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An Enzyme-Linked Immunosorbent Assay for Detection of Linear Alkylbenzene Sulfonate: Development and Field Studies

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An accurate immunoassay system was developed for the quantitative analysis of linear alkylbenzene sulfonates (LAS), the most widely used anionic surfactants among domestic detergents. To generate LAS-specific monoclonal antibodies (mAb), hybridoma cells were produced by the fusion of mouse myeloma cells and spleen cells from mice immunized with sulfophenyl-5-valeric acid coupled to bovine serum albumin. After screening using a competitive enzyme-linked immunosorbent assay (ELISA), a mAb with a high binding affinity for LAS was selected and used in the development of a sensitive competitive direct ELISA. The detection range is between 20 and 500 ppb LAS. The ELISA assay was optimized and validated by comparison with conventional methods for the analysis of LAS and anionic surfactants, such as high-performance liquid chromatography (HPLC) and methylene blue active substances (MBAS) methods, in river samples. The correlation coefficients between the assay values obtained using the ELISA and the HPLC and MBAS were 0.98 and 0.97, respectively. It is anticipated that this immunoassay system will be a useful monitoring technique for the detection of LAS in environmental water samples.

Introduction

Linear alkylbenzene sulfonates (LAS) (Figure 1) are some of the most widely used anionic surfactants today. They have a variety of industrial uses and are common ingredients in laundry detergents and household cleaning products (*1*), and approximately 2.8 million t of LAS have been produced in the world (*2*). LAS are more easily biodegraded than nonlinear alkylbenzene sulfonates. However, complete biodegradation still requires several days (*3*). Methylene blue active substances (MBAS) method (*4*) is generally used for anionic detergents. This method is subject to interference from other anionic detergents and is not suitable for the specific determination of LAS in the environment. Moreover, this method requires a harmful solvent (chloroform) for extraction. Unlike the MBAS method, gas chromatography/mass spectrometry (GC/MS) (*5*) and high-performance liquid chromatography (HPLC) (*6*−*8*) are suitable for specific determination of LAS and identify the homologue and isomer composition of LAS, thereby providing the basis for assessing the environmental behavior and risk associated to LAS. Whereas GC/MS, before the analysis, requires a time-consuming step that converts LAS into volatile derivatives. HPLC combined with solid-phase extraction and fluorescence detection is a desirable method for LAS analysis because no harmful solvent and conversion step are needed. For multisample and routine analysis for LAS, however, a more rapid, simple, and cost-effective method is strongly needed, such as an enzyme-linked immunosorbent assay (ELISA).

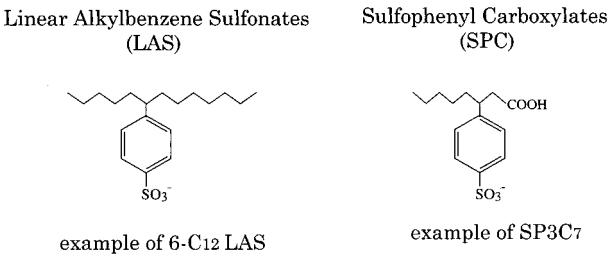

FIGURE 1. Structures of linear alkylbenzene sulfonates (LAS) and sulfophenyl carboxylates (SPC).

ELISAs are now available to measure a limited number of environmental contaminants, including industrial pollutants such as PCBs (*9*), pesticides (*10*−*12*), herbicides (*13*−*15*), petroleum (*16*), and heavy metals (*17*). These assays have significant advantages over more traditional analytical methods for environmental testing: they are quick, inexpensive, simple to perform, portable, and can be highly sensitive.

In this study, we generated a monoclonal antibody (mAb) that recognizes LAS but not sulfophenyl carboxylates (SPC, LAS metabolites) (Figure 1) nor other compounds and utilized it to assay LAS at concentrations as low as 20 ppb in water samples virtually without any pretreatment. An evaluation of the ELISA analysis for LAS in river samples was also carried out in terms of sensitivity, the time required for assay, and ease of analysis.

Experimental Section

Reagents. The commercial C_9−C_{13} LAS mixtures were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The exact percentage weight of each LAS homologue was 15.8%, 14.7%, 32.0%, 24.2%, and 13.3%, respectively. C_{12} LAS, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (ECDI), 3,3′,5,5′-tetramethylbenzidine (TMB), trifluoroacetic acid (TFA), and Dulbecco's phosphate buffered saline (PBS) were also purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's adjuvants, and horseradish peroxidase (HRP)-labeled goat anti-mouse IgG were purchased from Sigma Chemical Co. HRP was obtained from Boehringer-Mannheim Biochemicals. Block Ace was obtained from Yukijirushi Co. (Tokyo, Japan). Polystyrene 96-well plates were from Coaster Co. Daigo-T medium (Dainippon Pharmaceuticals, Osaka, Japan) was supplemented with 10% fetal calf serum (Biowhittaker). For HAT selection, hypoxanthine, aminopterin, and thymidine were added according to the procedure of Ando (*18*). Glass fiber filters (GS-25, 25-mm diameter, pore size 1.0 μm) were purchased from Advantec Toyo Co. (Tokyo, Japan). All other reagents were of the highest purity grade available.

Equipment. A microplate reader (Multiscan Bichromatic) with Deltagraph curve-fitting software was purchased from

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Dainippon Pharmaceuticals (Osaka, Japan). HPLC measurements were made using an AS-8020 automatic sample injector, CCPE pumps, and FS-8020 fluorometric detector (all from Tosoh Co. Ltd., Tokyo, Japan). The fluorometric detector was set at an excitation wavelength of 225 nm and an emission wavelength of 295 nm. A 25 cm × 4.6 mm i.d. column filled with 5-μm particle size, C_8 reverse-phase packing (Tosoh) was used. Seventy six percent of MeOH with 24% water (acidified with 0.2% (v/v) TFA) was used for solvent system at a flow rate of 0.8 mL/min.

Preparation of Hapten−Protein and Hapten−Enzyme Conjugate. The hapten, 5-sulfophenyl valeric acid, was synthesized by a sulfonation procedure as previously described (*19, 20*). Briefly, concentrated sulfuric acid (4 mL) and phenylcarboxylic acid (1 g) were reacted at 60 °C for 24 h. The mixture was carefully diluted with 20 mL of distilled water, washed with 2 × 20 mL diethyl ether to remove unsulfonated material, and neutralized to pH 7−9 by the addition of saturated aqueous sodium hydroxide solution. A 3-fold volume excess of 2-propanol was added causing sodium sulfate to precipitate out. The remaining solution was filtered, and the filtrate was evaporated to dryness. The white solid was purified by recrystallization from MeOH/diethyl ether mixtures. The synthesized hapten was coupled to the carrier protein BSA, OVA, and enzyme (HRP) by the ECDI procedure (*21*) with a few modifications. Fifty milligrams of hapten and 10 mg of protein were dissolved in 2 mL of PBS. A total of 160 mg of ECDI dissolved in 0.5 mL of PBS was added dropwise to the hapten−protein solution. The reaction mixture was stirred gently at 4 °C for 20 h and ultrafiltrated (MW 30 000 cutoff). After the carrier proteins and enzyme concentrations were determined, the conjugates were stored at 4 °C.

Immunization and Fusion Protocol. Five female BALB/c 12−24-week-old mice were injected with 100 μg of the hapten−BSA conjugate in 400 μL of PBS that was emulsified 1:1 in Freund's complete adjuvant. Five injections of the hapten−BSA conjugate with Freund's incomplete adjuvant followed at 2-week intervals. Serum titers were measured by ELISA 1 week after the last injection. After a rest period of 4−5 weeks, the mice with high serum titers of LAS-specific antibodies received booster injections of 30 μg of the hapten−BSA conjugate in 200 μL of PBS 3 days prior to the cell fusion carried out according to the method of Ando (*18*). On the third day the mice were sacrificed, and the immune spleen cells were fused with the murine myeloma cells (P3X63Ag-8U1) at a ratio of 5:1 using PEG4000 as described previously (*18*). After a 2-week HAT selection period, the supernatants from wells with growing hybridomas were assayed for LAS-specific antibodies by a competitive ELISA.

Evaluation of Fusion Products by ELISA. Flat-bottomed polystyrene microtiter plates were precoated overnight with 100 μL/well of hapten−OVA conjugate (5 μg/mL PBS) at 4 °C. After washing with PBS, 150 μL/well of Block Ace (diluted 1:3 with PBS) was applied to the plate, which was incubated overnight at 4 °C. When required, the plate was thawed and washed with PBS. Duplicate samples of the above-mentioned supernatants were transferred (100 μL/well) to the microtiter plate, the wells of which contained water or water containing LAS. After incubation at 37 °C for 1 h, unbound antibodies were washed off with PBS. This was followed by the addition of 100 μL/well of HRP-labeled goat anti-mouse IgG. After incubation at 37 °C for 1 h, plates were washed and 100 μL/well TMB substrate was added. The TMB substrate consisted of two parts citrate−phosphate buffer (40 mmol/L, pH 5.0) containing 350 mg/L urea hydrogen peroxide and one part 10 mg/mL TMB in dimethyl formamide. The substrate reaction was stopped after 15 min by adding 100 μL/well of phosphoric acid (0.5 mol/L), and the absorbance was measured at 450 nm using a microplate reader. Hybridoma with the lowest detection limit for LAS in an ELISA were cloned and expanded for antibody production.

TABLE 1. Cross-Reactivity Pattern of the Monoclonal Antibody with LAS and LAS Related Compounds

compound	% reactivity (LAS = 100%)
linear alkylbenzene sulfonates (IC_{50}, ppb)	100 (40)
sulfophenyl valeric acid	<0.1
sodium dodecyl sulfate	1.0
sodium myristate	0.3
sodium laurate	0.1
disodium lauryl sulfosuccinate	<0.1
benzenesulfonic acid	<0.1
phenol	<0.1
tolene	<0.1
xylene	<0.1
nonylphenol ethoxylate (EO = 10)	<0.1
polyoxyethylene (EO = 20) sorbitan monolaurate	<0.1
sodium palmitate	<0.1
sodium stearate	<0.1

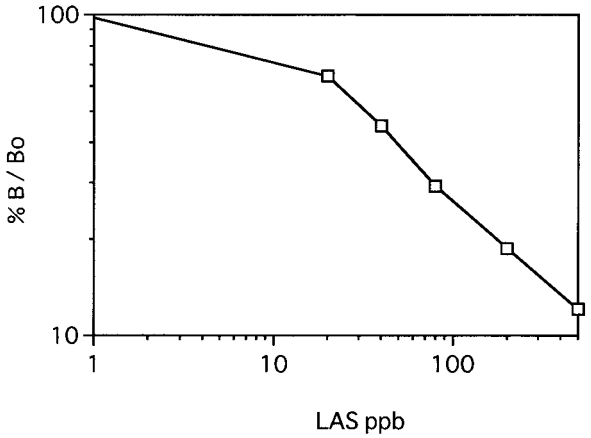

FIGURE 2. ELISA standard curve for LAS in distilled water. (absorbance value of zero control = 1.0.)

Production of Monoclonal Antibody. A priming injection of 0.5 mL of tetramethylpentadecane (pristane oil) was administered to female Balb/c mice. Ten days later, the mice received approximately 10^6 log-phase hybridoma cells in 0.5 mL of sterile PBS. Ascites fluids were harvested from the tenth day after the cells were introduced, and antibodies were purified by protein A affinity chromatography.

Competitive ELISA. Microtiter plates were coated with 100 μL/well of purified mAb (5 μg/ml) in coating buffer, incubated for 20 h at 4 °C, and then washed with PBS. A total of 150 μL of Block Ace (diluted 1:3 with PBS) was applied to the plate, which was then incubated for 20 h at 4 °C. When required, the plate was thawed and washed with PBS. Sixty microliters of sample (+LAS) and 60 μL of haptenated enzyme solution (2 μg/mL) were mixed in a separate uncoated well, and 100 μL aliquots were applied to triplicate wells of the antibody-coated plate, which was incubated for 90 min at room temperature and then washed. One hundred microliters of freshly prepared substrate (TMB) was added. The substrate reaction was stopped after 15 min with 100 μL of phosphoric acid (0.5 mol/L), and color was determined at 450 nm using a microplate reader. Data analysis was performed with the aid of a commercial ELISA software package (Deltagraph) using a four-parameter logistic equation for curve fitting and calculating the LAS concentrations of samples.

Determination of Antibody Specificity. The cross-reactivities of the selected mAb were determined with four LAS analogues and several nonanalogue compounds (Table 1). After the data were normalized by the % B/B_0 transfor-

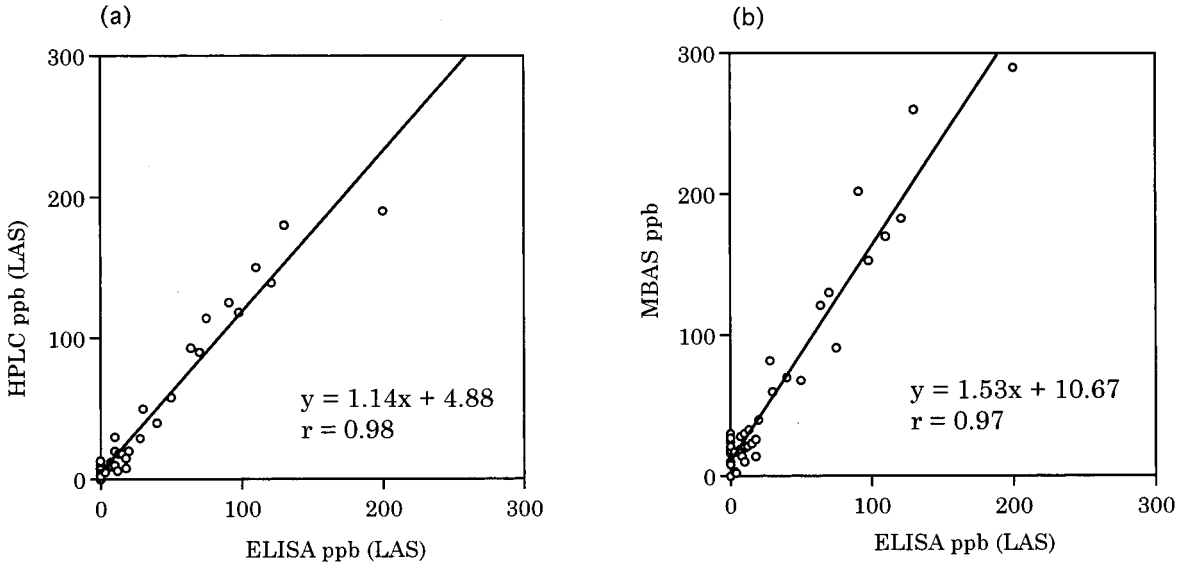

FIGURE 3. Comparison of analytical results obtained by ELISA and the conventional methods: (a) ELISA and HPLC. (b) ELISA and MBAS. The detection limits of the three methods were 20 ppb.

mation (eq 1, where A is the absorbance, A_0 is the absorbance at a zero dose of hapten, and A_{xs} is the absorbance at an excess of hapten), the LAS concentrations that caused 50% inhibition (Ic_{50}) were used to calculate cross-reactivities according to eq 2.

$$\% B/B_0 = (A - A_{xs}/A_0 - A_{xs}) \times 100 \quad (1)$$

$$\% \text{ cross-reactivity} = (\text{ppb of LAS at } Ic_{50})/(\text{ppb of cross-reacting LAS at } Ic_{50}) \times 100 \quad (2)$$

Sample Preparation and Analysis. River water samples were collected from 53 sites of 10 rivers or their branches in the Kinki districts (Osaka and Hyogo prefectures) in Japan from 1996 to 1997. Some of the rivers were markedly polluted while others were not. Raw water samples (500 mL) were filtered through a glass fiber filter (pore size 1 μm), and the residue on the filter was washed with 5 mL of MeOH, which was added to the filtrates. The filtrates were analyzed by ELISA without further pretreatment. On the other hand, for HPLC analysis, analytes were extracted by use of a solid-phase extraction according to Corcia et. al. (*6*). Briefly, the samples were passed through the graphtized carbon black (GCB) cartridge (obtained from Alltech Assosiates, Deerfield, IL) with the aid of a vacuum. After the cartridge was washed with water and the other solvents, LAS was eluted from the cartridge with 7 mL of eluant [10 mmol/L of tetramethyl-ammonium hydroxyde·$5H_2O$ in methylene chloride/MeOH (90:10, v/v)]. The extracts were dried and reconstituted with 500 μL of a 0.2% TFA/MeOH mixture (50/50, v/v) and injected to the LC column. The C_9-C_{13} LAS was used for calibration. The values of MBAS were determined as described in elsewhere (*4*).

Accuracy and Precision. The accuracy and precision of the ELISA method were determined by conducting standard fortification experiments with river water. The water samples were fortified with 50 and 100 ppb of LAS and concurrently analyzed pre- and post-fortification with five replicates. The mean net differences between neat and fortified samples were used to determine the percent recoveries at each level. Unspiked samples were also listed for comparison.

Results

Assay Working Range. The standard curve of C_9-C_{13} LAS was linear over the range from 20 to 500 ppb LAS when the logarithmic plot of optical density was constructed with respect to the logarithmic plot of the corresponding concentration (Figure 2) and exhibits a 50% binding value of approximately 40 ppb. The minimum detectable amount of LAS, defined as 80% B/B_0 was 20 ppb. This low value is well below the limits permitted in Japan for drinking water (200 ppb as MBAS, which are predominantly LAS). Furthermore, the standard curve of C_9-C_{13} LAS was coincided with that of C_{12} LAS (data not shown). It suggested that the response factor of each C_9-C_{13} LAS homologue was almost the same.

Cross-Reactivity of the LAS Antibody with Structurally Related Compounds. The ELISA may give erroneous LAS values due to interference compounds that may potentially bind or cross react with the LAS antibody due to some structural similarity with LAS, leading to an overestimation of the LAS concentrations. Standard solutions of potential cross-reactants were prepared, and the standard ELISA procedure was applied. The compounds were chosen on the basis of structural similarity as well as environmental occurrence. The results of cross-reactivity determinations are presented in terms of Ic_{50} values in Table 1. The data show that none of the potential cross-reactants, including SPC (LAS metabolites), exhibits more than 1% cross-reactivity relative to LAS, based on the ratio of the Ic_{50} values. These results suggested that the LAS ELISA can be used for environmental samples that may contain significant levels of these compounds. Furthermore, it is of interest that the antibody recognized only LAS (parent compound) but not SPC (LAS metabolites), although they have a similar structure to that of benzene sulfonate. This result indicates that the antibody recognized the alkyl chains as well as benzene sulfonate as epitopes.

Accuracy and Precision. The river samples were fortified with 50 and 100 ppb of LAS and analyzed before and after fortification. The initial levels of LAS in these samples ranged from approximately 0 to 200 ppb. The mean net increases in LAS levels are summarized in Table 2. Recoveries were approximately from 80−90% for the two concentrations tested (Table 2). The standard deviation (SD) and coefficient of variation (CV) of water analysis ranges were 4−6 and from 5 to 14, respectively. It is worth mentioning that the LAS concentrations were not affected by the different matrixes in any of the water samples tested. Therefore, no cleanup procedure for the water samples is necessary prior to measurement. The CV of unspiked samples containing 13

TABLE 2. LAS Estimated by ELISA in Blind-Coded Samples

sample no.	LAS fortification (ppb)	ELISA (ppb)	SD	CV (%)	LAS recovery (%)
1	50	44	6	12	88
2	50	50	6	14	100
3	50	42	3	7	84
4	50	41	2	5	82
1	100	80	6	8	80
2	100	94	5	5	94
3	100	83	4	5	83
4	100	81	4	5	81
5	0	13	3	23	
6	0	29	4	15	

and 29 ppb LAS were 23 and 15, respectively. These data showed that the reproducibility was maintained also at lower concentrations.

Comparison of ELISA with HPLC and MBAS Results. The results of ELISA and HPLC analysis of unspiked river samples are shown in Figure 3a. LAS contents estimated by ELISA were highly correlated with HPLC results at both lower and higher LAS concentrations with a correlation coefficient of 0.98, a slope of 1.14, and an intercept value close to 4.88. A slope of more than 1 indicates that the values obtained by HPLC are generally higher. Taking into consideration that ELISA shows an average recovery of ca. 85% (Table 2), these data indicated that the concentrations of LAS measured by ELISA may be a little underestimated.

The results of ELISA and MBAS analysis of river samples are shown in Figure 3b. The MBAS values were highly correlated with those of ELISA with a correlation coefficient of 0.97, a slope of 1.53, and an intercept value close to 10.67. A slope of 1.53 was expected because the MBAS values represented LAS and other anionic surfactants.

Discussion

The detection of LAS by immunoassay using a mAb represents a simple method for surfactant analysis, and to our knowledge, this is the first mAb generated for the detection of surfactants. Moreover, mAbs are analytical reagents with defined properties that can be produced in unlimited amounts.

The data from this study indicate that the mAb ELISA is sufficiently sensitive, accurate, and reproducible to be used in place of HPLC. Furthermore, the ELISA described here offers considerable advantages over the classical methods for trace amount analysis (GC, HPLC). The ease of handling (special skills are not required at all), the small sample volumes needed (60 μL; filtrated sample), nonrequirement of cleanup and concentration steps, relatively fast measurement (ca. 2.5 h with coated and washed plates), high sample turnover, low determination limits, and acceptable costs are important and attractive features.

The MBAS methods are generally used for measuring the concentrations of anionic surfactants. Kikuchi (*22*) et al. reported that the values obtained by MBAS were well correlated with those of LAS obtained by HPLC since LAS accounts for the majority of anionic surfactants (70−80% of anionic surfactants). In our case, the quantities of LAS measured by the ELISA were also found to be well correlated with those of MBAS (the correlation coefficient was 0.97) (Figure 3b). A slope of 1.53, probably due to the presence of other anionic surfactants, was similar in value to that of Kikuchi et al. (*22*). These data indicate that the values of MBAS can be estimated by the results of ELISA.

Although there is a limitation of ELISA that cannot provide homologue/isomer distribution of LAS contrary to HPLC, ELISA will contribute to the routine monitoring of LAS. The mAb, in the paper, has been converted into an ELISA test kit on a commercial basis.

Acknowledgments

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