Effects of various adjuvants on the physicochemical characteristics of the methyl salicylate/Tween® 20-isopropyl alcohol/water system

ผลของสารเสริมต่อคุณลักษณะทางกายภาพเคมีของระบบเมทิลซาลิไซเลต/ทวีน 20-ไอโซโพรพิลแอลกอฮอล์/น้ำ

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This research is supported by Huachiew Chalermprakiet University

Academic Year 2012

Research Title: Effects of various adjuvants on the physicochemical characteristics of the methyl salicylate/Tween 20-isopropyl alcohol/water system

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Year of Publication: 2016

Publisher: Huachiew Chalermprakiet University

Sources: Huachiew Chalermprakiet University

No. of Pages: 58 pages

Keywords: Methyl salicylate, Microemulsion, Menthol

ABSTRACT

The aim of this study was to investigate the use of methyl salicylate, the oily active substance, as oil phase in microemulsion preparation. The effects of types of adjuvants in the oil phase as well as their amounts on the phase behaviors and the physicochemical properties of the prepared formulations were also observed. In addition, the possibility of loading indomethacin into the investigated systems was also investigated. The pseudoternary phase diagram of methyl salicylate/Tween 20-IPA(1:1)/water system was first constructed as control. Then, other systems using methyl salicylate as oil phase in the presence of the studied adjuvants were investigated to illustrate their phase behaviors. Adjuvants in this study were eugenol, menthol, Plai oil, oleic acid, isopropyl palmitate, and isopropyl myristate. Each adjuvant was incorporated at three different weight ratios of methyl salicylate to adjuvant varying as 3:1, 2:1, 1:1. The pseudoternary phase diagram results showed that incorporation of menthol remarkably reduced the single phase area. Afterwards the formulations composed of 15% oil phase in the presence and absence of adjuvants, 35% water, and 50% the Tween 20-IPA (1:1) mixture were prepared and characterized. The effect of types of adjuvants and their amounts on the physicochemical properties of the prepared formulations varied considerably from adjuvant to adjuvant. All prepared formulations

were o/w microemulsions. They were isotropic transparent homogenous liquid mixtures with the nano-size particle droplets and narrow size distribution. They were of low viscosity with Newtonian flow. After long term stability for one year, the methyl salicylate microemulsion and the systems whose oil phases were composed of menthol, isopropyl palmitate or isopropyl myristate at the methyl salicylate-adjuvant weight ratios of 3:1 and 2:1 exhibited good physical stability without phase separation and precipitation. Colour remained unchanged. Indomethacin at the concentration of 0.75% was incorporated into the systems. All indomethacin-loaded methyl salicylate microemulsions in the presence and absence of proper adjuvants were transparent homogenous yellowish liquids with low viscosity values. They were still o/w microemulsions. They showed lower pH values in comparison with their indomethacin-free counterparts. After storage at ambient temperature for six months, the indomethacin-loaded methyl salicylate microemulsion whose oil phase was composed of menthol as adjuvant at the methyl salicylate-adjuvant weight ratios of 3:1 showed good stability.

ชื่อเรื่อง: ผลของสารเสริมต่อคุณลักษณะทางกายภาพเคมีของระบบเมทิลซาลิไซเลต/ทวีน 20-ไอโซโพรพิลแอลกอฮอล์/น้ำ
ผู้วิจัย: วารุณี ลี้สัจจะกูลสุนี ชาญณรงค์
สถาบัน: มหาวิทยาลัยหัวเฉียวเฉลิมพระเกียรติ
ปีที่พิมพ์: 2559
สถานที่พิมพ์: มหาวิทยาลัยหัวเฉียวเฉลิมพระเกียรติ
แหล่งที่เก็บรายงานฉบับสมบูรณ์: มหาวิทยาลัยหัวเฉียวเฉลิมพระเกียรติ
จำนวนหน้างานวิจัย: 58 หน้า
คำสำคัญ: เมทิลซาลิไซเลต ไมโครอิมัลชัน เมนทอล
ลิขสิทธิ์: มหาวิทยาลัยหัวเฉียวเฉลิมพระเกียรติ

บทคัดย่อ

งานวิจัยนี้เป็นงานด้านวิทยาศาสตร์ประยุกต์ที่ดำเนินการวิจัยโดยการทดลองในห้องปฏิบัติการ มีวัตถุประสงค์การวิจัยเพื่อศึกษาการใช้สารเมทิลซาลิไซเลตเป็นวัฏภาคน้ำมันในการเตรียมไมโครอิมัลชันและศึกษาถึงผลของชนิดและปริมาณสารเสริมที่เติมลงในวัฏภาคน้ำมันของระบบซึ่งประกอบด้วยเมทิลซาลิไซเลต/ทวีน 20-ไอโซโพรพิลแอลกอฮอล์/น้ำต่อการเกิดพฤติกรรมระบบวัฏภาคเดี่ยวในแผนภาพไตรภาคเทียมและผลที่มีต่อคุณลักษณะทางกายภาพเคมีของตำรับไมโครอิมัลชันที่เตรียมขึ้น นอกจากนี้ยังศึกษาถึงความเป็นไปได้ในการบรรจุยาอินโดเมทาซินซึ่งเป็นแก้อักเสบที่ไม่ใช่สเตียรอยด์(NSAIDs) ลงไปในระบบวัฏภาคเดี่ยวที่เลือกเตรียมขึ้น สารเสริมที่ใช้ในการศึกษาได้แก่ ยูจีนอล เมนทอล น้ำมันไพลารดโอเลอิก ไอโซพรอพริลปาลมิเทตและไอโซพรอพริลมัยริสเทต ปริมาณเมทิลซาลิไซเลตที่ใช้ในการศึกษาคิดเป็นอัตราส่วนต่อสารเสริมเท่ากับ 1:1, 2:1 และ 3:1

ผลจากการวิจัยพบว่าเมทิลซาลิไซเลตสามารถใช้เป็นวัฏภาคน้ำมันในการเตรียมเมทิลซาลิไซเลตไมโครอิมัลชันจากการศึกษาการสร้างแผนภาพไตรภาคเทียมพบว่าสารเสริมเมนทอลมีผลลดขนาดระบบวัฏภาคเดี่ยวในแผนภาพไตรภาคเทียมอย่างชัดเจน จากผลในแผนภาพไตรภาคเทียม

ทำให้ได้ความเข้มข้นของส่วนประกอบในการเตรียมระบบวัฏภาคเดี่ยว (ไมโครอิมัลชัน) ซึ่งประกอบด้วยวัฏภาคน้ำมัน 5% วัฏภาคน้ำ 35% และ 50% สารละลายผสมระหว่างทวีน 20-ไอโซโพรพิลแอลกอฮอล์ จากการศึกษาคุณลักษณะทางกายภาพเคมีของระบบวัฏภาคเดี่ยวที่เตรียมขึ้น พบว่า ทุกระบบวัฏภาคเดี่ยวที่เตรียมได้ทั้งตำรับที่มีสารเสริมและไม่มีสารเสริมเป็นไมโครอิมัลชันชนิดน้ำมันในน้ำมีลักษณะเป็นของเหลวใส ความหนืดต่ำ มีรูปแบบการไหลแบบนิวโตเนียน วัฏภาคภายในมีขนาดเล็ก มีการกระจายขนาดอนุภาคต่ำ ภายหลังการเก็บรักษาในอุณหภูมิแวดล้อมปกติเป็นเวลานาน 1 ปี พบว่า ตำรับเมทิลซาลิไซเลตไมโครอิมัลชันทั้งในกรณีที่ไม่มีสารเสริมและในตำรับที่มีสารเสริมเมนทอล ไอโซพรอพริลปาลมิเทตและไอโซพรอพริลมัยริสเทตที่อัตราส่วนเมทิลซาลิไซเลตต่อสารเสริมเท่ากับ 2:1 และ 3:1 เป็นตำรับที่มีความคงตัวดีทางกายภาพ ไม่เกิดการเปลี่ยนสีเมื่อสังเกตด้วยตาเปล่า ไม่เกิดการแยกชั้นและไม่มีตะกอนเมื่อนำมาบรรจุยาอินโดเมทาซินที่ระดับความเข้มข้น 0.75% พบว่าตำรับที่เตรียมได้มีลักษณะเป็นของเหลวใสสีอมเหลือง มีความหนืดต่ำ เป็นไมโครอิมัลชันชนิดน้ำมันในน้ำ ค่าพีเอชต่ำลงเมื่อเปรียบเทียบกับตำรับที่ไม่มีการบรรจุยาอินโดเมทาซิน ภายหลังการเก็บรักษาในอุณหภูมิแวดล้อมปกติเป็นเวลานาน 6 เดือน พบว่า อินโดเมทาซิน-เมทิลซาลิไซเลตไมโครอิมัลชันในตำรับที่มีสารเสริมเมนทอลที่อัตราส่วนเมทิลซาลิไซเลตต่อสารเสริมเท่ากับ 3:1 เป็นตำรับที่มีความคงตัวดี

Acknowledgements

This research was supported by a grant from Huachiew Chalermprakiet University.

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List of Abbreviations

AOAC	Association of Official Analytical Chemists
cm^3	cubic centimeter (s)
CPP	critical packing parameter
cPs	centipoise
$^{\circ}C$	Celsius degrees
g	gram (s)
HLB	hydrophile-lipophile balance
IPA	isopropyl alcohol
IPM	isopropyl myristate
IPP	isopropyl palmitate
µS/cm	microsiemens per centimeter
nm	nanometer (s)
o/w	oil in water
%	percent
pH	the negative logarithm of the hydrogen ion concentration
pKa	log of the reciprocal of the acid dissociation constant
rpm	revolutions per minute
S.D.	standard deviation
w/o	water in oil
w/v	weight by volume
w/w	weight by weight

Chapter I

Introduction

A miroemulsion is a single macroscopically homogeneous liquid solution which is a mixture of oil, water and surfactant but usually a second surfactant so-called cosurfactant is required. Microemulsions are optically transparent and thermodynamically stable. Microemulsions can form spontaneously after mixing the proper concentration of components together without the need of more energy input and sophisticated machines.

The microstructures of miroemulsions can be divided into three types: water-in-oil (w/o), bicontinuous structure, and oil-in water (o/w). It is well established that the transitions from one microstructure to another microstructure can occur over a wide range of compositions which depend on the properties of the oil and the surfactant used. Several factors were known to affect the microstructure, including chemical natures as well as concentrations of components, and sample environment investigated such as temperature and pressure. In addition, the presence of solubilized drug molecules are also important and should be concerned.

Microemulsions were found to be the promising topical drug delivery system for drugs and cosmetic substances. Many microemulsion researches were carried out in the aspects of its superior transdermal application to solutions or conventional dosage forms [1-4]. It is known that microemulsion compositions are crucial for skin permeation of microemulsions. More attention was paid for finding either the appropriate inert oil or the appropriate surfactant-cosurfactant system for preparing the microemulsion system good enough to the selected model drugs and cosmetic substances.

In this study, we studied the possibility of using a drug substance with oily characteristic as lipophilic phase in preparing the topical microemulsion system. Microemulsion study to date has never dealt with this point. We would like to gain insight into whether an oil drug substance itself can be used as oil phase in preparing the microemulsion. In an attempt, methyl salicylate was used as oil phase. Most common substances found in topical formulations were added as adjuvant so called additive to oil phase at different weight ratios of methyl salicylate to adjuvant.

The success of this research provided a new aspect in microemulsion preparation. This study was carried out as follows: Firstly, the pseudoternary phase diagrams of the methyl salicylate/Tween® 20-IPA(1:1)/water system in the presence and absence of selected adjuvants were investigated. Secondly, the prepared formulations in the presence and absence of selected adjuvants were examined under the microemulsion characterization. Finally, indomethacin, as the selected anti-inflammatory model drug, was incorporated into the selected systems and their physicochemical properties were characterized.

The objectives of the study were:

1. to investigate the effect of different selected adjuvants in the oil phase as well as their amounts on the pseudoternary phase diagram of the methyl salicylate/Tween® 20-IPA(1:1)/water system.

2. to investigate whether methyl salicylate can be used as oil phase in microemulsion preparation.

3. to characterize the physicochemical properties of the single-phase mixtures obtained from the methyl salicylate/Tween® 20-IPA(1:1)/water system in the presence and absence of selected adjuvants at different weight ratios of methyl salicylate to adjuvant.

4. to determine the possibility of loading a selected anti-inflammatory model drug, indomethacin, into the investigated microemulsion systems and afterwards characterize the physicochemical properties of the obtained drug-loaded microemulsions.

The success in this study introduced a new alternative delivery system of methyl salicylate and gave formulators the opportunity for further development of this alternative delivery system which contain both topical analgesic and anti-inflammatory drug.

Chapter II

Literature Review

A microemulsion is a low viscous macroscopically homogeneous liquid solution, in which, on the microscopic level, there is individual domains of water and oil separated by a layer of amphiphile. Microemulsions unlike emulsions are the nano-size particles and should not be considered as emulsions with very small droplet size. The size of microemulsion droplets is in the range of 20-200 nm. When the size is less than 100 nm which is smaller than the wavelength of light, it looks transparent [5]. Microemulsions and emulsions are quite different. Microemulsions are thermodynamically stable but emulsions are kinetically stable and can undergo separation into the corresponding phases upon storage.

Microemulsions form spontaneously. This is attributed to a negative free energy of microemulsion formation whose thermodynamic rationalization can be simplified by the equation as follows:

$$\Delta G_f = \gamma \Delta A - T \Delta S$$

where ΔG_f is the free energy of formation, γ is surface tension of the oil–water interface, ΔA is the change in interfacial area on microemulsification, ΔS is the change in entropy of the system which is effectively the dispersion entropy, and T is the temperature. When microemulsion is formed, the interfacial area is increased due to occurrence of the large number of very small droplets. An essential requirement is that the interfacial tension between the oil and water phases γ, must be reduced to a very low value, providing a small but positive value. However, the mixing of one phase in the other produces the large dispersion of the small droplets in the continuous phase, favourably increasing the entropy of the system. When the change in entropy of the system is dominant, a negative free energy of formation is achieved. Thus microemulsions form [6]. To prepare microemulsions, it is recognized that the easy way to gain insight into the microemulsion formation is to construct a phase diagram of at least three main components: oil, water and surfactant. After a phase diagram is constructed, the isotropic clear region is subsequently defined where its microstructure can be identified.

Microemulsion compositions and ingredients

As was known, microemulsion is clear stable homogenous mixture of oil, water, and surfactant. In the pharmaceutical application, microemulsions more commonly contain additional components such as cosurfactant, cosolvent and drug. When microemulsions are formulated to be used as a topical delivery system, the selection of formulation compositions and their relative concentrations in the mixture are of importance. Some ingredients may have penetration enhancement property. The combination of these enhancers should have to a certain extent synergistic enhancing effect on the permeation behavior of drug through the skin.

Oil phase

The word "oil" is defined for an organic phase that is immiscible with water. Saturated and unsaturated fatty acids can be used as oil phase. Several saturated and unsaturated fatty acids possess penetration enhancing property of their own. Therefore, they can be used as oil phase and penetration enhancer. Oleic acid is the most popular. In addition to fatty acids, esters, terpenes or medium chain triglycerides are usually served as oil phase. Commonly used esters are, for example, isopropyl myristate, isopropyl palmitate, isostearylic isostearate, glycerin triacetate. R(+)-limonene and Mygliol 812® are examples of terpenes and medium chain triglycerides, respectively.

Methyl salicylate can be obtained by distilling the leaves of wintergreen (*Gaultheria procumbens* L.) or produced synthetically by the esterification of synthetic salicylic acid. Methyl salicylate obtained from the leaves of wintergreen is referred to as wintergreen oil. It is indistinguishable from that prepared synthetically. Methyl salicylate as a counterirritant is used topically for arthritis and musculoskeletal disorders in the concentration range of 10-60% in the forms of liniments, gels, lotions or ointments [7].

Oleic acid, an unsaturated long-chain fatty acid with cis- configuration, was reported to greatly increase the flux of salicylic acid 28-fold and 5-flurouracil flux 56-fold through human skin membrane in vitro [8]. It interacts with and modifies the lipid domains of the stratum corneum by decreasing the phase transition temperatures of the skin lipids with a subsequent increase in motional freedom or fluidity of these structure [9-10].

As was known, the microemulsion is one of the potential drug delivery systems for topical use. The solubilizate partitions from the microemulsion droplets to the external phase during the delivery process. In preparation, after solubilizate is soluble, there should be a preferred site of solubilisation. Lipophilic drugs are preferably solubilized in apolar oil phase and/or hydrophobic tail region of the surfactant molecule [6]. The selection of oil phase as well as surfactant in which drug should be soluble is of importance.

Isopropyl myristate (IPM), fatty acid ester, was noted to be used as percutaneous absorption enhancers. It markedly enhanced nicorandil permeation across the excised hairless rat skin. Aliphatic esters such as IPM penetrate into the stratum corneum and increase the lipid fluidity by disruption of lipid packing, thereby increasing the diffusivity of drug and solvent in the stratum corneum and/or partition coefficient between the vehicle and stratum corneum [11-12]. The recent study of Engelbrecht et al. [13] concluded that IPM's mode of action as penetration promoter is presumably based on incorporation into the stratum corneum lipid matrix, extraction of certain stratum corneum lipids into a separate phase and perturbation of the multilamellar lipid assembly.

Isopropyl palmitate (IPP) is also a fatty acid ester. It is considered to be safe and have been widely used as humectants in cosmetic industry. In addition, it was found to have penetration enhancing capacity. IPP was reported to promote the skin penetration of three lipophilic compounds such as gliclazide, nimesulfide, and oxaproxin and one hydrophilic compound such as ribavirin across excised rat abdominal skin. The effectiveness was concentration-dependent [11-14].

Menthol, a monocyclic monoterpene obtained from peppermint oil or other mint oil or produced synthetically, was observed to exhibit permeation enhancing property [15-16]. The study of Kunta et al. [16] suggested the potential use of menthol as effective penetration enhancer. It significantly enhanced the permeability across the mouse skin of propranolol delivered from the hydrogel-based patches containing menthol as enhancer in comparison with that from the control patch (no menthol). They also suggested that its permeation enhancement mechanism could involve its distribution preferentially into the intercellular spaces of stratum

corneum and the possible reversible disruption of the intercellular lipid domain. In addition, menthol was also found to be an effective permeation enhancer for imipramine hydrochloride, a polar and water-soluble drug. The proposed mechanism for enhanced permeation of imipramine hydrochloride is the disruption of the hydrogen bond network at the head of ceramides in the lipid bilayer [17].

Menthol is known to have not only antipruritic properties but also cooling effect; therefore, it is effective in topical preparation as an antipruritic in the concentration of 0.1-1.0%. When applied to the skin, menthol causes vasodilation followed by a feeling of numbness, coolness and mild local anesthesia [18]. When used in higher concentrations in the concentration of 1.25-16%, menthol is used as a counterirritant [7].

Eugenol obtained from oil of cloves. Eugenol is colorless or pale yellow liquid with odor of cloves and spicy, pungent taste. It is practically insoluble in water, miscible with alcohol, chloroform, and oils. It darkens and thickens on exposure to air [19]. Eugenol was found to act on contact to depress cutaneous receptors involved in pain perception. Pronounced inhibition of prostaglandin and leukotrine biosynthesis provide additional analgesic activity [20]. Eugenol was usually found in topical analgesic formulation.

Plai oil is the essential oil obtained from rhizome extracts of *Zingiber cassumunar* Roxb. Plai oil has a pale amber color and a green peppery odor with a touch of a bite. The main active chemical constituents of plai oil are sabinene (27-34%), γ-terpinene (6-8%), α-terpinene (4-5%), terpinen-4-ol (30-35%), and (E)-1-(3,4- dimethoxyphenyl) butadiene (DMPBD) (12-19%) [21]. The Essential Plai oil has anti-inflammatory effect and analgesic action. Therefore, Plai oil is the essential oil of choice that has proven to be useful in general for folklore medicine in Thailand for the treatments of inflammation, sprains and strains, rheumatism, muscular pain and so on. [22].

Surfactants

Surfactants so-called surface-active agents are amphiphilic molecules in which a polar water-soluble head group is attached to a water-insoluble hydrocarbon chain. Surfactants are divided into four groups based on the characteristic of polar head group as follows: anionic, cationic, amphoteric and nonionic. The nature structure of a surfactant make it possible to adsorb

to surfaces or interfaces to reduce surface or interface tension. Therefore, surfactants are used as emulsifying agent, wetting agent, solubilizing agent, and detergent. The lowering of the interfacial tension between oil and water phases facilitates the formation of emulsions, nanoemulsions and microemulsions. However, the amount of surfactant in emulsion formulations is lower than that in microemulsions. This is because microemulsions have more interface areas between the aqueous and oil phases. In addition, surfactants help to solubilise low water-soluble active substances in the formulation and therefore make drug incorporation easier. The more surfactant there is, the higher solubilization capacity it is. Moreover, surfactants were reported to have potential to enhance skin permeation. However, the skin permeation property of surfactants is different from each other. It seems that anionic surfactants have a more penetration enhancement effect on human skin than nonionic surfactant. The use of ionic surfactants is confined because of concern about their toxicities. Zwitterionic surfactants are less toxic than ionic surfactants. Naturally occurring lipids used as surfactant phase are dioleylphosphatidyl ethanolamine, phosphatidylcholine, and distearoylphosphatidylcholine. Nonionic surfactants especially Tweens are widely used in pharmaceutical and cosmetic applications. The key to success in formulation development is surfactant selection. The hydrophile–lipophile balance (HLB) and the critical packing parameter (CPP) are the useful guide.

Tween®20 was reported to enhance penetration of the hydrophilic molecule to partition across the skin by affecting the skin barrier function [23-24]. Tween®20 was also found to improve the permeation of lidocaine across hairless mouse skin [25].

Cosurfactants

Cosurfactants are mostly short (ethanol) and medium-chain (propanol to octanol) alcohols; however, some long chain alcohols such as decanol and lauryl alcohol were also used [26-27]. The cosurfactant itself cannot form microstructure. The cosurfactant not only helps surfactant to further decrease the interfacial tension to zero but also makes the interfacial surfactant film fluidize by penetrating into the surfactant film and consequently creating a disordered film due to the void space among surfactant molecules [28]. The reduction in interfacial tension approaching zero is necessary for the system to achieve thermodynamic

stability. The flexibility of interfacial film is associated with different curvatures of the oil-water interface required to form microemulsions.

Indomethacin

Indomethacin, an indole acetic acid derivative, is an nonsteroidal anti-inflammatory drug (NSAID). Indomethacin appears as a pale yellow to yellow-tan, odourless or almost odourless, crystalline powder. Its pKa is 4.5. It is practically insoluble in water; soluble 1 in 50 of alcohol. It is sensitive to light. It is stable in neutral or slightly acidic media and unstable in alkaline solution. It decomposes by strong alkali. Indomethacin is used in the treatment of musculoskeletal and joint disorders. Preparations of indomethacin are commercially available in various dosage forms such as conventional capsules, suppositories, oral suspension and extended-release capsules. However, for external supportive treatment of pain, indomethacin is now also commercially available in the form of a topical spray at the strength of 0.8%w/v (Elmetacin®) and topical gel at 0.75%w/w (SATOGESIC® GEL) [19,29].

Microemulsion characterisation

As microemulsions form spontaneously under specific conditions and compositions, the aggregate structures can be altered by changes in system compositions and condition, especially temperature. The direct examination of microemulsion microstructure is of difficulty. Therefore, a combination of many indirect techniques are used to characterize microemulsion systems. Particle size distribution is one of most important parameters of microemulsions to characterize. It give essential information for the evaluation of its stability. For particle size characterization, many technologies including dynamic light scattering (DLS), small angle neutron scattering(SANS), small angle X-ray scattering (SAXS), cryo transmission electron microscopy, and pulsed field gradient spin echo (self-diffusion) NMR have been in use. At the microscopic level, viscosity, conductivity, electrical birefringence, and dielectric methods are employed to get information about the internal physiochemical states of systems. Viscosity measurement indicate the presence of rod-like or worm-like reverse micelles. Conductivity measurement give a means of determining whether a microemulsion is water-continuous or oil-continuous as well as give a

means of monitoring phase inversion phenomena. Dielectric measurements are a means of probing both the structure and dynamic features of the systems [3,6,30].

Chapter III

Materials and Methods

Materials

The chemical substances used in this study are listed below:

1. Methyl salicylate (Lot number #BCBJ9799V, Sigma-Aldrich, Inc., USA)
2. Tween® 20 (Lot S6386784206, Merck Schuchardt OHG, Germany)
3. Isopropyl alcohol (Emsure®, Lot K43851634239, Merck KGaA, Germany)
4. Eugenol (purity 99%, Lot number #STBC6086V, Sigma-Aldrich, Inc., USA)
5. Menthol (BP/USP grade, Batch number Y1208009, S.Tong Chemical Co. Ltd., Bangkok, Thailand).)
6. Plai oil (Code No. E064-T, Make Scents Limited, Thailand)
7. Oleic acid (Lot. 0000334096, Panreac, Panreac Quimica S.A.U., Spain)
8. Isopropyl palmitate (IPP) (DUB IPP, Batch number 11039005, Stearinerie Dubois Fils, France)
9. Isopropyl myristate (IPM) (Ph Eur, NF, Lot. K43907566304, Merck KGaA, Germany)
10. Water with conductance of 6.39 μS/cm.
11. Brilliant blue (The Government Pharmaceutical Organization, Bangkok, Thailand)
12. Indomethacin (CSPC Ouyi Pharmaceutical Co., Ltd., China)
13. Acetonitrile (HPLC grade, LiChrosolv®, Merck KGaA, Germany)
14. Methanol (HPLC grade, Merck KGaA, Germany)

Equipment

1. Electrical balance (Pioneer™ Ohaus®, USA)
2. Particle analyser (Delsa™ Nano C Particle Analyzer, Beckman Coulter®, USA)
3. Conductivity meter (SevenEasy, Mettler Toledo, Germany)
4. Cross-polarized light microscope (Nikon Microscope, Eclipse 50i, Japan)

5. pH meter (Lab 850, Schott® Instrument, Germany)
6. Brookfield® Digital Rheometer (Model DV-II+ Visometer, Brookfield Engineering Laboratory, USA)
7. Vortex mixer (Julabo® Paramix III, Julabo Labortech GmbH, Seelbach, Germany)
8. Ultracentrifuge (Universal 320R, Hettich, UK)
9. Magnetic stirrer (Heidolp Instruments, Germany)
10. Magnetic stirring bar
11. A high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) consists of a CBM-20A (system controller), a LC-20AD (solvent delivery unit), a DGU-20A5R (degassing unit), a SPD-20 A(UV-VIS detector), and a SiliaChrom® XDB1 C8 column (4.6 mm x 250 mm, 5µm).

Methods

1. Construction of the pseudo-ternary phase diagrams

Methyl salicylate was used as the oil phase. Tween® 20 and isopropyl alcohol (IPA) were selected as the surfactant and cosurfactant, respectively. To study the effects of investigated adjuvants on the pseudo-ternary phase diagrams, the investigated adjuvants in this study were nonvolatile substances and essential oils or volatile substances. Nonvolatile substances included oleic acid, isopropyl palmitate and isopropyl myristate. Phai oil and eugenol were selected as essential oils and menthol as volatile subatance. It should be noted that methyl salicylate and the studied adjuvant such as oleic acid, isopropyl palmitate or isopropyl myristate were miscible. Menthol can be soluble in methyl salicylate. The system of methyl salicylate/ Tween® 20-IPA(1:1)/water was used as control and the effect of each adjuvant on the system was investigated.

The pseudo-ternary phase diagrams were constructed using water titration method at ambient temperature to obtain the proper concentration range of components for the range of the single phase. The weight ratio of surfactant to cosurfactant so called K_m was fixed at 1:1. When an adjuvant was added to the oil, the oil phase was prepared with the weight ratio of methyl salicylate to adjuvant at 1:1, 2:1 and 3:1. The oil phase containing methyl salicylate with or without the studied adjuvant and the mixture of Tween® 20 and isopropyl alcohol were weighed

with the weight ratio of oil phase to the mixture of Tween® 20 and isopropyl alcohol at 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. Water was added dropwise to each mixture under magnetic stirring until the mixture became turbid. The concentrations of components were recorded to complete the pseudo-ternary phase diagrams. The contents of methyl salicylate with or without the studied adjuvant, the mixture of Tween® 20 and IPA, and water in the area of the single phase were selected based on these results.

2. Microemulsions

2.1 Preparation of microemulsions

According to the single phase region defined in the pseudo-ternary phase diagrams, the formulations of the single-phase mixture designated as microemulsions were defined and prepared according to the composition shown in Table 1 and 2. The variables were the presence or absence of the studied adjuvants in the oil phase. Formulation without adjuvant designated by the letter A was used as control. The letters 'E', 'M', 'P', 'O', 'IP', and 'IM' indicated the type of adjuvants added which were eugenol, menthol, Plai oil, oleic acid, isopropyl palmitate, and isopropyl myristate, respectively. The letters were followed by a number, the first digit corresponed to the ratio of methyl salicylate to adjuvant. The microemulsions were obtained by mixing the mixture of Tween® 20 and IPA with the oil phases with or without the adjuvant before adding the certain amount of water under magnetic stirring. The prepared microemulsions were kept in well-closed container at ambient temperature for future investigation.

Table 1. The composition of defined microemulsion formulations

Compositions	%w/w
Oil phase with and without adjuvant	15.0
Tween® 20:IPA (1:1)	50.0
Water	35.0

Table 2. Codes of different microemulsion formulations

Type of adjuvants	Formulations	Ratios of methyl salicylate to adjuvant
No adjuvant	A	-
Eugenol	E3	3:1
	E2	2:1
	E1	1:1
Menthol	M3	3:1
	M2	2:1
	M1	1:1
Plai oil	P3	3:1
	P2	2:1
	P1	1:1
Oleic acid	O3	3:1
	O2	2:1
	O1	1:1
Isopropyl palmitate (IPP)	IP3	3:1
	IP2	2:1
Isopropyl myristate (IPM)	IM3	3:1
	IM2	2:1

2.2 Microemulsion characteriazation

2.2.1 Appearance observation

The physical appearance including colour, clarity, the occurrence of phase separation and/or precipitation of the prepared microemulsions were observed visually. After preparation, the samples were stored at ambient temperature for at least 24 hours to achieve equilibrium before visual inspection. The optical isotropy of the resulting formulations was investigated using cross-polarized light microscopy (Nikon Microscope, Eclipse 50i, Japan).

2.2.2 Particle size measurements

The average droplet size and polydispersity index (PI) of the prepared formulations were determined by photon correlation spectroscopy instrument (Delsa™ Nano C Particle Analyzer, Beckman Coulter®, USA) at a temperature of 25°C. Each formulation was run in triplicate. The results were recorded as average±standard deviation.

2.2.3 Viscosity measurements

The viscosities of the prepared formulations were determined by a Brookfield® Digital Rheometer (Model DV-II+ Visometer, Brookfield Engineering Laboratory, USA) using a S18 spindle at 60 rpm. The measurement was run in triplicate. In addition, the correlation coefficients (R_{xy}) between shear rate (x) and shear stress (y) was also observed to indicate the flow property of the prepared formulations. The results were recorded as average±standard deviation.

2.2.4 pH measurements

The pH values of all the prepared formulations were measured at 25°C using a pH meter (Lab 850, Schott® Instrument, Germany). The results were recorded as average±standard deviation.

2.2.5 Electrical conductivity measurements

The electrical conductivity was measured at 25°C using a conductivity meter (SevenEasy, Mettler Toledo, Germany) which was calibrated using the standard solution of 1413 μS/cm before testing. The measurement was run in triplicate. The results were recorded as average±standard deviation.

2.2.6 Stability evaluation

Physical appearances were evaluated by visual inspection of the samples after preparation and after storage for one year at ambient temperature. Stable systems were

determined as those free of any physical change such as colour, clarity, phase separation and/or precipitation. The mean particle sizes of formulations upon storage at ambient temperature for one year were measured to assess whether droplet coalescence and/or aggregation occurred. The pH and conductivity values were also investigated.

3. Preparation and characterization of indomethacin-loaded methyl salicylate microemulsions

3.1 Preparation of indomethacin-loaded methyl salicylate microemulsions

Indomethacin was chosen as a model compound in this study because it is frequently used in the treatment of musculoskeletal and joint disorders. Based on the stability results (2.2.6), seven indomethacin-loaded microemulsions were prepared. The formulation compositions were shown in Table 3. Formulation F1 without adjuvant was used as a control. The indomethacin-loaded methyl salicylate microemulsions were obtained after combining appropriate amounts of all components under magnetic stirring at 800 rpm (Heidolp Instruments, Germany). The obtained indomethacin-loaded methyl salicylate microemulsions were kept in well-closed container at ambient temperature for future investigation.

Table 3. The compositions of indomethacin-loaded microemulsion formulations (%w/w)

Compositions	F1	F2	F3	F4	F5	F6	F7
Methyl salicylate	15.00	11.25	10.00	11.25	10.00	11.25	10.00
Menthol	-	3.75	5.00	-	-	-	-
Isopropyl palmitate	-	-	-	3.75	5.00	-	-
Isopropyl myristate	-	-	-	-	-	3.75	5.00
Tween® 20:IPA (1:1)	50.00	50.00	50.00	50.00	50.00	50.00	50.00
Water	34.25	34.25	34.25	34.25	34.25	34.25	34.25
Indomethacin	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

3.2 Characterization of indomethacin-loaded microemulsions

3.2.1 Appearance observation

See method 2.2.1

3.2.2 Particle size measurements

See method 2.2.2

3.2.3 Viscosity measurements

See method 2.2.3

3.2.4 pH measurements

See method 2.2.4

3.2.5 Conductivity measurements

See method 2.2.5

3.2.6 Centrifugation

Physical stability of indomethacin-loaded microemulsions was tested by centrifugation (Universal 320R, Hettich, UK) at 12,000 rpm for 30 min at 25°C.

3.2.7 Stability evaluation

Physical appearances were evaluated by visual inspection of the samples after preparation and after storage for six months at ambient temperature. Stable systems were determined as those free of any physical change such as colour, clarity, phase separation and/or precipitation. The mean particle sizes of microemulsions upon storage were measured to assess whether droplet coalescence and/or aggregation occurred. The viscosity, pH and conductivity values were also evaluated.

3.2.8 HPLC assay for quantification of methyl salicylate and indomethacin

The contents of two active ingredients were simultaneously quantified using a high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) consisting of a CBM-20A (system controller), a LC-20AD (solvent delivery unit), a DGU-20A5R (degassing unit), a SPD-20 A (UV-VIS detector), and a SiliaChrom® XDB1 C8 column (4.6 mm x 250 mm, 5μm). The chromatographic data analysis was performed with the LCsolution Program (Shimadzu, Japan). The injection volume was 20 μL. The mobile phase was prepared by three solvents A, B and C: solvent A was DI water pH 3.2 (adjusted with phosphoric acid), solvent B was acetonitrile and solvent C was methanol. The separation process followed a gradient elution procedure in which

concentration ratios of solvent A and B changed linearly. The run time program for HPLC step gradient method was as follows: time (min)/solvent A:B:C (%): 0.01-3.00/45:35:20, 3.01-20.00/45-20%A:35-60%B:20%C, 20.01-25.00/20:60:20, 25.01-30.00/20-45%A:60-35%B:20%C and 30.01-35.00/45:35:20. Flow rate was 1.0 mL/min. The retention times of methyl salicylate and indomethacin were about 15.869 and 22.080 minutes, respectively. The detection wavelength was 320 nm. The developed method was validated by determining selectivity, linearity, accuracy, and precision [31-32].

3.2.9 Preparation of reference standard

For analysis of drug contents, the stock solution of reference standard was prepared by dissolving 0.2 g of each drug in 100 ml of methanol.

3.2.10 Sample preparation

The 0.2 g of microemulsion samples was accurately weighted in 10-ml volumetric flask and then diluted to volume with methanol.

3.2.11 Validation of HPLC analysis

The developed method was validated by determining selectivity, linearity, accuracy, and precision. For specificity, the developed HPLC method was tested by injecting placebo with the same concentrations as those in the microemulsion formulations as well as reference standard solution of drugs. The peak of methyl salicylate and indomethacin at the specific retention time must be separated from those of other components.

For analysis of methyl salicylate, the reference stock solution of methyl salicylate in the methanol (2.0 mg/ml) was prepared. Calibration solutions of methyl salicylate were prepared to obtain the concentration range of 500.0-4000.0 µg/ml. The peak area of each concentration was the average of three determinations. The coefficient of determination (r^2) for methyl salicylate was found to be 0.9976.

For analysis of indomethacin, the reference stock solution of indomethcin in the methanol (2.0 mg/ml) was prepared. Calibration solutions of indomethacin were prepared to obtain the concentration range of 25.0-200.0 µg/ml. The peak area of each concentration was the average of three determinations. The coefficient of determination (r^2) for indomethacin was found to be 0.9968.

To assay the known added amount of the analyte in microemulsions, accuracy of the method indicated by the percent recovery was carried out by standard spiking method at three different concentration levels: 80%, 100% and 120%. According to the AOAC guidelines, the limits on the percent recovery of methyl salicylate and indomethacin were in the range of 97-103% and 95-105%, respectively. In this study, the mean percentage recovery of methyl salicylate and indomethacin were 102.7 and 96.6, respectively.

The precision indicated by the percent relative standard deviation (%RSD) was evaluated by determination of five replicate analysis of a fixed amount of methyl salicylate (3000 μg/ml) and indomethacin (150 μg/ml). The accepted value of the %RSD should be not more than 2%. The %RSD of methyl salicylate and indomethacin were 1.13 and 1.62, respectively.

The detection limit was the lowest amount of the analyte in the sample that could be detected. A signal-to-noise ratio of 3:1 was considered acceptable for estimating the detection limit. For quantitation limit, determination of the signal-to-noise ratio established the minimum concentration at which the analyte can be reliably quantified. A signal-to-noise ratio was 10:1.

3.2.12 Statistical analysis

All experiments were carried out in triplicate. Data were expressed as the mean value ± standard deviation (S.D.). Statistical significance of difference was analyzed by Student's *t*-test at the level of p=0.05.

Chapter IV

Results and Discussion

1. Construction of the pseudoternary phase diagram

Because microemulsions form spontaneously by simply mixing suitable mixtures of system compositions. In this study, methyl salicylate was primarily used as oil phase. The effects of six different adjuvants as well as three ratios of methyl salicylate to adjuvant on the single-phase area were plotted using the pseudoternary phase diagram in order to identify the compositions that resulted in the formation of microemulsions. Methyl salicylate is miscible with the Tween® 20-IPA(1:1) mixture. Methyl salicylate in this study was successfully used as oil phase in the phase behavior study. The pseudoternary phase diagram of methyl salicylate/Tween® 20-IPA(1:1)/water system was observed and used as control for comparison with those of adjuvant containing systems. The phase behavior showed a two-phase region and a single-phase region. The area of a single-phase system was designated as microemulsion formation. No attempt was made to identify the other association structures. Therefore, the ranges of appropriate amounts of components in the area of a single-phase system could be defined for microemulsion formulation.

Figure 1 showed the pseudoternary phase diagram of the methyl salicylate, Tween® 20-IPA(1:1), water system in the absence of adjuvant. A single-phase system was observed in an area of the surfactant-cosurfactant rich region. This diagram was used as control to investigate the effects of any adjuvant incorporation.

The effects of the addition of an adjuvant, i.e. eugenol, menthol, Plai oil, oleic acid, isopropyl palmitate and isopropyl myristate into the oil phase on the pseudoternary phase diagram were investigated in comparison with that of the methyl salicylate/Tween® 20-IPA(1:1)/water system. The ratio of methyl salicylate to an adjuvant ranged from 1:1 to 3:1. The results were shown in Figure 2-7. In Figure 2, the pseudoternary phase diagram of the methyl salicylate-eugenol/Tween® 20-IPA(1:1)/water systems were shown. The single-phase zones as observed

were comparable to that of the control system and remained unchanged as the ratio of methyl salicylate to eugenol increased from 1:1 to 3:1.

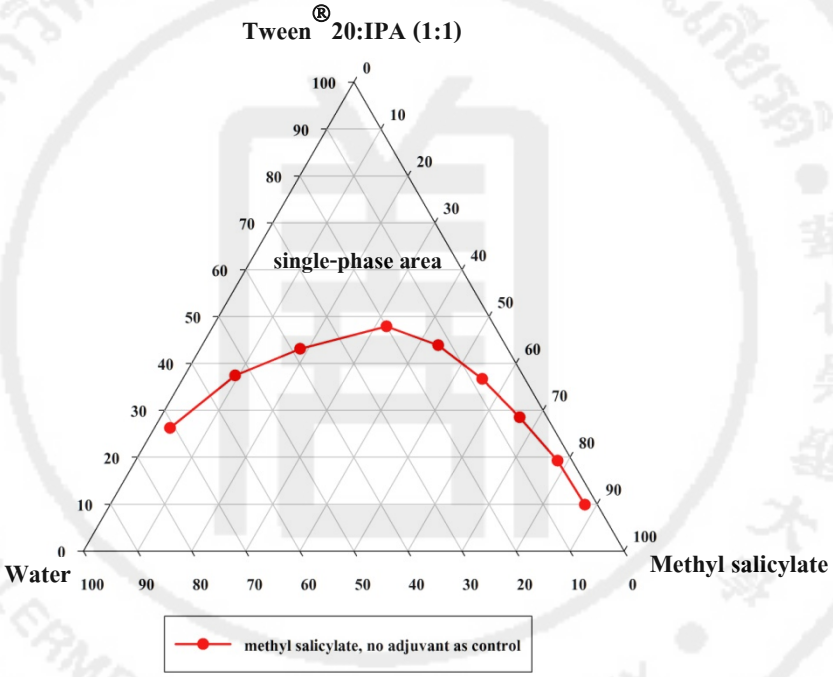

Figure 1. The pseudoternary phase diagram of the methyl salicylate/Tween® 20-IPA(1:1)/water system.

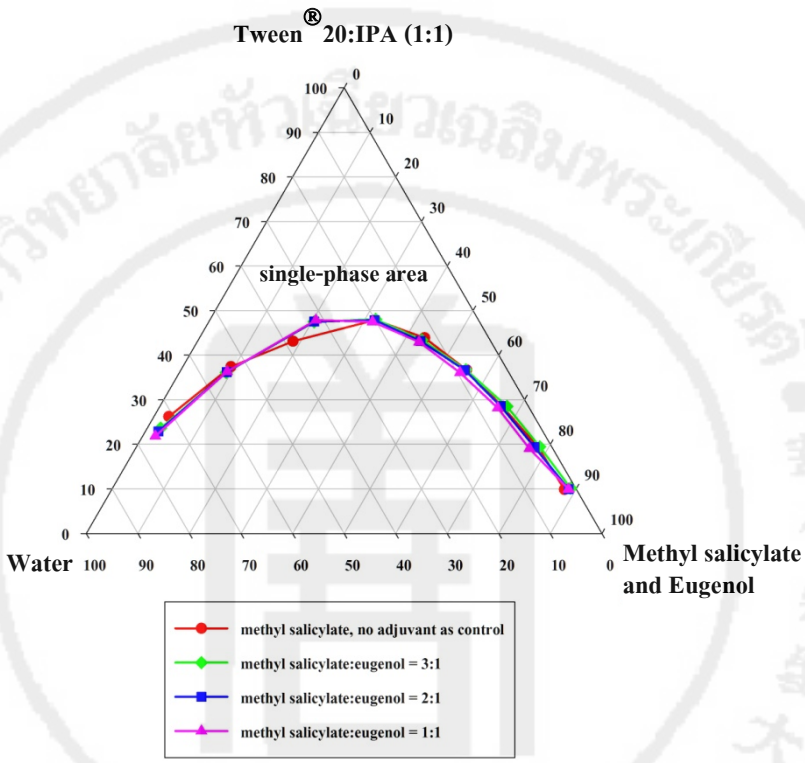

Figure 2. The pseudoternary phase diagrams of the methyl salicylate-eugenol/Tween® 20-IPA(1:1)/water systems at 3:1, 2:1, and 1:1 weight ratios of methyl salicylate to eugenol.

Figure 3 demonstrated the pseudoternary phase diagram of the systems containing menthol an adjuvant. The addition of menthol led to the smaller single-phase areas than that of control system. When the ratios of methyl salicylate to menthol changed from 1:1 to 3:1, the single-phase areas seemed to be unchanged.

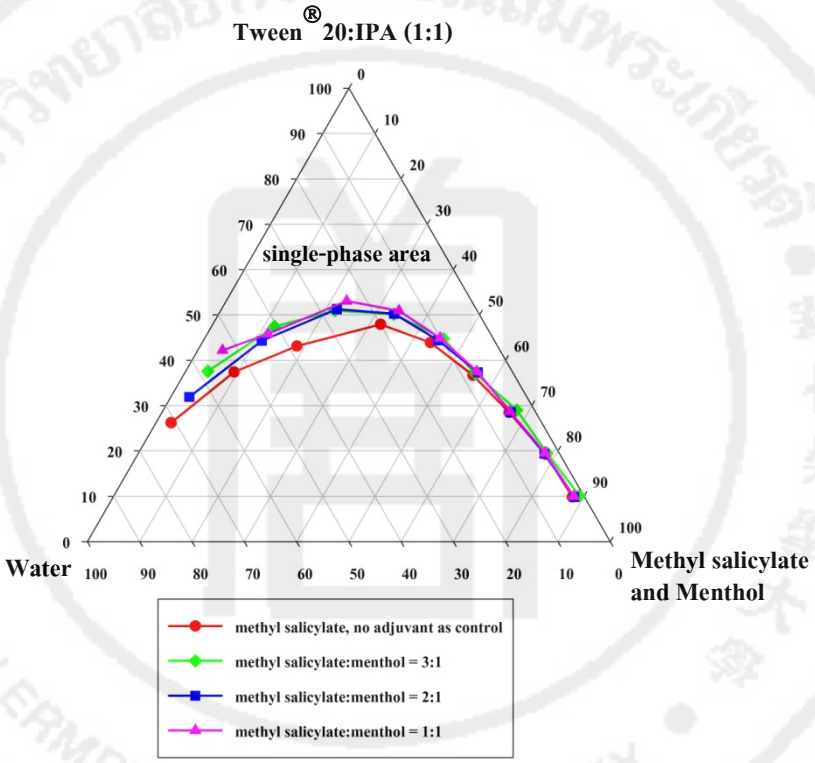

Figure 3. The pseudoternary phase diagrams of the methyl salicylate-menthol/Tween® 20-IPA(1:1)/water systems at 3:1, 2:1, and 1:1 weight ratios of methyl salicylate to menthol.

Figure 4 exhibited the pseudoternary phase diagrams of the methyl salicylate-Plai oil/Tween® 20-IPA(1:1)/water systems. The systems with a methyl salicylate-Plai oil ratio of 3:1 showed the comparable size of the single-phase area in comparison with that of control. In contrast, the systems containing the methyl salicylate-Plai oil ratios of 2:1 and 1:1 showed the smaller the single-phase regions.

Incorporation of oleic acid as an adjuvant at the methyl salicylate-oleic acid ratios of 3:1, 2:1, and 1:1 seemed to have no influence on the single-phase areas in the pseudoternary phase diagram in comparison with that of control as presented in Figure 5.

Figure 6 elucidated the effect of the amount of isopropyl palmitate (IPP) incorporated on the single-phase region in the pseudoternary phase diagram. The phase study obviously illustrated the pronounced effect of the 1:1 ratio of methyl salicylate to IPP which significantly reduced the size of the single-phase area.

Addition of IPM to the methyl salicylate in the oil phase resulted in the phase behavior as shown in Figure 7. It could be noted that the phase behaviors of the combination of isopropyl myristate (IPM) with methyl salicylate were similar to those of the methyl salicylate-IPP/Tween® 20-IPA(1:1)/water systems. The 1:1 ratio of methyl salicylate to IPM showed the most drastic change in the single-phase area.

It is the fact that the study of phase diagram is primarily used as the guideline on the microemulsion formation. It is important to determine whether the interesting substances could be used as oil phase in a given surfactant system to form microemulsions. In general, long-chain triglycerides, such as vegetable oils, medium chain triglyceride and fatty acid esters are often used as pharmaceutical oil excipients in microemulsion formulation. In the previous published studies, it was found that the lower the molecular volume of oils, the greater the surfactant efficiency [33]. In this study, methyl salicylate, eugenol, and menthol are of comparable low molecular volumes of 215.1, 255.5, and 291.5 cm^3/mol, respectively. In contrast, oleic acid, IPM, and IPP have larger molecular volumes of 523.9, 528.2, and 581.2 cm^3/mol, respectively. Small molecular volume substances should be solubilized completely in the tail of surfactant. Even though oleic acid, a polar substance, has about twice the molecular volume of menthol, it unexpectedly showed less effect on the single phase region of the pseudoternary phase diagram. It was confirmed that solubilization of substances used as oil phase depended not only on the

molecular volume but also, to a certain extent, on other physicochemical properties (i.e. chemical structure) of the solubilisate. In addition, the amount of the adjuvant added into the oil phase also played a role in the solubilzation as observed in the cases of incorporation of IPM and IPP. It was possible that the more addition of IPM or IPP at the 1:1 weight ratio of methyl salicylate to IPM or IPP made less efficient the solubilization of a given Tween® 20-IPA system for oil phase, thereby resulting in the smaller single phase area.

Based on the pseudoternary phase diagram results, the appropriate concentrations of three components were selected to obtain the single phase mixtures for further investigation. The desired characteristics of the preparations as well as the maximum and minimum acceptable levels of components for a topical application were also taken into consideration. Therefore, the prepared formulations were composed of oil phase, the surfactant-cosurfactant mixture and water phase at the concentration of 15%, 50%, and 35%, respectively. For systems containing eugenol, menthol, Plai oil, or oleic acid as an adjuvant, the effect of three levels of the methyl salicylate-adjuvant weight ratios (3:1, 2:1, and 1:1) on the physicochemical properties of the formulations were studied whereas the effect of two weight ratios (3:1and 2:1) were investigated for the systems containing IPP or IPM. Therefore, there were 17 formulations for further microemulsion characterization.

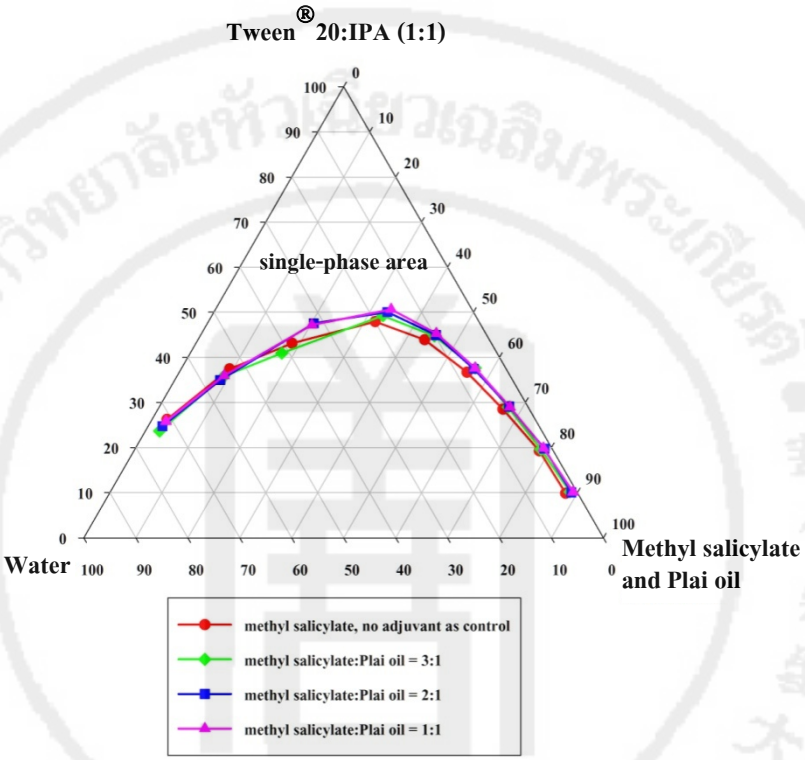

Figure 4. The pseudoternary phase diagrams of the methyl salicylate-Plai oil/Tween® 20-IPA(1:1)/water systems at 3:1, 2:1, and 1:1 weight ratios of methyl salicylate to Plai oil.

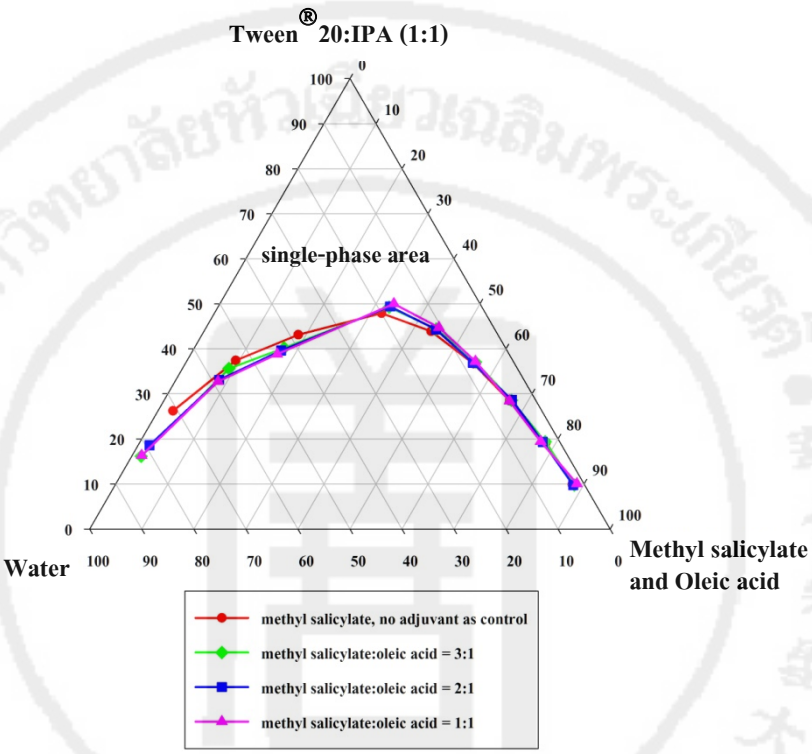

Figure 5. The pseudoternary phase diagrams of the methyl salicylate-oleic acid/Tween® 20-IPA(1:1)/water systems at 3:1, 2:1, and 1:1 weight ratios of methyl salicylate to oleic acid.

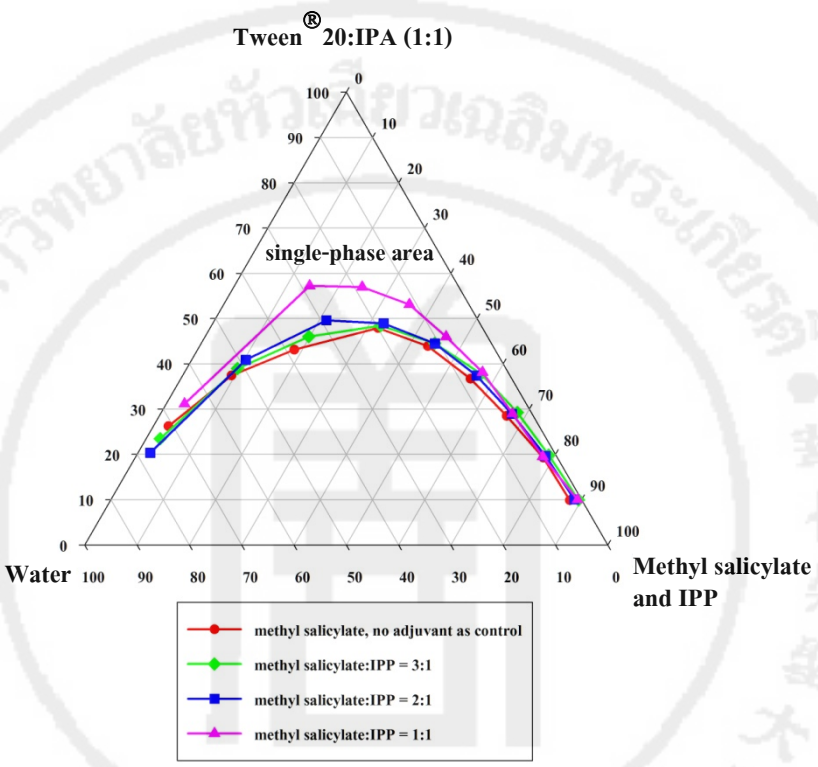

Figure 6. The pseudoternary phase diagrams of the methyl salicylate-IPP/Tween® 20-IPA(1:1)/ water systems at 3:1, 2:1, and 1:1 weight ratios of methyl salicylate to IPP**.**

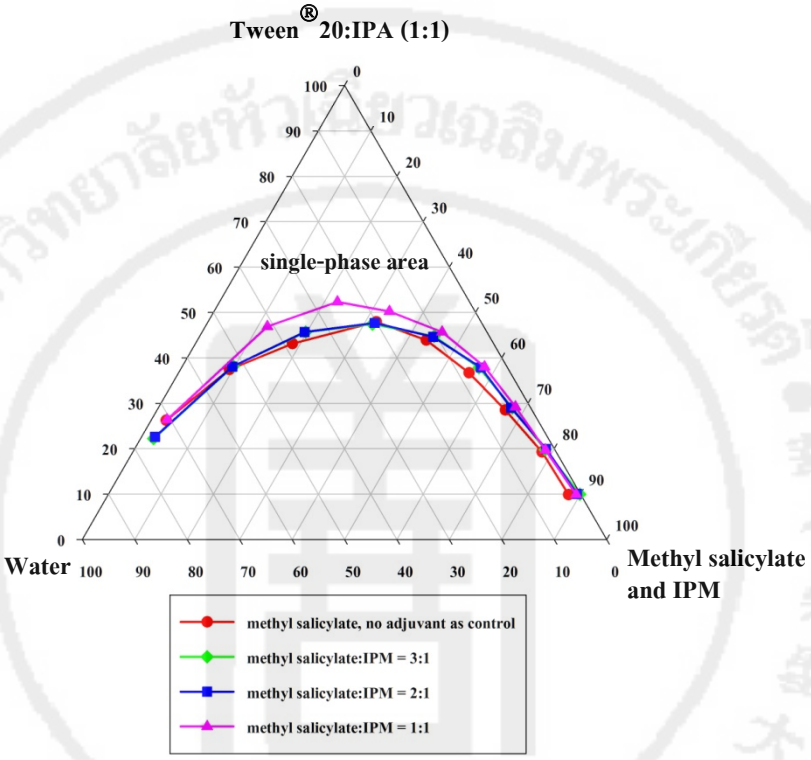

Figure 7. The pseudoternary phase diagrams of the methyl salicylate-IPM/Tween® 20-IPA(1:1)/ water systems at 3:1, 2:1, and 1:1 weight ratios of methyl salicylate to IPM**.**

2. Characterization of the methyl salicylate/Tween® 20-IPA(1:1)/water system in the presence and absence of adjuvants in the oil phase

Based on the results obtained from the pseudoternary phase diagram as mentioned before, the single-phase region of each system in the pseudoternary phase diagram was defined and then all 17 formulations were prepared according to Table 1. Each adjuvant, i.e. eugenol, menthol, Plai oil, or oleic acid at three different weight ratios of methyl salicylate to adjuvant was combined with methyl salicylate to serve as oil phase in the formulation preparation whereas IPP and IPM at two different weight ratios of methyl salicylate to adjuvant were carried out. The physicochemical properties of the prepared formulations were investigated as below.

2.1 Appearance

The appearances of all 17 formulations were shown in Table 4 and Figure 8. All were the transparent homogenous single phase liquids. Under the cross-polarized light microscopy, no birefringence was found in all studied samples and the view of the system looked dark as shown in Figure 9, indicating isotropic property [34]. Isotropic characteristic is one of the important properties of microemulsions.

Table 4. Appearance observation of formulations after preparation

Formulations	Appearances after preparation
A	clear homogenous liquid
E3	clear slightly light brown homogenous liquid
E2	clear light brown homogenous liquid
E1	clear brown homogenous liquid
M3	clear homogenous liquid
M2	clear homogenous liquid
M1	clear homogenous liquid
P3	clear light yellowish homogenous liquid
P2	clear light yellowish homogenous liquid
P1	clear light yellowish homogenous liquid
O3	clear slightly yellowish homogenous liquid
O2	clear slightly yellowish homogenous liquid
O1	clear slightly yellowish homogenous liquid
IP3	clear homogenous liquid
IP2	clear homogenous liquid
IM3	clear homogenous liquid
IM2	clear homogenous liquid

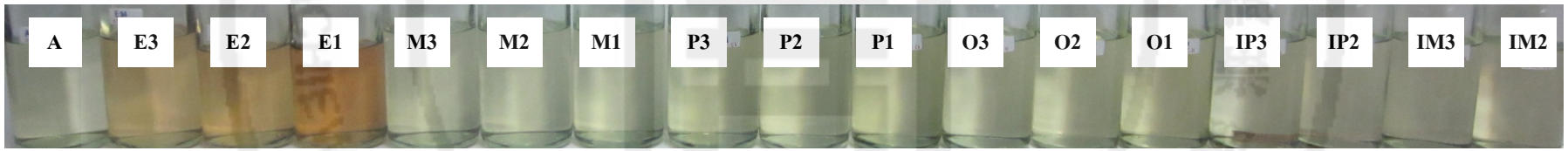

Figure 8. The appearance of all prepared formulations after preparation.

Figure 9. The picture under cross polarized-light microscopy of formulation A as representative of all 17 formulations.

2.2 Particle size measurements

The average droplet sizes of all 17 formulations ranged from 67.5 to 163.6 nm as shown in Figure 10 and their polydispersity index values as reported in Table 5 indicated that all formulations had narrow size distribution. The mean droplet diameter of formulation A, as control without an adjuvant, was 73.0 nm with a polydispersity index of 0.246. The addition of different adjuvants as well as the different weight ratios of methyl salicylate to adjuvant affected the particle size. Obviously, the incorporation of menthol into the oil phase led to the significant increase in the particle size. The more the menthol incorporated, the bigger the particle size observed. It should be noted that the presence of oleic acid at 1:1 weight ratio of methyl salicylate to oleic acid, formulation O1, resulted in a remarkable increase in the droplet size up to 120.8 nm. From the particle size data, these could be explained on the basis of physicochemical characteristic of adjuvants. All adjuvants chosen in this study are water-insoluble hydrophobic compounds. After the microemulsion was formed, the chemical nature of each adjuvant made it possible to locate itself within the core or at surfactant-cosurfactant film of the microemulsion droplet. The non polar compound was surely in the core whereas the molecule of water-insoluble substance with intermediate hydrophobicities was orientated at the interface. The droplet size became larger possibly due to the enlargement of the core or due to an increase in the number of surfactant and cosurfactant molecules per microemulsion droplet in an attempt to cover the core. In case of oleic acid, our study was in agreement with the finding of Paolino et al. [35]. They

reported a significant increase in particle size due to the presence of oleic acid in the formulation and indicated that oleic acid was anchored at the surfactant-cosurfactant film.

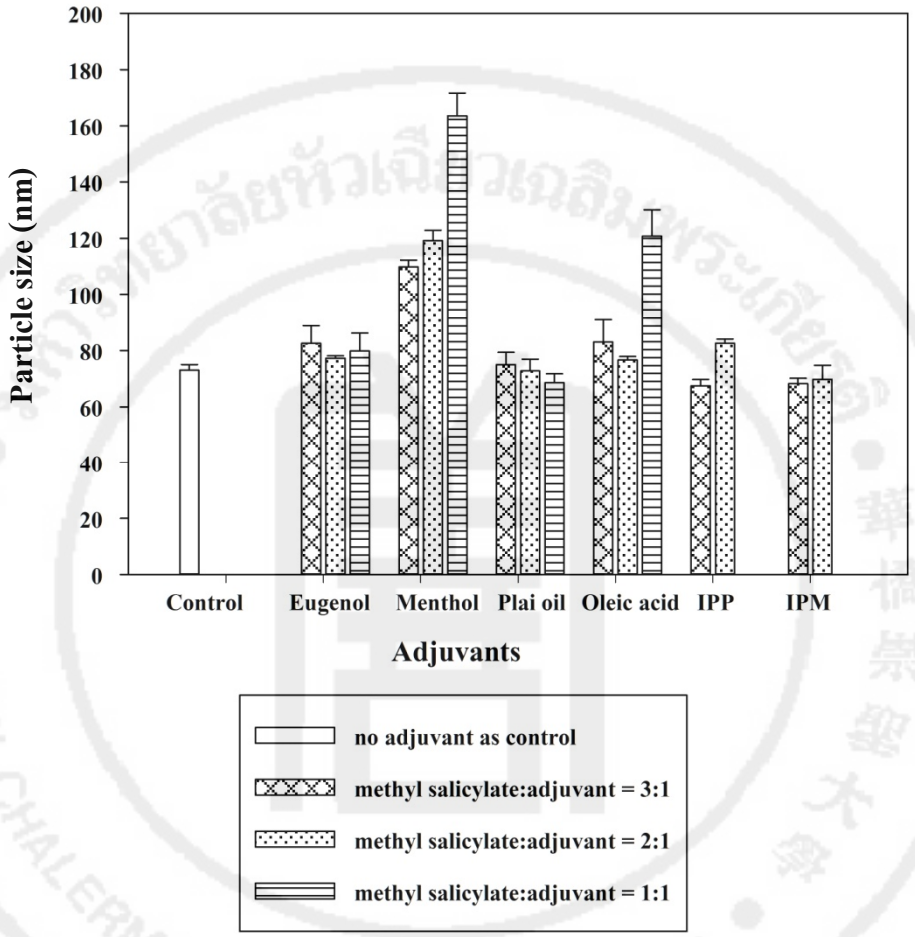

Figure 10. The mean droplet sizes of all 17 formulations (n=3).

Table 5. The polydispersity index (PI) values of all prepared formulations

Formulations	Polydispersity index (PI) (mean±S.D.)
A	0.246±0.043
E3	0.146±0.094
E2	0.202±0.028
E1	0.234±0.033
M3	0.071±0.001
M2	0.152±0.003
M1	0.088±0.049
P3	0.192±0.052
P2	0.165±0.047
P1	0.217±0.014
O3	0.197±0.016
O2	0.164±0.015
O1	0.279±0.013
IP3	0.212±0.014
IP2	0.235±0.016
IM3	0.181±0.019
IM2	0.167±0.080

2.3 Viscosity

As presented in Table 6, all 17 formulations showed low viscosity values in the range of 12.0–16.1 cPs. It suggested that the addition of adjuvant into the oil phase showed no effect on the apparent viscosity. This might be because all used adjuvants are soluble in or miscible with methyl salicylate. The correlation coefficients between shear rate and shear stress were high approaching 1.0, suggesting Newtonian flow behavior. The low viscosity with Newtonian flow behavior is typical of microemulsion system [36].

2.4 pH

The pH resulted as shown in Figure 11 showed that the pH of formulation A in the absence of adjuvant was 6.58. All formulations except for the oleic acid-containing formulations showed pH values above 6. It was obvious that a noticeable decrease in pH was observed in formulations containing oleic acid. This was because oleic acid is a weak acid substance. The higher the oleic acid content, the more decrease the pH value as observed. Formulation O1 had the lowest pH value about 5.32.

2.5 Electrical conductivity

Electrical conductivity measurements can be used to reveal the type of microemulsions. The electrical conductivity results of all 17 formulations were elucidated in Figure 12. The high electrical conductivities ranged from 43.1 to 55.6 μS/cm, suggesting the formation of o/w structure. The high conductivity value more than 10 μS/cm suggested the water as external pseudophase [6, 37].

All physicochemical results mentioned above suggested that all 17 prepared formulations were o/w microemulsions.

Table 6. The viscosity values of all prepared formulations

Formulations	Viscosity (cPs) (mean±S.D.)	Correlation coefficient between shear rate (x) and shear stress (y)
A	12.0±0.06	0.9989
E3	12.2±0.06	0.9996
E2	12.7±0.10	0.9990
E1	12.7±0.06	0.9995
M3	12.9±0.06	0.9997
M2	12.9±0.10	0.9988
M1	14.4±0.06	0.9995
P3	12.4±0.12	0.9988
P2	13.3±0.17	0.9995
P1	13.3±0.06	0.9993
O3	14.0±0.06	0.9991
O2	14.0±0.15	0.9991
O1	16.1±0.17	0.9999
IP3	14.4±0.15	0.9995
IP2	15.1±0.10	0.9997
IM3	14.2±0.15	0.9995
IM2	15.1±0.06	0.9998

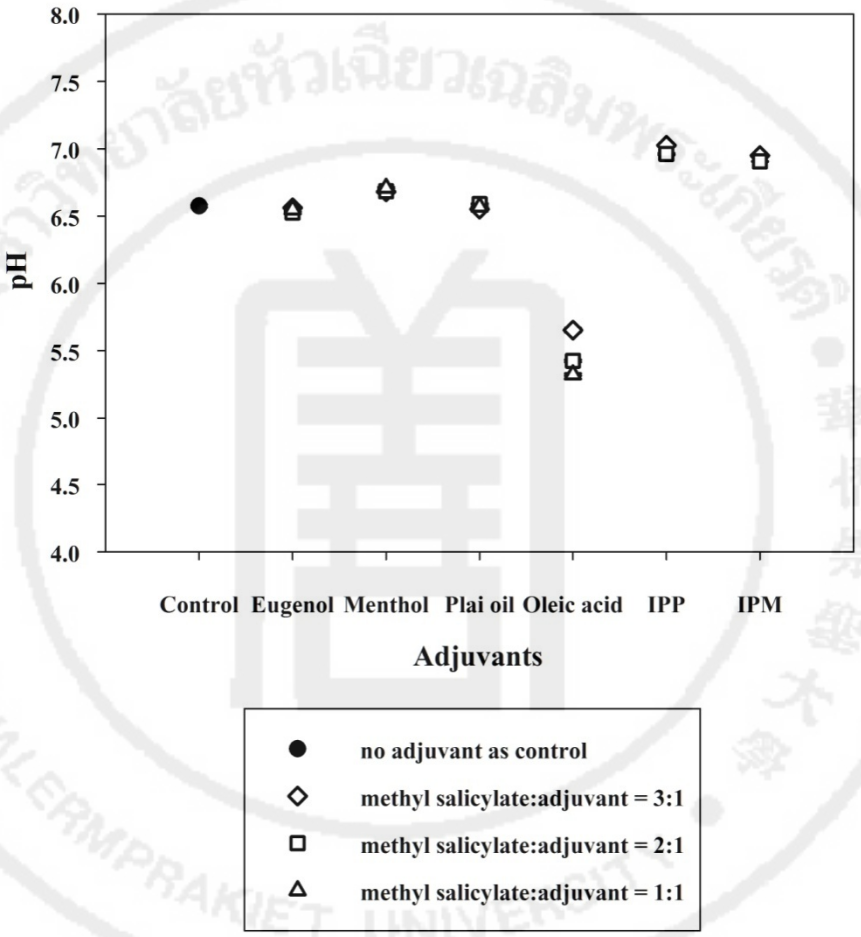

Figure 11. The pH values of all prepared formulations (n=3).

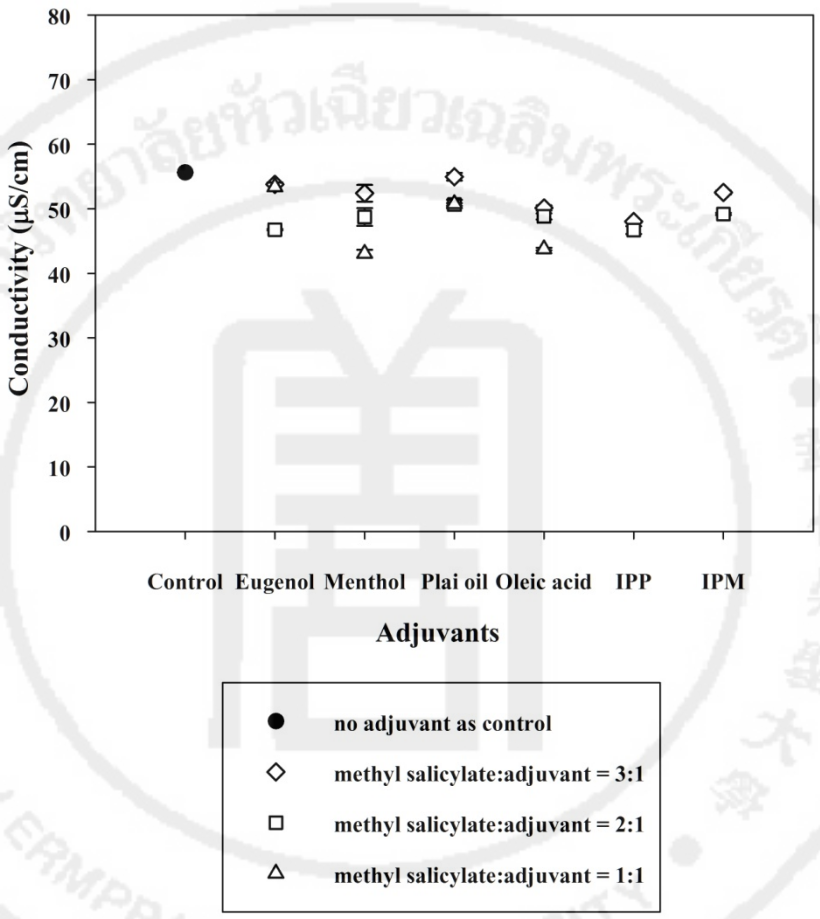

Figure 12. The conductivity results of all prepared formulationns (n=3).

2.6 Stability evaluation

Because the change in physical stability take time to occur, the long term stability at ambient temperature for one year was carried out to ensure the changes. After long term stability, the appearances of all 17 formulations were shown in Table 7 and Figure 13. Phase separation and precipitation were not found in all samples. It was found that the colour of 8 formulations including formulations A, M3, M2, M1, IP3, IP2, IM3, and IM2 visually remained unchanged. In contrast, the colour of the eugenol-containing formulations changed considerably. The oleic acid-containing formulations gained yellowish colour. In case of formulations containing Plai oil, the colour of formulation P3, P2 and P1 changed slightly from light yellowish to yellowish. The colour change suggested the physical instability. It should be noted that in this study, no stabilizer was added into the formulations. Even though eugenol was usually found together with methyl salicylate in various topical analgesic formulations, eugenol posed problems in these studied systems, resulting in the drastic change in colour. This might be attributed to the property of eugenol itself which darkens on exposure to air [19]. In case of oleic acid, it oxidized and acquired colour on exposure to air [19]. Plai oil is an essential oil composed of various substances. Oxidation of some substances might be expected to occur, thereby resulting in colour change to a certain degree.

The average droplet size and the polydispersity index values of all 17 microemulsion formulations were shown in Figure 14 and Table 8, respectively. The pH data and electrical conductivity results of all formulations were elucidated in Figure 15. Taking 8 formulations showing good physical appearance as mentioned above into consideration, the average droplet size of most formulations became slightly bigger upon storage for one year. It was found that the mean particle size of the menthol-containing formulations increased significantly. The more the menthol, the larger the particle size. It was obvious that formulation M1 had the mean particle size more than 200 nm. This mean particle size substantially increased in comparision with that after preparation. In contrast, formulations M2 and M3 had bigger particle sizes than those after preparation, but their particle sizes were below 150 nm. Formulations M2 and M3 were selected to further study. The other formulations had the mean droplet size below 100 nm and their polydispersity index values suggested the narrow size distribution. The pH of the formulations

decreased slightly in comparison with those after preparation. For electrical conductivity results, all formulations showed slightly higher electrical conductivity values than those after preparation.

Based on the stability results, there were seven microemulsion formulations for further study.

Table 7. The appearances of all prepared microemulsions after storage at ambient temperature for one year

Formulations	Appearances after storage for one year
A	clear homogenous liquid
E3	clear light brown homogenous liquid
E2	clear brown homogenous liquid
E1	clear dark brown homogenous liquid
M3	clear homogenous liquid
M2	clear homogenous liquid
M1	clear homogenous liquid
P3	clear yellowish homogenous liquid
P2	clear yellowish homogenous liquid
P1	clear yellowish homogenous liquid
O3	clear yellowish homogenous liquid
O2	clear yellowish homogenous liquid
O1	clear yellowish homogenous liquid
IP3	clear homogenous liquid
IP2	clear homogenous liquid
IM3	clear homogenous liquid
IM2	clear homogenous liquid

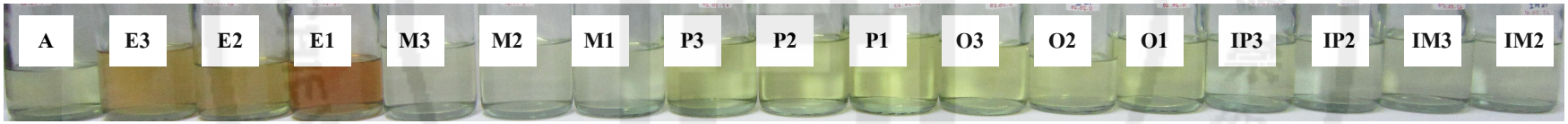

Figure 13. The appearance of all prepared microemulsions after storage at ambient temperature for one year.

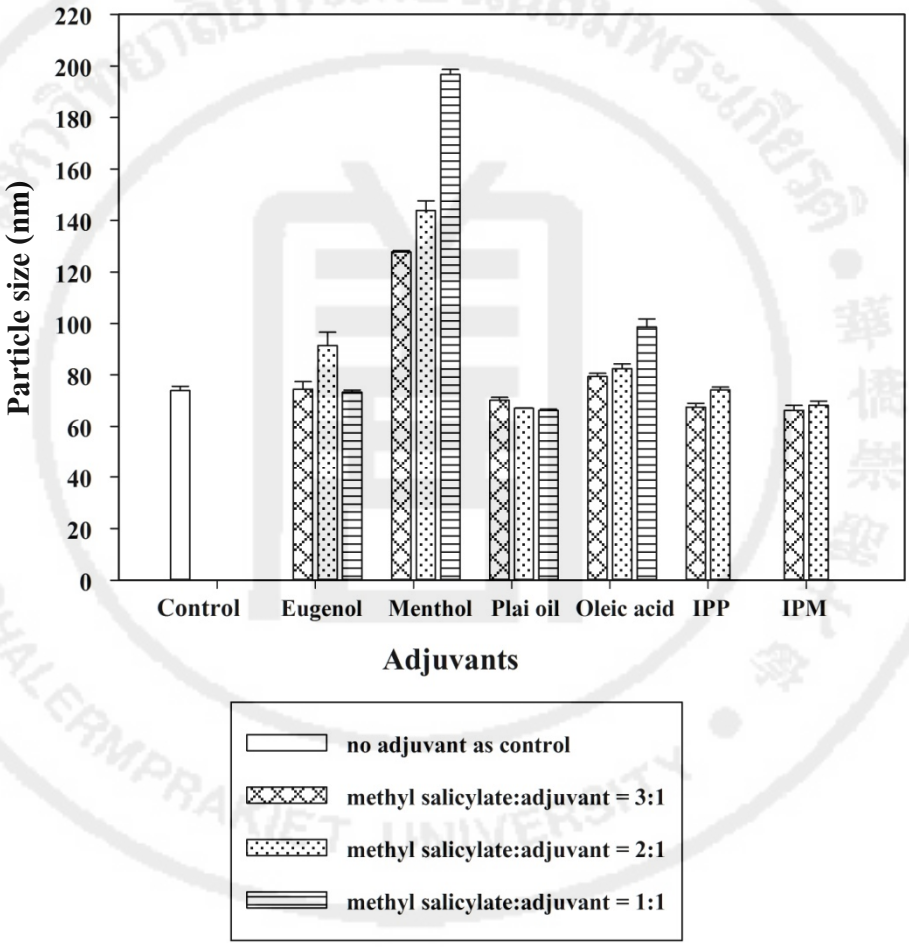

Figure 14. The mean droplet size of all prepared microemulsions after storage for one year (n=3).

Table 8. The polydispersity index (PI) values of all prepared microemulsions after storage for one year.

Formulations	Polydispersity index (PI) (mean±S.D.)
A	0.097±0.023
E3	0.170±0.061
E2	0.151±0.064
E1	0.168±0.066
M3	0.065±0.010
M2	0.091±0.033
M1	0.119±0.007
P3	0.236±0.039
P2	0.199±0.041
P1	0.164±0.012
O3	0.155±0.017
O2	0.167±0.017
O1	0.175±0.041
IP3	0.180±0.005
IP2	0.199±0.028
IM3	0.227±0.037
IM2	0.192±0.028

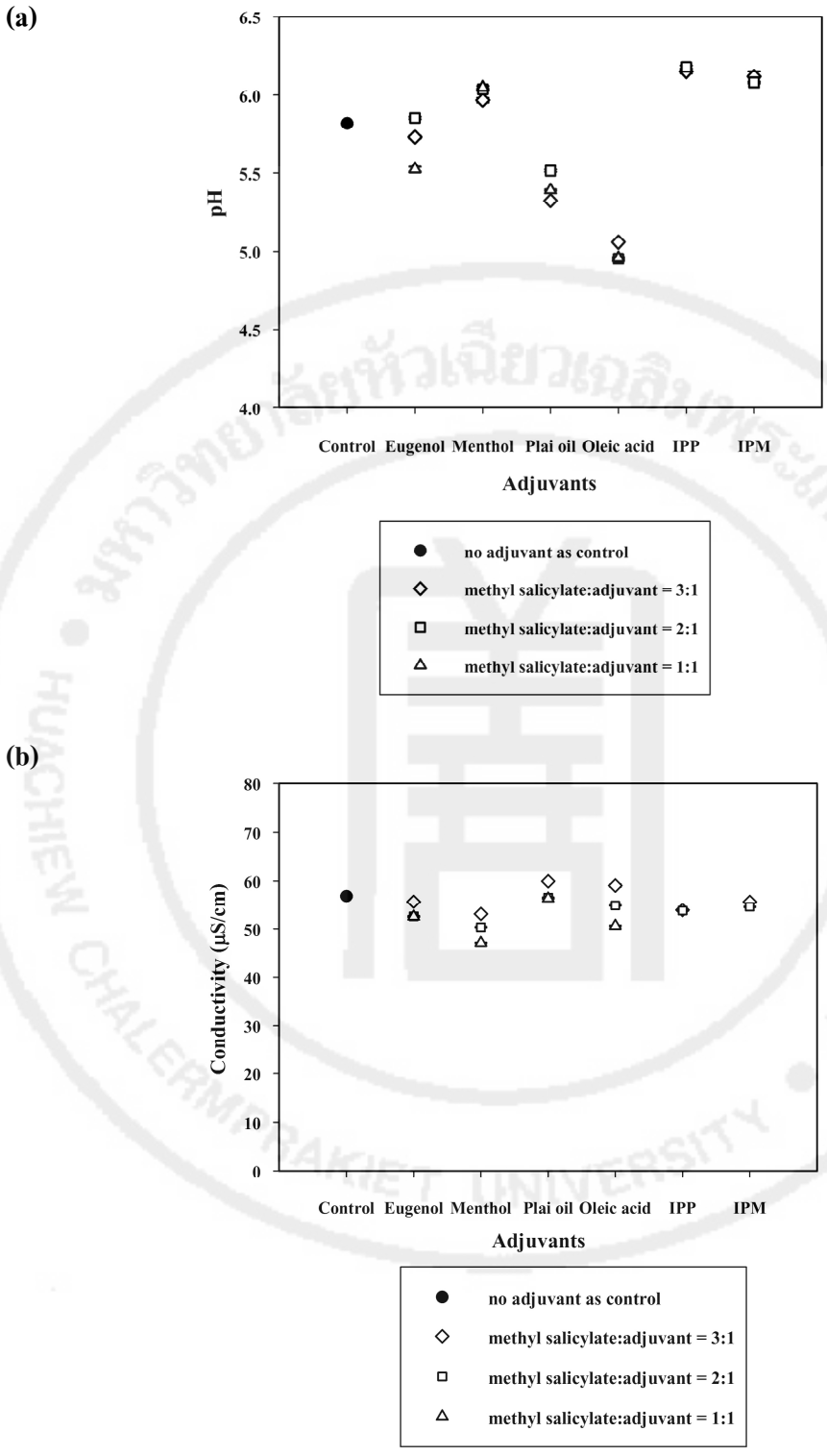

Figure 15. The pH values (a) and electrical conductivity (b) of all 17 prepared microemulsions after storage for one year (n=3).

3. Characterization of indomethacin-loaded microemulsions in the presence and absence of the adjuvant in the oil phase

In this study, indomethacin was chosen as model drug at 0.75%w/w as a general strength of indomethacin incorporated in the commercial topical gel formulation. Taking the stability results of indomethacin-free microemulsion systems into consideration, seven indomethacin-loaded formulations were prepared as previously shown in Table 3. The incorporation of drug into the microemulsion system can affect the system microstructure. The possibility of NSAIDs drug molecules to take part in the microstructure of microemulsion system was issued by Kriwet and Mueller-Goymann [38]. To determine whether the addition of selected drug affected the microstructure of the microemulsion system or not, physicochemical properties of the indomethacin-loaded methyl salicylate microemulsions were investigated.

All seven indomethacin-loaded methyl salicylate microemulsions after preparation were clear homogenous yellowish single phase liquids as shown in Figure 16. Under the cross-polarized light microscopy, the pictures viewed of all indomethacin-loaded formulations were also black similar to the indomethacin-free microemulsion systems, indicating isotropic characteristic. This suggested that incorporation of indomethacin had no effect on microstructure of the methyl salicylate microemulsion system. After centrifugation test, all showed good physical stability. No phase separation and drug precipitation were observed. In addition, after storage at ambient temperature for 6 months, the appearance of all seven indomethacin-loaded microemulsions remained unchanged and had the same isotropic property as those after preparation. No phase separation and drug precipitation were found in all samples.

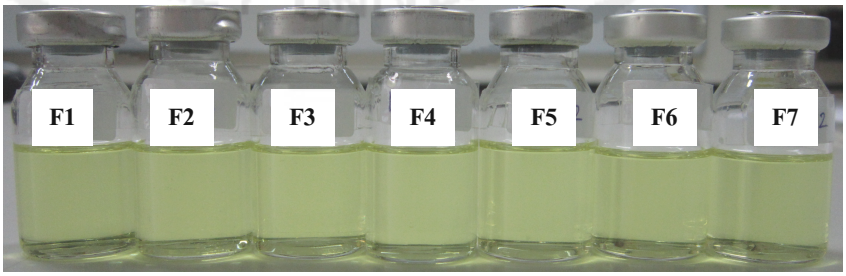

Figure 16. The appearance of all indomethacin-loaded methyl salicylate microemulsions after preparation.

For particle size measurements, all indomethacin-loaded formulations had the particle sizes in the range of 69.1-90.2 nm as elucidated in Figure 17 and the polydispersity index values in the range of 0.003-0.04. After storage at ambient temperature for six months, it was found that the average particle sizes of all formulations, except for formulation F2, increased significantly (p<0.05). It should be noted that particle sizes after storage of formulations F3, F5, and F7 whose oil phases were composed of a 2:1 weight ratio of methyl salicylate to adjuvant changed considerably. The larger particle size might be attributed to the occurrence of droplet coalescence and/or aggregation. The results revealed that the amount of adjuvant incorporated in the oil phase should be optimized. The more adjuvant in the oil phase provided a tendency for particle droplets to coalesce and/or aggregate.

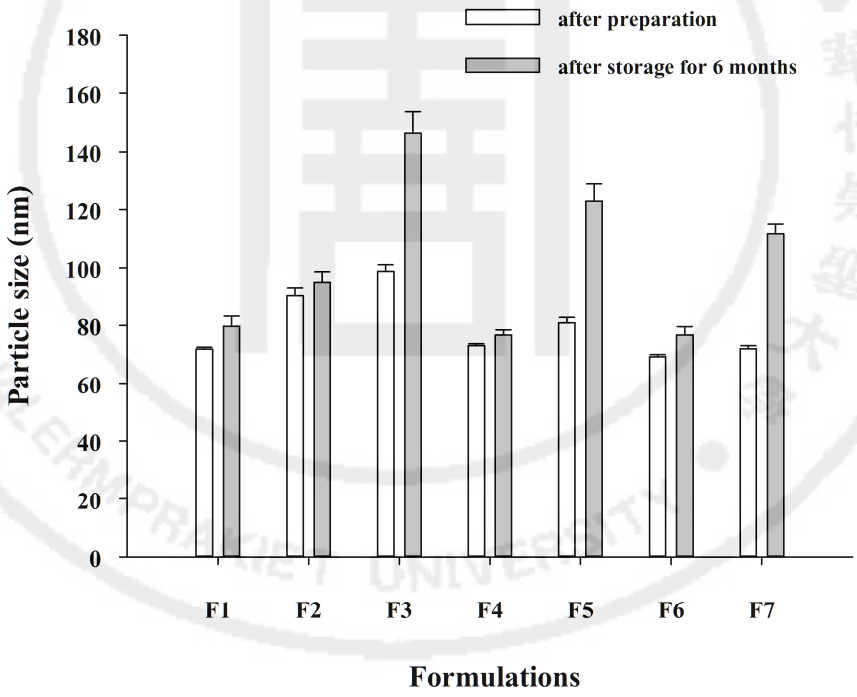

Figure 17. The mean particle size of all seven indomethacin-loaded methyl salicylate microemulsions after preparation and after storage for six months (n=3).

As shown in Figure 18, all indomethacin-loaded formulations showed the low viscosity values. In addition, the correlation coefficients between shear rate and shear stress of all formulations approached 1, suggesting the Newtonian flow behavior. The results suggested that

the presence of indomethacin neither had any influence on viscosity nor changed the flow behavior. After storage, the viscosity values were still low and comparable to those after preparation.

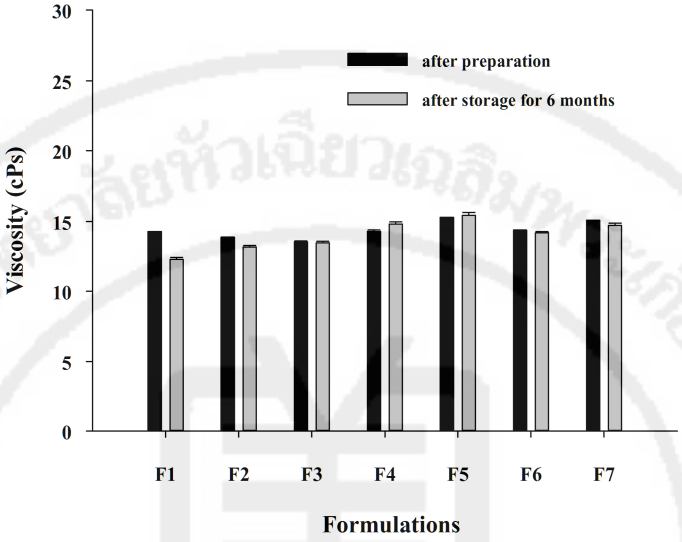

Figure 18. The viscosity values of seven indomethacin-loaded methyl salicylate microemulsions after preparation and after storage for six months (n=3).

For pH investigation, the pH values of seven indomethacin-loaded methyl salicylate formulation after preparation were in the range from 5.555 to 5.607 as elucidated in Figure 19. It was observed that the presence of indomethacin was found to remarkably lower the pH of formulations compared with their blank counterpart formulations. This was because indomethacin is a weak acidic drug and its pKa is 4.5. The addition of indomethacin did lower the pH of the formulations. After storage, the pH values were more or less the same as those after preparation.

The effect of indomethacin incorporation on the conductivity of the indomethacin-loaded formulations was illustrated in Figure 20. The conductivity values varied from 42.1 to 45.4 μS/cm. The results suggested that they were the o/w microemulsions. After storage for six months, the conductivity values were comparable to those after preparation.

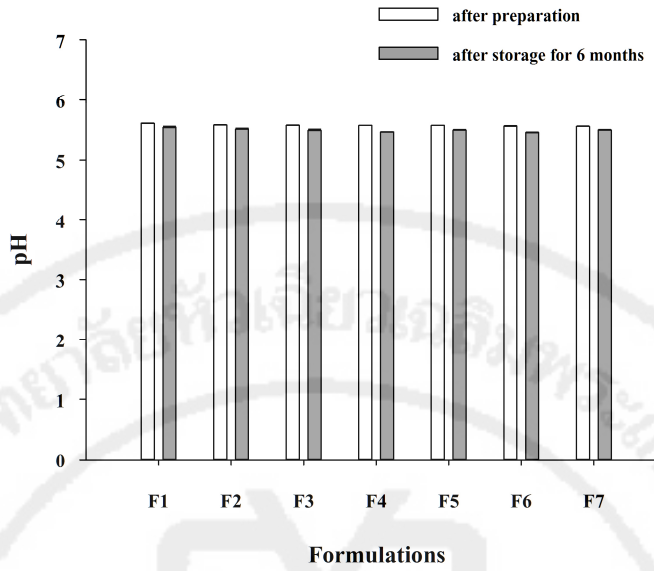

Figure 19. The pH of seven indomethacin-loaded methyl salicylate microemulsions after preparation and after storage for six months (n=3).

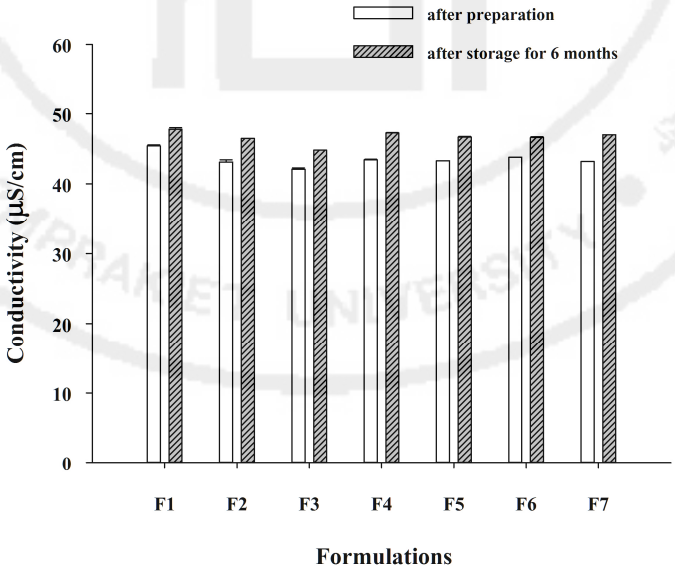

Figure 20. The conductivity results of seven indomethacin-loaded methyl salicylate microemulsions after preparation and after storage for six months (n=3).

For HPLC quantitative analysis of methyl salicylate and indomethacin after storage of indomethacin-loaded methyl salicylate formulations at ambient temperature for six months, the results were shown in Table 9. It was found that the amounts of methyl salicylate and indomethacin in all prepared formulations after storage were close to those after preparation, suggesting that methyl salicylate and indomethacin had good chemical stability. The content of indomethacin in formulation F1, as control, in the absence of an adjuvant remained high, suggesting that indomethacin had good compatibility with methyl salicylate. All other adjuvant-containing formulations irrespective of both types and amounts of components in the oil phase also showed high drug remaining for methyl salicylate and indomethacin, suggesting that the presence of investigated adjuvant in the oil phases had no effect on the drug stability. It should be noted that more IPM in the oil phase as in formulation F7 might affect chemical stability of methyl salicylate and indomethacin, thereby resulting in the lowest drug remaining for methyl salicylate and indomethacin. Indomethacin was subject to alkaline hydrolysis. It has maximum stability at room temperature near pH 3.75 with a calculated shelf life of 8.4 days [39]. Its half-life at room temperature was reported to be about 200 hours in pH 8.0 buffer. When the pH of solutions increased, the half-life decreased [40]. However, in the aqueous surfactant solutions the acid and base catalysed hydrolysis of indomethacin was decreased [41]. In addition, the study of Krasowska [42] also reported that the degradation of solubilized indomethacin decreased with the increasing surfactant concentration. A high level of indomethacin remaining in this study could be explained on the basis of micellar solubilized systems. This was because the rate of drug hydrolysis in the dispersed phase is generally smaller than that in the continuous bulk phase; therefore, the solubilized drug should be protected from attacking ions. As a consequence, indomethacin solubilized in the studied systems composed of high concentration of Tween® 20 should be preserved from degradation so that it should be more stable.

Taking the physicochemical properties regarding the particle size and size distribution and the drug remaining after storage of all indomethacin-loaded methyl salicylate microemulsions into consideration, it was found that formulation F2 whose oil phase was composed of menthol as adjuvant at the methyl salicylate-adjuvant weight ratio of 3:1 showed good physicochemical properties and stability.

Table 9. The percent of drug remaining after storage of indomethacin-loaded methyl salicylate formulations for 6 months

Formulations	%Methyl salicylate remaining	%Indomethacin remaining
F1	99.5±0.7	98.5±1.2
F2	99.1±0.4	99.1±0.4
F3	98.2±0.5	97.4±0.8
F4	98.6±0.3	98.8±0.7
F5	99.0±0.6	98.1±1.1
F6	100.0±0.1	100.0±0.5
F7	96.8±0.9	94.5±1.1

Conclusions

Methyl salicylate, the medicative substance with oily characteristic, was successfully used as oil phase in the microemulsion preparation. The pseudoternary phase diagram of the methyl salicylate/Tween® 20-IPA(1:1)/water system was used as control. The addition of eugenol, Plai oil, or oleic acid did not significantly change the size of single phase area whereas menthol showed the pronounced effect and obviously reduced the single phase area. For IPP or IPM containing systems, only the 1:1 weight ratio of methyl salicylate to IPP or IPM significantly decreased the single phase area. The single phase mixtures, composed of 15% oil phase in the presence and absence of various weight ratios of studied adjuvants, 50% the Tween® 20-IPA (1:1) mixture, and 35% water, were o/w microemulsions. However, upon storage for one year, the methyl salicylate microemulsion system and the systems containing menthol, isopropyl palmitate or isopropyl myristate as an adjuvant at the methyl salicylate-adjuvant weight ratios of 3:1 and 2:1 showed good physical appearance and physicochemical properties. It was noteworthy that in case of menthol, the amount of methyl salicylate and menthol at the weight ratio of 2:1 was comparable to that in the commercial analgesic cream, Counterpain®. Incorporation of 0.75% indomethacin into the methyl salicylate microemulsions in the presence and absence of proper adjuvants also produced clear homogenous yellowish liquids with low viscosity values and o/w microemulsion characteristic. In comparision with their drug-free counterparts, the presence of indomethacin resulted in lower pH values. After long term stability at ambient temperature for six months, the indomethacin-loaded methyl salicylate microemulsion whose oil phase was composed of menthol as adjuvant at the methyl salicylate-adjuvant weight ratio of 3:1 exhibited good physicochemical properties, especially the particle size and size distribution and also showed good chemical stability regarding the content of methyl salicylate and indomethacin remaining.

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APPENDIX

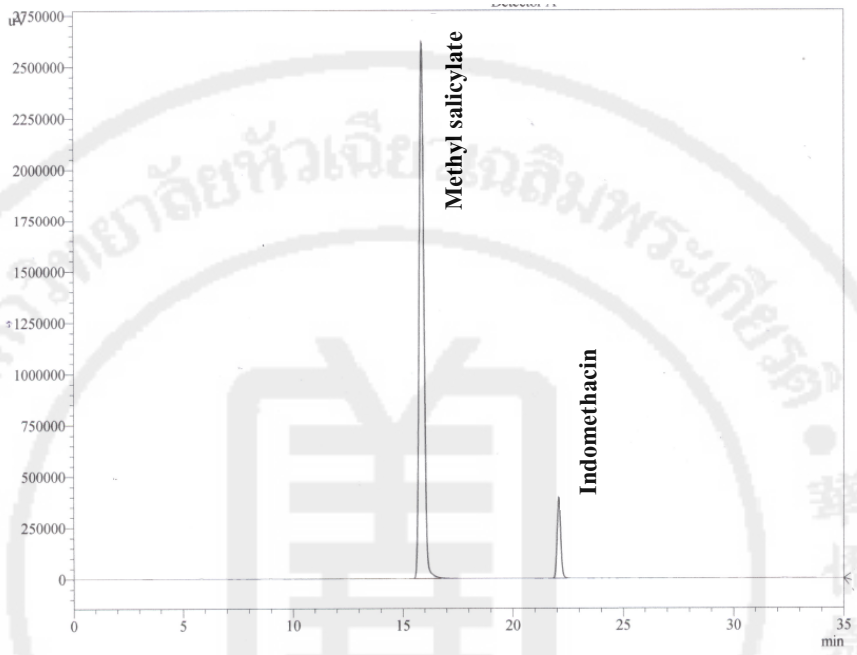

Figure 21. The HPLC chromatogram of formulation F6, for example, after storage for six months.

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