$ (0.8 nmol heme) in labeled 0.05% Emulgen 911 buffer (220 µl, 120 µg Emulgen 911, 71 142 dpm) was chromatographed as above with 0.5% sodium cholate buffer. Total recovery of radioactivity was 96%. The distribution of label was: sodium cholate buffer (73%), water (13%), and methanol (14%). Fractions 5-22 (10 to 19 min) were associated with cytochrome $P\text{-}450_1$ (determined by absorbance at 405 nm) and contained 0.96% of total eluted radiolabeled detergent.

RESULTS AND DISCUSSION

A preliminary indication of the potential application of HPLC for removal of nonionic detergents was obtained during the analysis of cytochrome P-450 fractions from a DE-52 column under conditions previously described (8). A late eluting peak with strong absorbance at 280 nm, too intense in relation to the amount of protein injected, was observed. (Fig. 1). This peak was identified as Emulgen 911 by comparing retention characteristics and

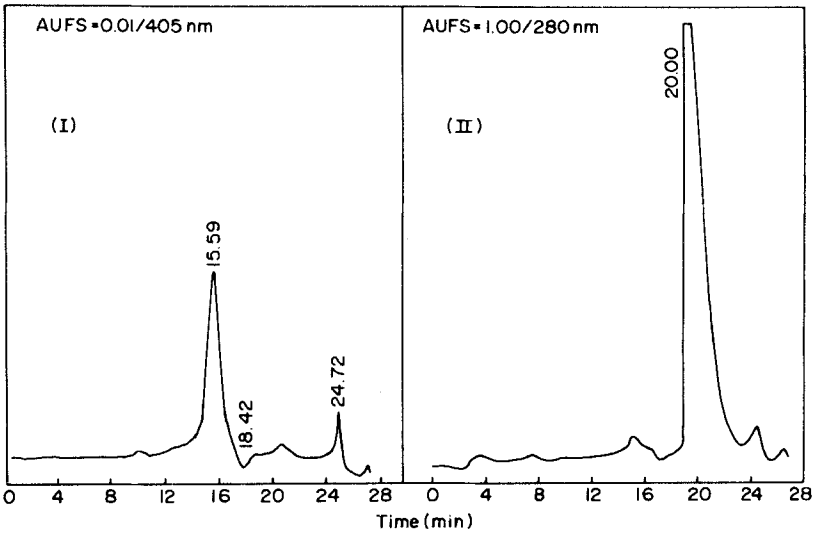

Figure 1. Partially purified cytochrome P-450 from a DE-52 column chromatographed on a Spherogel TSK-3000SW column eluted at 1 ml/min with 0.1M phosphate (pH 7.5) containing 0.2% Lubrol WX and 0.5% sodium cholate. Trace I, absorbance at 405 nm, Trace II, absorbance at 280 nm.

coelution on HPLC with an authentic standard. This particular sample of cytochrome P-450 from the ion-exchange column (DE-52) had been treated with Amberlite XAD-2 for a period of one hour following literature procedures (2). Elution of the HPLC column with a Lubrol WX-sodium cholate combination (8) allowed monitoring of the separation at 280 nm. At this point it became clear that the HPLC column was efficient in removing tightly bound detergent and that this approach could have a useful complementary role in the final stages of protein purification.

For the removal of Emulgen 911, two sets of conditions were explored. First, elution with a buffer containing 0.5% sodium

cholate and, second, elution with a buffer containing 0.2% Lubrol WX and 0.5% sodium cholate. The removal of detergent from the protein fractions was quantitated by incorporation of [^{3}H]-Emulgen 911 in the detergent mixture used to solubilize the microsomes. Because of our particular interest in the behavior of cytochrome P-450 under HPLC conditions, the profiles of the Emulgen 911 solubilized microsomal samples used in the experiments is shown in Fig. 2. The number 1 identifies the peak associated with the major cytochrome P-450 fraction. It is interesting to observe that in the absence of a nonionic detergent in the buffer eluent the cytochrome P-450 proteins aggregate readily and elute near the void volume (10-13 min, Fig. 2). The cytochrome P-450 is not denatured under these conditions, and, as in the case of elution with Lubrol WX, the isolated fraction from HPLC provided a carbon monoxide binding difference spectrum after reduction with sodium dithionite. The elution profile with Lubrol WX was as previously described (8), the cytochrome P-420 elutes near the exclusion volume (10-13 min), and the major cytochrome P-450 fraction elutes between 12-18 min peaking at 16 min (Fig. 2).

The elution profiles, radioactive and 280 nm absorbance, obtained by chromatography of the [^{3}H]-Emulgen 911 buffer are shown in Fig. 3A. Three radioactive peaks designated as A, B, and C are distinguishable. The distribution of radioactivity among these peaks is shown in Table 1. Peak A eluted at the

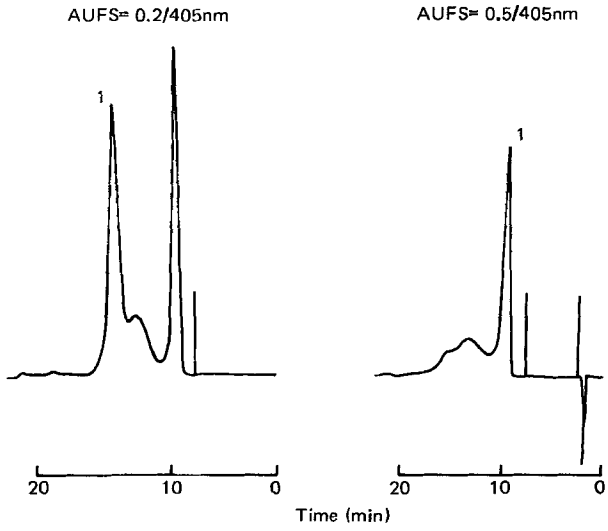

Figure 2. HPLC profile for the microsomal sample in the presence (right-hand trace) and absence (left-hand trace) of the nonionic detergent Lubrol WX. The number 1 identifies the fraction corresponding to cytochrome P-450.

inclusion volume (25 ml) as determined with tritiated water. Peak B is a broad, disperse band, and peak C is highly retained and eluted well beyond the inclusion volume. Peak C contained 44% of the eluted radioactivity and was associated with the major absorbance peak at 280 nm. Obviously, the mechanism of retention of Emulgen 911 in this column is not based exclusively on size, perhaps involving hydrophobic interactions with the bonded phase. It was also clear that the labeled detergent was not strictly pure; however, since other studies have been conducted with [^{3}H]-Emulgen 911 from the same source, a comparison would be possible only by using the original detergent sample (2,10).

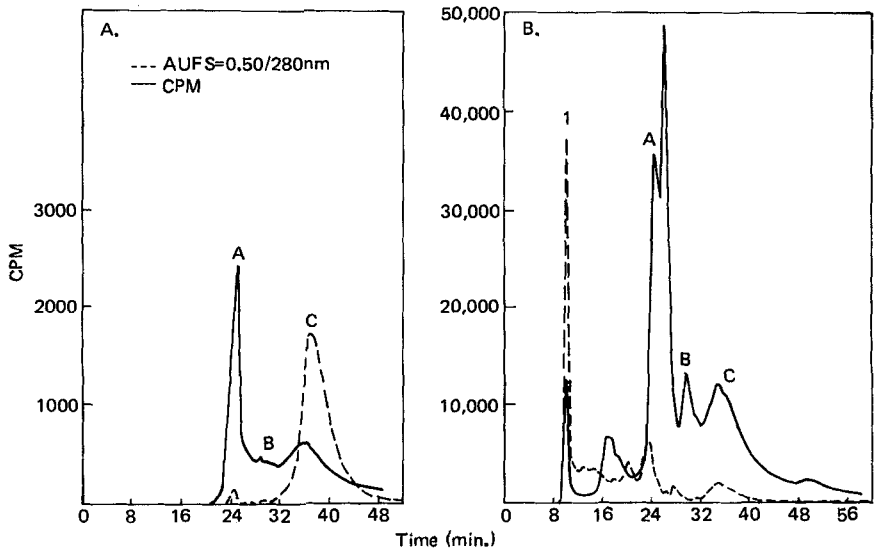

Figure 3. HPLC profile for samples eluted with buffer/0.5% sodium cholate. Trace A, [^{3}H]-Emulgen 911 buffer solution. Trace B, microsomal sample solubilized in [^{3}H]-Emulgen 911 buffer. The number 1 designates the cytochrome P-450 fraction.

The elution profile obtained from a sample of rat liver microsomes solubilized in [^{3}H]-Emulgen 911 and eluted with 0.5% cholate buffer is shown in Fig. 4B. Two distinct areas were established for the protein sample. Peak 1, from 10 to 13 min., corresponds to the major cytochrome P-450 peak (labeled 1, in Fig. 3B) as determined by absorbance at 405 mm (Fig. 2); peak 2, 13-16 min, contains other proteins with lesser amounts of heme proteins, including cytochrome b_5. As shown in Table 1, the total amount of radioactivity emerging in the protein fractions was 12% of the recovered radioactivity. By examining the amounts

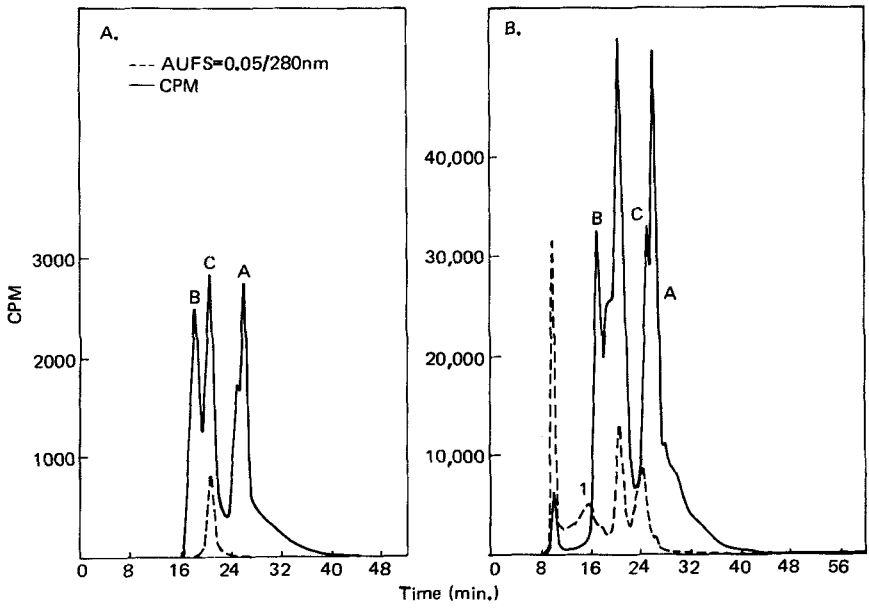

Figure 4. HPLC profile for samples eluted with buffer/0.5% sodium cholate 0.2% Lubrol WX. Trace A, [^{3}H]-Emulgen 911 buffer solution; Trace B, microsomal sample solubilized in [^{3}H]-Emulgen 911 buffer. The number 1 designates the cytochrome P-450 fraction.

recovered for the detergent peaks (Table 1), peak C shows a significant decrease, relative to blank, in cpm content. Peak C is the major detergent component observable by UV and it is tempting to speculate that the detergent eluting in the protein fractions is derived largely from this particular pool. As a whole, 88% of the [^{3}H]-Emulgen 911 is removed in one pass through the column; if we consider only the cytochrome P-450 fraction (peak 1), close to 96% removal is achieved. The efficiency of this approach to the removal of large amounts of nonionic detergents may be best visualized by considering the effective

Table 1

Distribution of Radioactivity Obtained by Chromatography of [^{3}H]-Emulgen 911 and Microsomes Solubilized in [^{3}H]-Emulgen 911

Sample	Eluent Buffer	Relative Distribution (%) Of Recovered Radioactivity				
	Peak:	1	2	A	B	C
Blank	A	-	-	41.4	14.4	44.2
Microsomes	A	4.2[a]	8.0[b]	40.2	12.0	35.7
Blank	B	-	-	43.4	25.3	31.3
Microsomes	B	2.1	-	47.0	13.7	37.3

[a]Fraction collected between 10-13 min, Figures 2 and 3B.

[b]Fraction collected between 13-16 min, Figures 2 and 3B.

[c]Fraction collected between 10-13 min, Figures 2 and 4B.

load of detergent and the ratio of detergent to protein before and after HPLC. For the microsomal sample, the initial ratio was 1 µg detergent/5µg protein; the recovered fraction contained 12% (30 µg) of the original detergent load and, assuming 70% as an average protein recovery (7,8), a final ratio of 1 µg detergent/30 µg protein.

This remaining 4% [^{3}H]-Emulgen 911 cannot be considered as representative of the extent of binding of this detergent to cytochrome P-450 since there are numerous other proteins eluting in the same fraction. This aspect was investigated with purified cytochrome P-450$_1$ from rabbit lung. The cytochrome P-450 (0.8 nmol) in 0.05% [^{3}H]-Emulgen 911 (220 µl, 120 µg detergent) gave

after HPLC, using cholate buffer, a solution containing heme protein (0.48 nmol by UV analysis (6)) and [^{3}H]-Emulgen 911 (1% of recovered label, 1.2 μg) for a final ratio of 2.5 μg Emulgen 911/nmol P-450. For Emulgen 911 the average molecular weight is ca. 900 (3) which indicates that after HPLC 2.7 nmol of detergent remain bound to one nmol of cytochrome P-450.

The elution profile of [^{3}H]-Emulgen 911 when Lubrol WX is incorporated in the buffer (buffer B) is shown in Fig. 4A. As with buffer A (Fig. 3A), three radioactive peaks are discernible, except that now all components are eluted within the inclusion volume of the column. Peak A still designates the peak eluting at the inclusion volume; for consistency we designate C the peak associated with strong absorbance at 280 nm, and in this case it elutes before peak A. The distribution of radioactivity in Table 1 shows an increase in the cpm in peak B with a concomitant decrease in peak C. The elution experiment with Lubrol WX was carried out largely in order to gain information on the nature of the interaction between Emulgen 911 and the HPLC bonded phase, and about the possible exchange between nonionic detergents under the chromatographic conditions used. Chromatography of the microsomal sample with buffer B is shown in Fig. 4B. Under these conditions only peak 1 (Table 1) could be quantitated because of the strong tailing of peak B. As shown in Fig. 4B the peak corresponding to cytochrome P-450, identified as 1 on the trace, overlaps partially with a detergent peak. As shown in Table 1, only 2% of the

eluted radioactivity remains associated with this protein fraction. There are changes in the relative amounts of peak B and peak C but our inability to quantitate protein peak 2 makes analysis rather uncertain. The elution experiment with Lubrol WX does provide a clue as to the mechanism of action of the HPLC column towards nonionic detergents. In the absence of Lubrol WX, i.e., buffer A, Emulgen 911 interacts strongly with the column and elutes beyond the inclusion volume (Fig. 3A). In the presence of Lubrol WX, i.e., buffer B, Emulgen 911 is unable to compete for hydrophobic sites on the column and elutes considerably faster (Fig. 4A). Supporting evidence for this mechanism was found in the distribution of [^{3}H]-Emulgen 911 among buffer, water, and methanol fractions (see experimental). With Lubrol WX, 91.2% of the radiolabel was eluted in the buffer fraction compared to 51% with sodium cholate; in the latter water (23%) and methanol (26%) accounted for a significant portion. Differences in the distribution of [^{3}H]-Emulgen 911 among peaks A, B, and C (Table 1) are also suggestive of the formation of mixed aggregates with Lubrol WX. In all cases the formation of multiple high molecular aggregates observed in the LH-20 procedure does not materialize (*?*).

Under both sets of conditions, the removal of [^{3}H]-Emulgen 911 from the microsomal preparation compares favorably with optimal conditions reported for LH-20 and XAD-2 treatments which range from 50-60%. For the purified cytochrome P-450 examined, the

ratio obtained (2.5 µg detergent/nmol P-450) closely approaches the optimal values reported for hydroxylapatite (6). The recoveries of heme protein from HPLC columns have been demonstrated to be high (6,7), and since methods are available for the efficient removal of sodium cholate and Lubrol WX (5), the approach described here has practical potential in the removal of tightly bound nonionic detergents from proteins. Experimental factors to bear in mind are the load of detergent and concentration of protein solution, both of which may affect the efficiency of the procedure.

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