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Ultrafiltration Method for Measuring Free Preservatives in Aqueous Phase of Oil-in-Water Emulsions[1]

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The antimicrobiological activity of preservatives incorporated in oil-in-water emulsion systems has been stated to be controlled by the concentration of the undissociated free species in the aqueous phase. A novel technique of ultrafiltration using the Diaflo membrane was investigated in order to measure the free methyl *p*-hydroxybenzoate in the aqueous phase of the oil-in-water emulsion. The method was found to differentiate between preservative that was bound, or solubilized, by the surfactant and preservative that was free in the aqueous phase. Emulsions containing various amount of methyl *p*-hydroxybenzoate were centrifuged and water rich layers were transferred to be ultrafiltration for the measurement of preservative concentrations. This technique could provide, in experiments of short duration, the direct estimation of the total preservative concentration in the emulsion needed to maintain a minimum inhibitory concentration of microbiologically active free preservative in the aqueous phase. Results from the ultrafiltration method were in good agreement with those from the microbiological method.

Microbiological activity of preservatives in heterogeneous systems such as pharmaceutical and cosmetic emulsions is extremely complex. A preservative added to an oil-water mixture partitions between the two phases, its antimicrobial activity being governed by the concentration in the aqueous phase.[3] Moreover, it has been shown by many authors[4] that the preservative activity of organic acids in the aqueous phase is controlled by the concentration of the undissociated acid and not of the ion. In oil-in-water emulsion systems, the third components are the emulsifying agents which usually are able to form the micelle in the aqueous phase. Previous investigations[5] have reported that many of the commonly used preservatives in emulsions are solubilized by, or bound to surfactants in aqueous solutions. The antimicrobial activity of preservatives in such a system has been shown to be directly related to the concentration of free unbound preservatives.[6]

A basic integrated model for the quantification of preservative action in emulsion system was reported by Garrett.[7] It is a troublesome process to determine the required constants

1) Presented at the 91th Annual Meeting of Pharmaceutical Society of Japan, Fukuoka, April, 1971.
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3) *a*) M.G. deNavarre and H.E. Bailey, *J. Soc. Cosmet. Chem.*, **7**, 427 (1956); *b*) M. Aoki, M. Matsumoto, I. Yoshioka, and Y. Isa, *Yakuzaigaku*, **17**, 231 (1957); *c*) H.S. Bean and S.M. Heman-Ackah, *J. Pharm. Pharmacol.*, **16**, *Suppl.*, 58T (1964); *d*) S.M. Heman-Ackah and G.H. Konning, *ibid.*, **19**, *Suppl.*, 189S (1967).
4) *a*) O. Rahn and J.E. Conn, *Ind. Eng. Chem.*, **36**, 185 (1944); *b*) E.R. Garrett and O.R. Woods, *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 736 (1953).
5) *a*) M. Aoki, A. Kamata, I. Yoshioka, and T. Matsuzaki, *Yakugaku Zasshi*, **76**, 939 (1956); *b*) N.K. Patel and H.B. Kostenbauder, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 289 (1958); *c*) F.D. Pisano and H.B. Kostenbauder, *ibid.*, **48**, 310 (1959); *d*) G.M. Miyawaki, N.K. Patel, and H.B. Kostenbauder, *ibid.*, **48**, 315 (1959); *e*) F.W. Goodhart and A.N. Martin, *J. Pharm. Sci.*, **51**, 50 (1962); *f*) W.P. Evans, *J. Pharm. Pharmacol.*, **16**, 323 (1964).
6) *a*) M.G. deNavarre, *J. Soc. Cosmet. Chem.*, **8**, 68 (1957); *b*) S.M. Blaug and S.S. Ahsan, *J. Pharm. Sci.*, **50**, 138 (1961); *c*) N.K. Patel and J.M. Romanowski, *ibid.*, **59**, 372 (1970); *d*) A.G. Mitchell, *J. Pharm. Pharmacol.*, **16**, 533 (1964).
7) E.R. Garrett, *J. Pharm. Pharmacol.*, **18**, 589 (1966).

for calculating the total preservative concentration necessary for the maintenance of a minimum inhibitory concentration of the microbiologically active species in the aqueous phase. Furthermore, it has not been elucidated whether results calculated using this mathematical models correspond with those obtained experimentally. From a practical viewpoint, it is important to develop a direct method to measure the amount of free preservative in the aqueous phase of the emulsion, and accordingly, the total preservative required to attain the desired concentration in the phase. A three chambered dialysis method using a Millipore VS membrane and a nylon membrane to quantify the preservative in the aqueous phase was presented by Kazmi and Mitchell.[8] Its main disadvantage is that it is time consuming to attain the equilibrium and the nylon membrane is stated to bind phenolic preservatives.[5b]

In this article, an ultrafiltration method using a dextran gel membrane will be described. It will also be demonstrated that results obtained by this method are related to the results by an *in vitro* microbiological procedure.

Experimental

Material——Methyl *p*-hydroxybenzoate(MP), riboflavin, lactose and light mineral oil were of J.P. VIII grade. Sodium dodecyl sulfate(SDS) was purified with isopropanol and petroleum ether as described by Tokiwa.[9] Polyoxyethylene (**8**)[10] lauryl ether (PLE), purely synthesized one-spot grade in thin-layer chromatography (TLC) and gas chromatography, was supplied by Nikko Chemicals Co., Tokyo. Polyoxyethylene (**2**) palmityl ether, polyoxyethylene (**4**) stearyl ether, polyoxyethylene (**6**) stearyl ether and polyoxyethylene (**10**) butyl ether were of commercial grade and supplied by Nihon Emulsion Co., Tokyo. The Diaflo membrane, UM-10, 43 mmϕ, the crosslinked dextran gel membrane, was commercially available from Amicon Corp., Mass., U.S.A. All other chemicals were of reagent grade.

Determination of the Critical Micelle Concentration (CMC)——The CMC of PLE and SDS were determined according to the conventional methods, using a Surface Tensometer type ST-1, Shimadzu Seisakusho, Kyoto, or a Conductivity Outfit model MY-7, Yanagimoto Mfg. Co., Kyoto.

Quantitative Analysis——The concentration of MP, aniline hydrochloride and riboflavin were determined spectrophotometrically at wavelengths of 256 mμ, 278 mμ and 445 mμ, respectively. Lactose was measured by the colorimetric method of Momose.[11] SDS was analyzed by an electric conductivity method, and PLE by a surface tension method.

Ultrafiltration Procedure——The ultrafiltration cell model No. 50 from Amicon Corp. was modified to avoid the adsorption of *p*-hydroxybenzoic acid ester by plastic materials. All plastics of the cell were exchanged for glass. The modified ultrafiltration cell was filled with 40 ml of the test solution. Using the pressure of 2 kg/cm^2 from the air compressor tank, the solution was forced to filter through the membrane into the vessel. The effluent was cut and collected in 2 or 4 ml portions in succession. After taking 24 ml of fractions in all, the pressure was released and residual contents of the cell was also collected. The membrane was rejected if there was a significant amount of surfactant leakage. Temperature was held at 25° during the entire operation. Usually the flow rate of distilled water through the membrane was 1.2 ml/min under the described conditions.

Table I. Formulation of Oil-in-Water Emulsions

Ingredients	%
Light mineral oil	30.0
Polyoxyethylene (**2**) palmityl ether	0.8
Polyoxyethylene (**4**) stearyl ether	0.9
Polyoxyethylene (**6**) stearyl ether	2.0
Polyoxyethylene (**10**) butyl ether	1.9
MP	0—0.5
Distilled water	add to 100.0

8) S.J.A. Kazmi and A.G. Mitchell, *J. Pharm. Sci.*, **60**, 1422 (1971).
9) F. Tokiwa, *J. Phys. Chem.*, **72**, 1214 (1968).
10) The number in parentheses denoted the nominal number of oxyethylene units per molecule.
11) T. Momose and A. Inaba, *Chem. Pharm. Bull.* (Tokyo), **9**, 263 (1961).

Preparation of Emulsion——The emulsions were prepared according to the formula shown in Table I. All surfactants were put into mineral oil and heated to 70°, meanwhile MP was dissolved in a sufficient amount of water at 70°. The hot water phase was poured into the oil phase little by little under stirring. Phase inversion was observed in this process, and finally an oil-in-water emulsion was obtained. The hot emulsion thus obtained were cooled and equilibrated at a temperature of 25° for a period of 7 days.

Separation of Aqueous Phase of Emulsion——The aqueous phase of the oil-in-water emulsion was separated by ultracentrifuge technique as illustrated in Chart 1 in detail. About 50 ml of the extremely thin emulsion available for the ultrafiltration was obtained from 200 ml of the original emulsion.

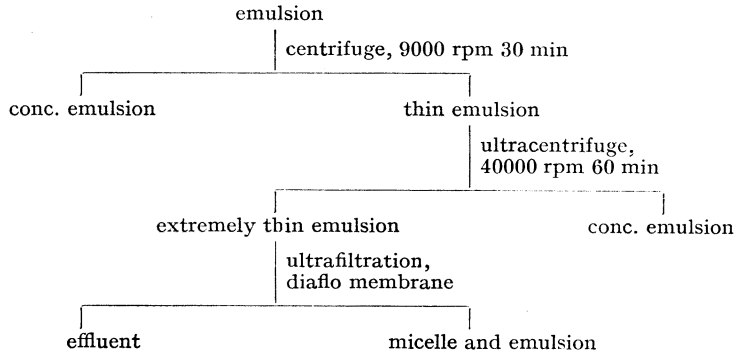

Chart 1. Separtion Procedure of Aqueous Phase of Emulsion

Microbiological Procedure——The test procedures were essentially the same as those employed for the parenteral products presented by Rdzok, *et al.*[12] The eight test organisms used were *Bacillus subtilis* PCI-219, ATCC 6633; *Aspergillus niger* ATCC 6275, IFO 6341; *Penicillium citrinum* (laboratory isolated); *Candida albicans* IFO 0601; *Saccharomyces cerevisiae* ATCC 9763; *Staphylococcus aureus* 209-P, IFO 3061; *Pseudomonas aeruginosa* IFO 3080; and *Escherichia coli* K-12, ATCC 14948, IFO 3301.

Result and Discussion

Critical Micelle Concentration

The curve represented the surface tension of PLE showed a sharp break at a surfactent concentration of 4.5×10^{-3}%. The value of this CMC is in good agreement with the data reported.[13] The CMC of SDS determined electroconductometrically was 0.23%. The obtained value shows also close agreement with the data presented.[14]

Ultrafiltration of Surfactant Solution

The two concentration levels of surfactant solutions, namely above and below the CMC, were employed for the ultrafiltration. Figures 1 and 2 show the surfactant concentration in each fraction from the ultrafiltration process. In the case of PLE, the concentrations of residual solutions of the cell after the ultrafiltration run were 5.6×10^{-3} and 83×10^{-3}% for the solutions below and above the CMC. Although a small amount of PLE permeates through the Diaflo membrane UM-10, as is shown in Fig. 1, its effluent concentration is far below the CMC under the experimental conditions. In other words, it is ascertained that the membrane concentrates PLE but dose not permit the passage of enough amount to form the micelle in the filtrate.

The filtration pattern of rather concentrated solution of SDS appeared to be somewhat different from that of PLE as illustrated in Fig. 2. The SDS concentrations of residual

12) E.J. Rdzok, W.E. Grundy, F.J. Kirchmeyer, and J.C. Sylvester, *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 613 (1955).
13) N. Ohba and A. Takahashi, *Chim. Phys. Appl. Prat. Ag. Surface, C.R. Congr. Int. Deterg.*, **5th**, 2, 481 (1969).
14) P. Mukerjee, *J. Phys. Chem.*, **62**, 1390 (1958).

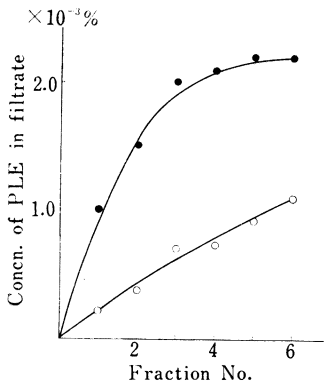

Fig. 1. Ultrafiltration of PLE Solutions
The filtrates were fractionated in 4 ml portions. The surfactant concentrations of feed solution were 2.0×10^{-3}% (—○—, below the CMC), and 20×10^{-3}% (—●—, above the CMC). The CMC of PLE was 4.5×10^{-3}%.

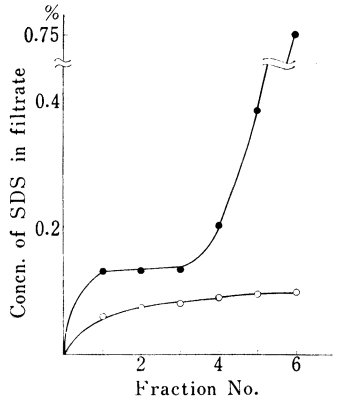

Fig. 2. Ultrafiltration of SDS Solutions
The filtrates were fractionated in 4 ml portions. The surfactant concentrations of feed solution were 0.096% (—○—, below the CMC), and 0.6 % (—●—, above the CMC). The CMC of SDS was 0.23%

solutions in the ultrafiltration cell were 5.6×10^{-3} and 1.3% for the solutions below and above the CMC. While the curve of SDS concentration in the filtrate is quite similar to that of PLE at the concentration below the CMC, the curve shows a rather complicated shape above the CMC, and a significant amount of surfactant leakage is observed during the latter half of the filtration process. The fact that the membrane treated with the SDS solution above the CMC has shown about ten times faster rates of flow than the untreated suggests the deterioration of the UM series of membranes by ionic surfactants. However, concerning nonionic surfactants such as PLE the ultrafiltration using a Diaflo membrane is a valid technique to remove micelles from the surfactant solution.

Ultrafiltration of MP Solution and Others

The simple solution of MP was filtered through the Diaflo UM-10 membrane. The cuve of MP concentration in each fractionated portion from the ultrafiltration is shown in Fig. 3. In an early stage of the effluence the concentration of MP is fairly low, but the filtrate concentration reaches its constant level after 8 ml filtration and is 3.9×10^{-3}%. The MP concentration of residue in the cell, on the other hand, was 3.9×10^{-3}%. Compared the filtrate with feed and residual solution in MP concentration, the membrane is considered to allow MP to pass freely. The cause of the lower concentration of MP at the beginning of the ultrafiltration may be accounted for by the dilution effect with water which is necessary to prevent the membrane from the dehydration damage.

The ultrafiltration curves for MP in the PLE solutions are presented in Fig. 4. The MP concentrations of the residual solutions were 9.3×10^{-3} and 30×10^{-3}% for the PLE concentrations below and above the CMC. The free passage of MP through the Diaflo membrane at a PLE concentration below the CMC indicates that all of MP is found to exist in the unbound form, while the retention of MP above the CMC shows that about 80% of MP is in the solubilized or bound form.

Markers of known molecular weight, aniline hydrochloride, lactose and riboflavin were filtered to know the extent of markers permeated through the Diaflo membranes under the described conditions. As shown in Fig. 5, aniline hydrochloride and MP with molecular weight below 152 permeated freely, whereas lactose and riboflavin having molecular weight around 350 were partially retained and PLE at 538 molecular weight was almost completely retained. These results suggest that the Diaflo membrane functions as a diffusion membrane relating to the molecular weight of substances, and is useful in the measurement of binding characteristics for MP-surfactants interactions.

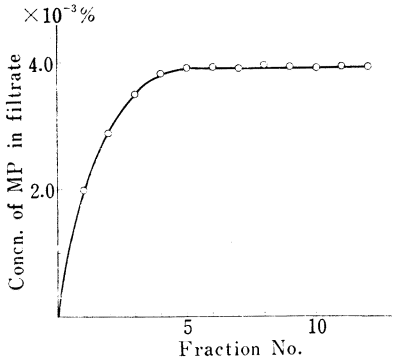

Fig. 3. Ultrafiltration of Simple MP Solution

The filtrates were fractionated in 2 ml portions. The MP concentration of feed solution was 3.9×10^{-3}%.

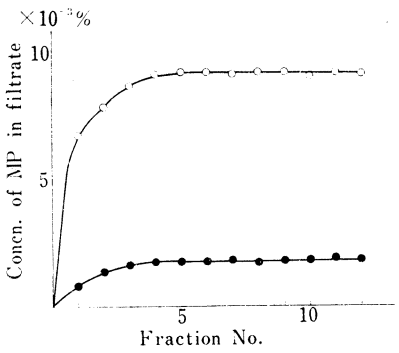

Fig. 4. Ultrafiltration of MP in the PLE Solution

The filtrates were fractionated in 2 ml portions. The MP concentration of feed solution was 10×10^{-3}%. The PLE concentrations were 1.0×10^{-3}% (—○—, below the CMC), and 30×10^{-3}% (—●—, above the CMC).

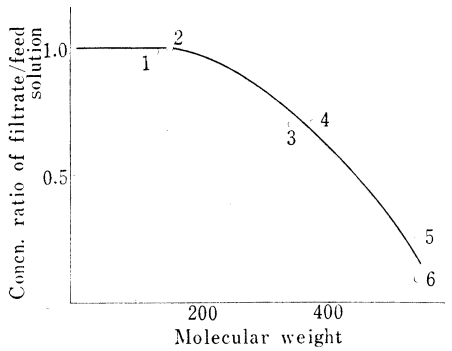

Fig. 5. Solute Permeation through Diaflo Membrane UM-10

Solute and its concentration of feed solution, 1: aniline hydrochloride 3×10^{-3}%, 2: MP 2×10^{-4}%, 3: lactose 5×10^{-3}%, 4: riboflavin 7.4×10^{-2}%, 5: PLE 2×10^{-3}% (below the CMC), 6: PLE 2×10^{-2}% (above the CMC).

Free MP Concentrations in Aqueous Phase of Oil-in-Water Emulsions and Microbiological Activities

The aqueous phases separated ultracentrifugally from oil-in-water emulsions were filtered through the Diaflo membranes to quantify the free MP concentrations. As the pH values of sample emulsions ranging from 5.0 to 5.6 were far below 8.5, a pK_a of MP,[15] all of MP in the effluent through the membrane was considered to be in undissociated active form. As can be seen in Table II, the free MP concentration in the aqueous phase depends on the total amount of MP incorporated in the emulsion. However, the ratio of the free species to the total MP in the emulsion varies from 0.196 to 0.386 suggesting that the distribution of MP in the complex emulsion system does not obey the simple partition low. Available discussion on this phenomenon has been partially presented by Kazmi, *et al.*,[8] and the association of preservative in the oil phase may be one of the possible cause of the complicated distribution. Further evidence on mechanism will be reported shortly.

The data determined the efficacy of a preservative in the emulsion are presented in Table III. The antimicrobial action of the emulsions containing MP of less than 0.2% was considered unsatisfactory because of the lack of bacteriostatic or bactericidal effects on *Aspergillus niger* and *Penicillium citrinum*. Emulsions containing 0.3 and 0.5% MP had satisfactory preservative properties. Bandelin[16] has reported that the minimum concentration of MP required to inhibit *Aspergillus niger* is 0.1% at pH 5. The preservation of an emulsion requires that there must be a minimum inhibitory concentration of free undissociated MP in the aqueous phase. Therefore, the total amount of MP required in the emulsion to provide

15) T.R. Aalto, M.C. Firman, and N.E. Rigler, *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 449 (1953).
16) F.J. Bandelin, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 691 (1958).

TABLE II. Free MP Concentration in Aqueous Phase of Emulsion

% of MP in emulsion (T)	% of free MP in aqueous phase (F)	F/T
0.0051	0.0010	0.196
0.0205	0.0043	0.210
0.0512	0.0116	0.227
0.102	0.0242	0.237
0.203	0.0566	0.279
0.307	0.0958	0.312
0.508	0.196	0.386

TABLE III. Results of Preservative Test Applied to Test Emulsions

MP (%)[a]	Days	Plate count (cells/ml)							
		B. sub.	*A. niger*	*P. citri.*	*C. alb.*	*S. cere.*	*Stap. aure.*	*Ps. aeru.*	*E. coli*
0	0	110000	2800	34000	13000	6700	98000	260000	340000
	10	150000	6600	62000	9000	2500	<10	1500	<10
	20	240000	1500	28000	44000	<10	<10	4200	<10
	30	89000	3500	30000	22000	<10	<10	55000	<10
0.005	0	110000	2800	34000	13000	6700	98000	260000	340000
	10	160000	5000	34000	500	<10	<10	4900	<10
	20	170000	3500	38000	500	<10	<10	1800	<10
	30	86000	4500	25000	750	<10	<10	13000	<10
0.02	0	110000	2800	34000	13000	6700	98000	260000	340000
	10	110000	6000	46000	6000	<10	<10	1000	<10
	20	200000	3500	37000	13000	<10	<10	11000	<10
	30	54000	6000	27000	17000	<10	<10	73000	<10
0.05	0	110000	2800	34000	13000	6700	98000	260000	340000
	10	160000	2000	32000	1100	<10	<10	4300	<10
	20	340000	4000	36000	1500	<10	<10	1100	<10
	30	79000	4000	2500	780	<10	<10	59000	<10
0.1	0	110000	2800	34000	13000	6700	98000	260000	340000
	10	150000	1500	19000	310	<10	<10	<10	<10
	20	210000	2500	35000	25	<10	<10	<10	<10
	30	79000	2000	11000	<10	<10	<10	<10	<10
0.2	0	110000	2800	34000	13000	6700	98000	260000	340000
	10	110000	3500	36000	30	<10	<10	<10	<10
	20	34000	3500	25000	<10	<10	<10	<10	<10
	30	74000	1500	7500	<10	<10	<10	<10	<10
0.3	0	110000	2800	34000	13000	6700	98000	260000	340000
	10	140000	2500	6500	<10	<10	<10	<10	<10
	20	24000	150	3500	<10	<10	<10	<10	<10
	30	95000	<10	3500	<10	<10	<10	<10	<10
0.5	0	110000	2800	34000	13000	6700	98000	260000	340000
	10	160000	<10	<10	<10	<10	<10	<10	<10
	20	90000	<10	<10	<10	<10	<10	<10	<10
	30	83000	<10	<10	<10	<10	<10	<10	<10

a) concentration in the total emulsion
B. sub.=*Bacillus subtilis* PCI-219, *A. niger*=*Aspergillus niger* ATCC6275, *P. citri.*=*Penicillium citrinum*, *C. alb.*=*Cardida albicans* IFO 0601, *S. cere.*=*Sacharomyces cerevisiae* ATCC 9763, *Stap. aure.*=*Staphylococcus aureus* 209-P, *Ps. aeru.*=*Pseudomonas aeruginosa* IFO 3080, *E. coli*=*Escherichia coli* K-12

a minimum inhibitory concentration of preservative in the aqueous phase, namely 0.1%, should exceed 0.3% in the total emulsion as can be seen in Table II.

Agreement between the values of the two methods, physicochemical and microbiological procedures, shows that the ultrafiltration technique provides a relatively simple direct method to estimate the total concentration of preservative necessary for the satisfactory preservetion. This direct method should be useful for complex emulsions in which the presence of liquid crystalline phase or of reversed micelle in the oil phase would make the calculation using a mathematical model derived by Garrett[7] difficult or impossible.

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