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Reversed-phase liquid chromatographic retention and membrane activity relationships of local anesthetics

Hironori Tsuchiya*, Maki Mizogami, Ko Takakura

Asahi University School of Dentistry, 1851-1 Hozumi, Mizuho, Gifu 501-0296, Japan

Abstract

The chromatographic retention and membrane activity relationships of local anesthetics were studied to address the possible mechanisms for structure specificity and inflammation-associated decrease of their effects. Five representative drugs (3 mM for each) were reacted with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine liposomes in 25 mM potassium phosphate buffer (pH 5.9–7.9, containing 100 mM NaCl and 0.1 mM EDTA) for 10 min at 37 °C and the membrane fluidity changes were analyzed by measuring fluorescence polarization with 1,6-diphenyl-1,3,5-hexatriene. Their capacity factors were determined on octadecyl-, octyl- and phenyl-bonded silica columns with a mobile phase consisting of 25 mM potassium phosphate buffer (pH 5.9–7.9, containing 100 mM NaCl and 0.1 mM EDTA)–methanol (30:70, v/v) at a flow rate of 1.0 ml/min and at a column temperature of 37 °C and diode-array detection. Mepivacaine, prilocaine, lidocaine, ropivacaine and bupivacaine fluidized membranes in increasing order of intensity, which agreed with their clinical potency. The relative degree of membrane fluidization correlated with that of retention on an octadecyl stationary phase more significantly than the other phases. Both membrane-fluidizing effects and capacity factors decreased by lowering the reaction and mobile phase pH, being consistent with the hypothesis that anesthetic potency is reduced in inflammation because of tissue acidity. Reversed-phase liquid chromatography appears to be useful for estimating the structure-specific and pH-dependent membrane-fluidizing effects of local anesthetics.

Keywords: Reversed-phase liquid chromatography; Local anesthetics; Membrane fluidization; Retention and membrane activity relationships

1. Introduction

The mode of action for anesthetics has been still controversial despite their clinical uses over one century. The proposed theories and/or hypotheses include the possible interaction of drugs with nerve cell membranes. In addition to membrane proteins, such as receptors and enzymes, anesthetics are known to act on membrane lipid bilayers to modify their fluidity [1]. Especially, local anesthetics induce the fluidization of bio-membranes, including liposomal, synaptosomal and cellular membranes [2,3]. Local anesthesia would occur by the direct mechanism, in which the fluidized lipid bilayers influence the membrane functions responsible for nerve transmission, or by the indirect mechanism, in which more fluid membranes change the conformation of receptor proteins, and consequently block sodium channels, or by both [4–6].

Amide-type local anesthetics, which have been most frequently used in clinical anesthesia, are lidocaine, prilocaine, bupivacaine, ropivacaine and mepivacaine (Fig. 1). These drugs show different clinical properties depending on their structures [7,8]. In 1-alkyl-2′,6′-pipecoloxylidide compounds, the anesthetic potency increases in the order of mepivacaine, ropivacaine and bupivacaine with lengthening the alkyl chains. Lidocaine and prilocaine are less potent than bupivacaine. Such structure-dependence of pharmacological effects is accounted for not only by the selective affinity of drugs to membrane receptors and enzymes but also by the specific hydrophobic interaction of drugs with membrane lipids [9]. The intensity of anesthetic and membrane interaction is usually related to the partitioning of

* Corresponding author. Tel.: +81 58 3291266; fax: +81 58 3291266.
E-mail address: hiro@dent.asahi-u.ac.jp (H. Tsuchiya).

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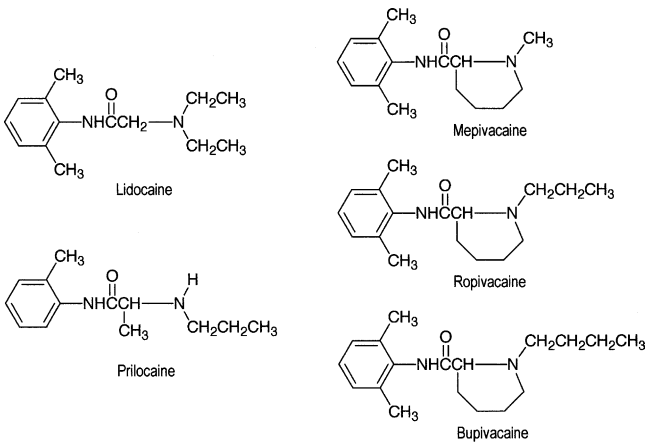

Fig. 1. Chemical structures of local anesthetics.

drugs between non-polar (lipid) and polar (aqueous) phase [7].

As a clinical phenomenon concerning local anesthesia, the failure to obtain satisfactory pain relief has been well recognized in inflammation [7]. Acidic by-products lower the pH of inflamed tissues. Therefore, as represented by the Henderson–Hasselbalch equation ($pH = pK_a + \log$ [non-ionized anesthetic]/[ionized anesthetic]), acidic conditions reduce the concentrations of active anesthetics in non-ionized forms which can diffuse into and interact with membrane lipid bilayers. The hypothetic mechanism indicates that tissue acidity is responsible for the local anesthetic failure associated with inflammation.

Since the classical work of Overton and Meyer showing a correlation between anesthetic action and lipid solubility [10], many researchers have tried to relate the lipid/water partitioning of hydrophobic drugs to their pharmacological activity. Partition coefficients of local anesthetics between *n*-octanol and buffer correlate with potency, onset-time and duration of action [9,11,12]. They are usually determined by shake- and stir-flask methods [12,13]. However, their experimental conditions are remarkably influenced by used liquid phases, temperature, equilibration time and stability during analysis. In addition to such methodological problems, the biological partitioning is not necessarily identical to the bulk-phase hydrocarbon/water partitioning [14]. Instead of butch methods, chromatographic techniques, especially high-performance liquid chromatography (HPLC), have been increasingly used to investigate the hydrophobic interaction of membrane-acting drugs as well as their pharmaceutical properties [15,16]. Most of previous HPLC studies have focused on the retention and structure relationships of solutes to predict their chromatographic behaviors and partition coefficients from the obtained capacity factors [14]. However, the concept to correlate HPLC characteristics with pharmacological effects, being referred to as the retention and activity relationship, has not been applied to local anesthetics except for anti-bacterial and insect-repellent drugs [17,18].

The aim of the present work was to study the retention and membrane activity relationships of local anesthetics by reversed-phase HPLC for addressing the possible mechanisms underlying structure specificity and inflammation-associated decrease of their effects. The membrane effects of structurally different local anesthetics (Fig. 1) were comparatively determined with varying the pH of reaction media. Structure- and pH-dependent potency to modify membrane fluidity was related to the capacity factors obtained from different stationary phases.

2. Experimental

2.1. Chemicals and materials

Lidocaine hydrochloride, prilocaine hydrochloride and bupivacaine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Ropivacaine hydrochloride and mepivacaine hydrochloride were supplied by AstraZeneca (Södertälje, Sweden). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Avanti Polar Lipids (Birmingham, AL, USA) and Molecular Probes (Eugene, OR, USA), respectively. Methanol and water of liquid chromatographic grade (Kishida, Osaka, Japan) were used for preparing reagent and mobile phase solutions. All other reagents were of the highest analytical grade available (Wako, Osaka, Japan).

2.2. Membrane fluidity measurement

Liposomal membranes with the lipid bilayer structure were prepared by the method of Okimoto et al. [19] with some modifications. In brief, an aliquot (250 μl) of the ethanol solution, containing 10 mM DPPC and 50 μM DPH, was injected four times into 199 ml of 25 mM potassium phosphate buffer (pH 5.9, 6.9, 7.4 and 7.9, containing 100 mM NaCl and 0.1 mM EDTA) under stirring above a phase transition temperature of DPPC. With this procedure, unilamellar vesicles are prepared and DPH is localized exclusively in the hydrocarbon core of membrane lipid bilayers [20,21]. The aqueous solution of mepivacaine, prilocaine, lidocaine, ropivacaine or bupivacaine was added to liposomal suspensions placed in a cuvette controlled at 37 °C to give a final concentration of 3 mM for each drug. After the reaction for 10 min with stirring, fluorescence polarization was measured at 360 nm for excitation and 430 nm for emission by an RF-540 spectrofluorometer (Shimadzu, Kyoto, Japan) equipped with polarizers and a temperature-controlled cuvette holder as reported previously [22,23]. Polarization values were calculated by the formula $(I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$, in which I was the fluorescence intensity and the subscripts V and H referred to the vertical and horizontal orientations of the excitation and emission polarizers, respectively [24]. The grating correction factor was calculated as $G = (I_{HV}/I_{HH})$. Compared with

control (liposomes treated with water alone), the decreased polarization means an increase of membrane fluidity (membrane fluidization).

The membrane structures of liposomes were confirmed after their preparation and treatment with local anesthetics by a transmission electron microscopic method [25].

2.3. HPLC analysis

The chromatographic system consisted of an LC-10ADVP liquid chromatograph (Shimadzu, Kyoto, Japan) connected to an SIL-10ADVP autosampler (sample volume of 5 μl), a DGU-4A degasser (Shimadzu), a CTO-6A column oven (Shimadzu) and an SPD-M10AVP diode-array detector (Shimadzu) controlled by an FMV-5133D5 personal computer (Fujitsu, Tokyo, Japan). The used reversed-phase columns (Shimadzu) were as follows: a Shim-pack CLC-ODS column (150 mm × 6.0 mm i.d., particle size 5 μm), a Shim-pack CLC-C_8 column (150 mm × 6.0 mm i.d., particle size 5 μm) and a Shim-pack CLC-Phenyl column (150 mm × 6.0 mm i.d., particle size 5 μm). The mobile phase, a mixture of 25 mM potassium phosphate buffer (pH 5.9, 6.9, 7.4 and 7.9, containing 100 mM NaCl and 0.1 mM EDTA)–methanol (30:70, v/v), was delivered at a flow rate of 1.0 ml/min and at a column temperature of 37 °C. Mepivacaine, prilocaine, lidocaine, ropivacaine and bupivacaine (1 mM for each) were dissolved in water, and then an aliquot (5 μl) of the resulting solutions was repeatedly injected onto three columns. Column eluates were detected at absorption wavelengths of 203 and 220 nm. The capacity factors of local anesthetics were calculated by the defined formula $(t_R - t_0)/t_0$, in which t_R was the obtained retention time of each drug and t_0 (the retention time of a non-retained compound) was determined as reported previously [26].

2.4. Statistical analysis

All results are expressed as mean ± S.E. ($n = 5$ or 6). Statistical analysis was performed with StatView 5.0 (SAS Institute, Cary, NC, USA). The data on membrane fluidity changes were analyzed with Student's *t*-test. A P-value < 0.05 was considered significant.

3. Results and discussion

Hydrophobic drugs like anesthetics are incorporated into lipid bilayers to alter the fluidity of liposomal membranes as well as that of synaptosomal and cellular membranes [27]. The membrane effects of local anesthetics were determined, using protein-free liposomes prepared with DPPC, phospholipid most frequently used for studying membrane-acting drugs, to focus on the interaction with membrane lipids. Five representative drugs (Fig. 1) were reacted with DPPC liposomes at an identical molar concentration to compare their membrane effects. The changes in membrane fluidity were analyzed with DPH, a fluorescence probe for the membrane hydrocarbon core, because the tested drugs are preferentially distributed into the deeper hydrophobic regions of lipid bilayers [2,3]. All anesthetics showed the membrane-fluidizing effects as indicated by the decrease of DPH polarization (Table 1). When reacted at pH 6.9, mepivacaine, prilocaine, lidocaine, ropivacaine and bupivacaine fluidized liposomal membranes in increasing order of intensity. The relative degree of membrane fluidization almost agreed with that of clinical anesthetic potency [7,8]. The membrane effects of local anesthetics decreased with lowering the reaction pH from 7.9 to 5.9. Ropivacaine could not be analyzed at pH 7.4 and 7.9 because it precipitated slightly in the present reaction system. The pH in inflamed tissues shows 6.9 or less [28]. A correlation of decreasing changes between membrane fluidization and pH is consistent with the speculation that the drug and membrane interaction reduced by tissue acidity is responsible for the local anesthetic failure in inflammation [7].

When correlating chromatographic retention with hydrophobicity or membrane activity, it is ideal to measure the capacity factors of solutes, using a mobile phase of 100% water or buffer. However, the direct measurement is difficult because of long retention times in actual chromatography [14]. In order to solve this problem, methanol has been added as an organic modifier most commonly, since it shows the water-like property and less influence on lipid-phase partitioning than other organic solvents, such as acetonitrile and tetrahydrofuran [29]. Reversed-phase HPLC systems with the mobile phase consisting of buffer and methanol have been applied to local anesthetics. Grouls et al. [15] estimated the relation between chromatographic retention and partition coefficients of different drugs. However, they neither related the capacity factors to the anesthetic activity nor used the conditions (mobile phase pH and column temperature) comparable to the physiological conditions producing anesthesia. While Mizogami et al. [2] discussed the hydrophobic interaction potency of local anesthetics based on their retention characteristics, the experimental conditions were different between chromatography and anesthetic effect analysis. In contrast to previous studies, the present HPLC was performed with the same mobile phase buffer and temperature as those used in membrane experiments.

Typical chromatograms obtained from an octadecyl-bonded silica column are shown in Fig. 2. The retention of local anesthetics varied by difference of their structures and the mobile phase buffer pH. Such variations were also found in chromatography, using octyl- and phenyl-bonded silica columns.

The capacity factors increased in the order of mepivacaine, prilocaine, lidocaine, ropivacaine and bupivacaine on octadecyl and octyl stationary phases, whereas the elution order of the former two drugs was reversed on a phenyl stationary phase (Table 2). The relative degree of retention on both alkyl phases was not necessarily compatible with that of partition from buffer to *n*-octanol [7,12]. The partition coefficients of

Table 1
Structure-specific and pH-dependent fluidizing effects of local anesthetics on liposomal membranes

pH	Polarization change from control				
	Mepivacaine	Prilocaine	Lidocaine	Ropivacaine	Bupivacaine
5.9	$-0.0018 \pm 0.0008^{*}$	$-0.0051 \pm 0.0010^{**}$	$-0.0045 \pm 0.0002^{**}$	$-0.0057 \pm 0.0012^{**}$	$-0.0092 \pm 0.0012^{**}$
6.9	$-0.0054 \pm 0.0007^{**}$	$-0.0066 \pm 0.0007^{**}$	$-0.0089 \pm 0.0006^{**}$	$-0.0117 \pm 0.0005^{**}$	$-0.0205 \pm 0.0006^{**}$
7.4	$-0.0094 \pm 0.0012^{**}$	$-0.0156 \pm 0.0011^{**}$	$-0.0160 \pm 0.0003^{**}$	Not determined	$-0.0222 \pm 0.0009^{**}$
7.9	$-0.0097 \pm 0.0015^{**}$	$-0.0204 \pm 0.0005^{**}$	$-0.0190 \pm 0.0007^{**}$	Not determined	$-0.0315 \pm 0.0010^{**}$

Mean ± S.E. ($n = 5$ or 6).
* $P < 0.05$.
** $P < 0.01$ compared with control.

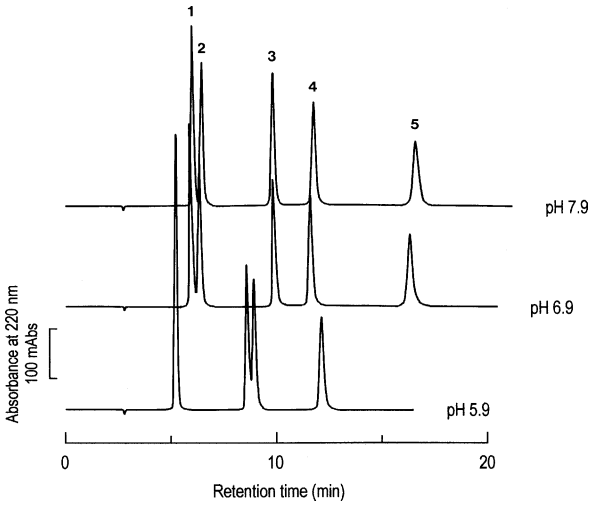

Fig. 2. Typical chromatograms of local anesthetics obtained by using octadecyl-bonded silica column and mobile phase buffer of different pH (5.9, 6.9 and 7.9). Peaks: (1) mepivacaine; (2) prilocaine; (3) lidocaine; (4) ropivacaine and (5) bupivacaine.

various anesthetics into membranes are much smaller than those into bulk lipids [10]. Reversed-phase HPLC retention is more suitable for studying the interaction between local anesthetics and membrane lipids than bulk-phase hydrocarbon/water partitioning because the alkyl chains of stationary phases are structured and the penetration of drugs into these membraneous chains is based on an entropy-driven process not associated with the transfer into bulk lipids [29,30]. In addition to structure-dependent changes, the capacity factors of local anesthetics decreased with lowering the mobile phase pH from 7.9 to 5.9.

The retention and membrane activity relationships were confirmed by plotting the capacity factors determined at mobile phase pH 6.9 against the degree of membrane fluidization (DPH polarization decrease) induced at reaction pH 6.9 (Fig. 3). Linear regression analyses provided $y = 0.0038x + 0.0005$ ($R^2 = 0.9585$, $P < 0.005$) for octadecyl, $y = 0.0055x - 0.0004$ ($R^2 = 0.9459$, $P < 0.01$) for octyl and $y = 0.010x - 0.004$ ($R^2 = 0.8873$, $P < 0.05$) for phenyl stationary phase. Previous HPLC studies correlating chromatographic retention with partition coefficients reported that the phenyl stationary phase gave a better correlation than the octadecyl phase [26,31,32]. However, the present results showed that a correlation between capacity factor and membrane activity was greatest in the order of octadecyl-, octyl- and phenyl-bonded silica column. Such results may be interpreted by the analogous mechanisms of membrane fluidization and chromatographic retention, that is, the interaction of local anesthetics with the hexadecanoyl chains of

Table 2
Capacity factors of local anesthetics determined by reversed-phase HPLC using different stationary phases and mobile phase pH

Column and pH	Capacity factors				
	Mepivacaine	Prilocaine	Lidocaine	Ropivacaine	Bupivacaine
Octadecyl-bonded silica					
5.9	0.9297 ± 0.0029	0.9297 ± 0.0029	2.1657 ± 0.0037	2.2759 ± 0.0055	3.4172 ± 0.0083
6.9	1.1884 ± 0.0023	1.3390 ± 0.0021	2.6242 ± 0.0031	3.2490 ± 0.0028	4.9883 ± 0.0036
7.4	1.2744 ± 0.0019	1.4439 ± 0.0022	2.7205 ± 0.0047	3.4184 ± 0.0042	5.2037 ± 0.0067
7.9	1.3036 ± 0.0006	1.4805 ± 0.0009	2.7877 ± 0.0016	3.5209 ± 0.0019	5.3745 ± 0.0033
Octyl-bonded silica					
5.9	0.7477 ± 0.0014	0.7477 ± 0.0014	1.6900 ± 0.0023	1.6900 ± 0.0023	2.4717 ± 0.0030
6.9	0.9532 ± 0.0006	1.0634 ± 0.0006	2.0106 ± 0.0007	2.3945 ± 0.0011	3.5425 ± 0.0017
7.4	0.9913 ± 0.0006	1.1185 ± 0.0006	2.0871 ± 0.0007	2.5236 ± 0.0009	3.7491 ± 0.0012
7.9	0.9972 ± 0.0070	1.1274 ± 0.0069	2.1040 ± 0.0069	2.5596 ± 0.0065	3.8082 ± 0.0069
Phenyl-bonded silica					
5.9	0.6763 ± 0.0010	0.5704 ± 0.0011	1.1790 ± 0.0008	1.1790 ± 0.0008	1.5166 ± 0.0006
6.9	0.9478 ± 0.0004	0.8768 ± 0.0004	1.5213 ± 0.0003	1.7507 ± 0.0003	2.2404 ± 0.0004
7.4	0.9736 ± 0.0006	0.9050 ± 0.0004	1.5569 ± 0.0006	1.8158 ± 0.0007	2.3284 ± 0.0008
7.9	0.9818 ± 0.0008	0.9089 ± 0.0011	1.5640 ± 0.0018	1.8313 ± 0.0018	2.3461 ± 0.0028

Mean ± S.E. ($n = 6$).

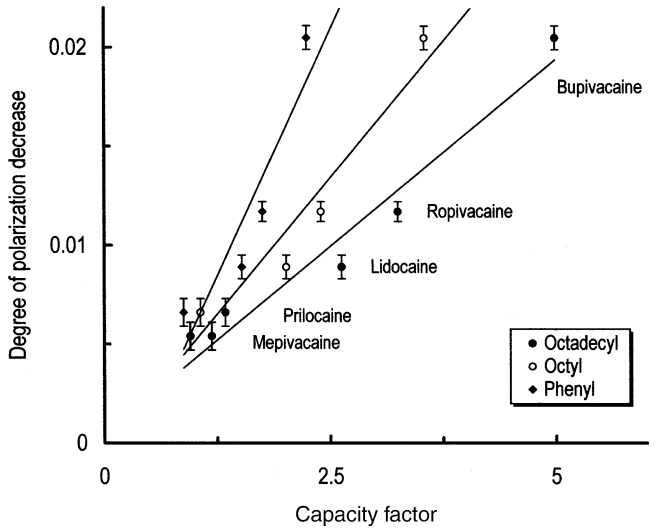

Fig. 3. Relation between chromatographic retention and membrane fluidization. Local anesthetics were chromatographed on different stationary phases with the mobile phase of pH 6.9 and reacted with DPPC liposomes at pH 6.9. The capacity factors were plotted against the degree of DPH polarization decrease.

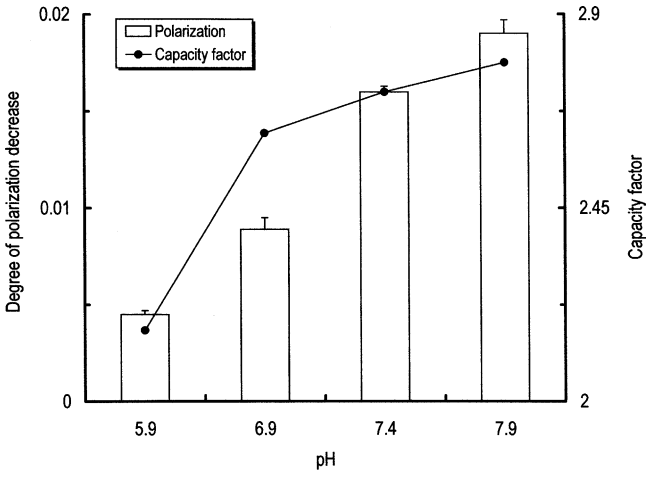

Fig. 4. pH-dependent chromatographic retention and membrane fluidization of lidocaine. Lidocaine was chromatographed on an octadecyl-bonded silica column and reacted with DPPC liposomes by varying mobile phase and reaction pH. The capacity factors were illustrated together with the degree of DPH polarization decrease.

membrane DPPC and with the octadecyl chains of stationary phase.

The deviation from linearity is considered to be caused by different pK_a values of drugs which influence their penetration into lipid bilayers and their potency to fluidize membranes. The reported pK_a is 8.19 for lidocaine, 8.16 for ropivacaine and 8.21 for bupivacaine, whereas being 7.92 for mepivacaine and 8.02 for prilocaine [12]. Compared at pH around neutrality, local anesthetics of smaller pK_a are likely to interact with membrane lipids more intensively than ones of larger pK_a.

When lowering the pH of both mobile phase and reaction buffer from 7.9 to 5.9, the capacity factors on an octadecyl-bonded silica column decreased together with the membrane-fluidizing effects of lidocaine (Fig. 4) and mepivacaine (Fig. 5). Similar results were also obtained from the other local anesthetics (data not shown). Although co-decreasing changes were apparent between chromatographic retention and membrane fluidization, a pH-dependent decrease of the former was not so steep as that of the latter. Such difference could be produced by the residual unreacted silanol groups of silica particles which interact with bases like local anesthetics and retain them more intensively than predicted. Local anesthetics are clinically known not to work well in inflamed tissues. Various pharmacological mechanisms for this phenomenon have been speculated: the increased blood flows promote the removal of drugs from the injection site, the increased proteinous exudates adsorb drugs to deactivate them, certain inflammatory chemical mediators influence nerve transmission and anesthetic activity, and the morphological changes occur in nerve trunks and even in the central nervous system [7]. In addition, it has been generally accepted that the anesthetic failure associated with inflammation is due to tissue acidity [28]. This hypothesis is supported by a correlating reduction of membrane fluidization and chromatographic retention.

Reversed-phase liquid chromatography, especially on the octadecyl stationary phase, appears to be useful for estimating the structure-specific and pH-dependent membrane-fluidizing effects of local anesthetics as the mode of action. An HPLC method may be the possible replacement for bioassays which are usually employed to study the anesthetic potency comparatively.

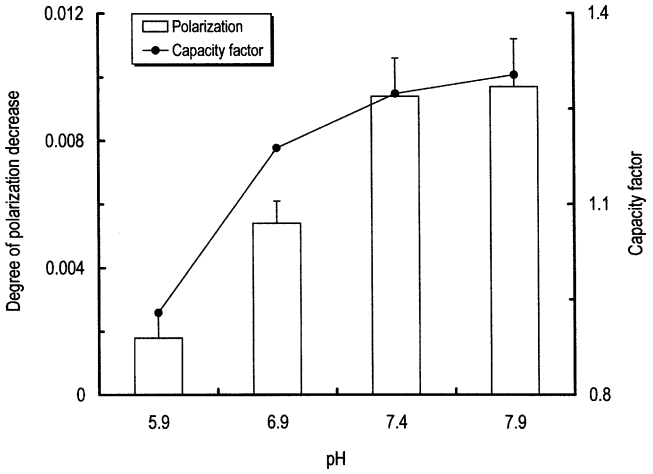

Fig. 5. pH-dependent chromatographic retention and membrane fluidization of mepivacaine. Mepivacaine was chromatographed on an octadecyl-bonded silica column and reacted with DPPC liposomes by varying mobile phase and reaction pH. The capacity factors were illustrated together with the degree of DPH polarization decrease.

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