
Regular article

Analysis of the stability of external-application dermatologic preparations

: Consideration from rheological measurements

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Abstract

The present study examined the stability of mixtures of various combinations of moisturizers, water in oil (w/o)-type or oil in water (o/w)-type cream preparations containing heparinoids, and steroidal ointments or creams (o/w-type) frequently used in children. Centrifugation at room temperature led to separation of mixtures of w/o-type moisturizers and steroidal ointments into three layers. Polarized microscopic observations, NIR spectroscopy, and dye-based analyses revealed the presence of oily components in the upper and middle layers and water-soluble components in the lower layer. Separation into three layers upon centrifugation was also observed for mixtures of o/w-type moisturizers and steroidal ointments. In contrast, neither the o/w-type moisturizer and steroidal cream nor the w/o-type moisturizer and steroidal cream mixtures separated into layers upon centrifugation. Consideration of the characteristics of each preparation is necessary when mixing external-application dermatologic preparations. Centrifugation at 4 °C did not result in layer separation of the w/o-type moisturizer and steroidal ointment mixture, suggesting that cold storage of such mixtures provides superior stability compared with room temperature storage. However, despite no obvious layer separation, the NIR spectra indicated that water movement was induced within the mixture. These results clearly indicate that methods such as NIR spectroscopy are useful for early determinations of the stability of mixed external-application dermatologic preparations.
Key word: heparinoids, steroidal preparation, stability, oil in water, water in oil, mixing
**Introduction**

Steroidal preparations for external application on the skin are used primarily for anti-inflammatory purposes and are classified into five groups according to the degree of absorption (i.e., “strongest,” “very strong,” “strong,” “medium,” and “weak”). The use of steroidal preparations in the “strong,” “medium,” or “weak” groups is particularly recommended in pediatric dermatology. The mixing of prescription compounds with steroidal preparations and moisturizers or bases according to patient needs is also common \(^1,^2\). The providing of instructions for mixing moisturizers or bases with steroidal ointments by physicians in Japan has improved compliance in pediatric patients\(^1\). Understanding the factors that influence the quality and stability of mixtures of external preparations is therefore very important for pharmacists. Nagelreiter et al. reported that the skin penetration of active pharmaceutical ingredients (APIs) is influenced by the type of cream base used\(^2\). Furthermore, numerous reports have described the influence of mixing on the release profile and skin permeation of APIs in external-application preparations\(^3,^4,^5,^6,^7\). Other studies have reported changes in the mixture formulation for a variety of combinations\(^8\). However, few studies have examined the physico-chemical changes in mixtures at the microscopic level.

In general, the mixing of external-application dermatologic preparations is determined by the type of the base used. The relationship between the type of base used in a preparation and the mixing possibilities according to the dispensing guidelines in Japan, Chozai-shishin\(^9\), is
shown in Table 1. The use of heparinoid-containing water in oil (w/o)-type cream preparations as moisturizers is increasing, and steroidal ointments are often mixed with these preparations. One possible explanation for this is that pharmacists are more comfortable with mixing such formulations because these combinations (i.e., w/o-type and o-type) are not defined as “impossible” to mix (Table 1).

We previously reported that centrifugation leads to the separation of w/o-type moisturizing creams and white petrolatum mixtures into three layers, although centrifugation does not induce the separation of cream-only preparations. These observations suggest that the stability of a preparation is diminished by mixing creams and ointments. It is therefore necessary to determine objectively and scientifically the types of blending variations that are induced by mixing different types of dermatologic preparations.

Near-infrared (NIR) spectroscopy utilizes electromagnetic waves in the wavelength region between the infrared and visible light regions. Because absorption in NIR spectroscopy is very low in comparison with the mid- and far-infrared regions, samples can be analyzed quickly in a non-destructive and non-contact manner without the need for prior sample processing. As the characteristics of spectra of biomolecules such as proteins, lipids, starches, and water in this wavelength range are known, NIR is frequently used for non-destructive analyses of food ingredients. NIR spectroscopy is also used in pharmaceutical sciences for applications such as (i) qualitative validation of the components
of dermatologic preparations, ointments, or creams\(^{12}\), (ii) assessment of the degree of mixing of powders prepared by mortar and pestle, fine granules, and dry syrups\(^{13}\), and (iii) assessment of the distribution of the primary drug and additives in tablets by microscopic infrared spectroscopy, which combines microscopy and NIR spectroscopy\(^{14,15}\).

The present study examined the stability of mixtures of various combinations of moisturizers, w/o- or oil in water (o/w)-type creams containing a heparinoid, and steroidal ointments or creams (o/w-type) of the “strong” and “medium” absorption categories frequently used in children. The mixtures were forcibly degraded by centrifugation and then evaluated for stability by analyzing the water distribution of the base using techniques such as polarized microscopic observation, NIR spectroscopy, and dye-based analyses.

(Table 1)

**Experimental Section**

1. **Reagents**

The heparinoid-containing moisturizing agents Hirudoid\(^{\circledR}\) Soft ointment 0.3% (w/o-type, lot 5A04P, HRD\(_{wo}\)) and Hirudoid\(^{\circledR}\) cream 0.3% (o/w-type, lot 23308, HRD\(_{ow}\)) were purchased from Maruho Co., Ltd (Osaka, Japan).

The following steroidal ointments were examined in this study: betamethasone valerate–
containing ointment Rinderon® V 0.12% (lot 5330, Shionogi & Co., Ltd., Osaka, Japan., RND\textsubscript{o}), clobetasone butyrate–containing ointment Kindavate® 0.05% (lot 14074, Glaxo SmithKline K. K., Tokyo, Japan., KND\textsubscript{o}), and diflucortolone valerate–containing ointment Nerisona® 0.1% (lot Y01U, Bayer Yakuhin, Ltd., Osaka, Japan., NRS\textsubscript{o}).

The following steroidal creams were examined in this study: betamethasone valerate–containing cream (o/w-type) Rinderon® V 0.12% (lot 5129, Shionogi & Co., Ltd., RND\textsubscript{ow}), and prednisolone valerate acetate ester–containing cream (o/w-type) Lidomex® Kowa 0.3% (lot EU4H, Kowa Company, Ltd., Aichi, Japan., LDM\textsubscript{ow}).

2. Preparation of mixtures consisting of moisturizing creams and steroidal external-application preparations

Equal mass mixtures of moister cream (HRD\textsubscript{wo} or HRD\textsubscript{ow}) and steroidal preparation (ointment or cream) were prepared using a rotation/revolution type mixer, NRJ-250 (2000 rpm, 30 s; Thinky Co., Ltd., Tokyo, Japan). An aqueous solution of 0.1 or 1.0 w/v% water-soluble dye methylene blue (MB) and a liquid paraffin solution of 0.1 w/v% fat-soluble dye Sudan III were prepared, and 1 drop was added by dropper to the appropriate preparations prior to mixing.
3. Centrifugation

Mixed samples were centrifuged at room temperature or 4 °C at 16500×g for 0.5, 1, 3, 5, or 7 min. The condition of centrifugation was determined by previous report to the reference10).

4. Polarization microscopy

The microscopic features of the preparations were characterized by applying a small amount of sample to a microscope slide, covering with a cover slip, and observing under an E-600-Pol polarizing microscope (Meiji-techno, Tokyo, Japan) in reflection mode at 200× magnification.

5. NIR spectroscopy

Mixed preparations consisting of a moisturizing cream and a steroidal external-application preparation were prepared and centrifuged as described above, and then small samples of each separated layer or site (Fig. 1) were collected for acquisition of NIR spectra.

(Fig. 1)
NIR transmission spectra (optical path length: 0.2 mm) were acquired using a Spectrum One NTS spectrometer (PerkinElmer, Inc., Waltham, MA, U.S.A.) equipped with an Omni Cell system used for mulls (Specac Inc., Cranston, U.S.A.) at a wavenumber resolution of 4 cm⁻¹, employing 32 scans across the wavelength range 4000–8000 cm⁻¹. NIR spectra of air were also acquired as background.

6. Determination of rheological characteristics

Flow curves of shear rate versus shear stress were obtained using a viscometer (TV-30; Toki Sangyo Co., Ltd., Tokyo, Japan). The temperature of the base plate was 25 ± 0.1 °C. The shear rate was varied from 0.38 to 9.58 s⁻¹.

Results

1. Change in appearance of mixtures consisting of moisturizing creams and steroidal external-application preparations after centrifugation

When mixtures of HRD_w/o, w/o-type heparinoids, and RND_o or KND_o steroidal ointments (HRD_w/o&KND_o, HRD_w/o&RND_o) were centrifuged at room temperature (16500×g, 7 min), separation into three layers (upper, middle, and lower) was observed (Fig. 2 for HRD_w/o&RND_o only, Fig. S1 for the other mixtures). In the sample to which 1 drop of MB aqueous solution (1.0 w/v%) was added prior to mixing, color was observed only in the lower
layer following centrifugation (Fig. 2 for HRD<sub>wo</sub>&RND<sub>o</sub> only). In samples to which Sudan III liquid paraffin solution (1.0 w/v%) was added, color was observed in the upper and middle layers (Fig. 2 for HRD<sub>wo</sub>&RND<sub>o</sub> only). In contrast, minimal separation was observed for HRD<sub>wo</sub>&NRS<sub>o</sub> (Table 2). NRS<sub>o</sub> is a steroidal ointment of the “very strong” absorption class, and therefore, preparations containing this agent are rarely used in pediatric dermatology. However, since the different results were obtained for KND<sub>o</sub> and RND<sub>o</sub>, which are used in treating children, we selected NRS<sub>o</sub> as the target drug for subsequent experiments in this study.

(Fig. 2)

In mixtures of HRD<sub>ow</sub>, o/w-type heparinoids, and RND<sub>o</sub> or KND<sub>o</sub> (HRD<sub>ow</sub>&RND<sub>o</sub> or HRD<sub>ow</sub>&KND<sub>o</sub>), separation into three layers was observed following centrifugation, as seen with HRD<sub>wo</sub>&RND<sub>o</sub> and HRD<sub>wo</sub>&KND<sub>o</sub> (Table 2). However, separation into layers following centrifugation was not observed in mixtures consisting of HRD<sub>wo</sub> and RND<sub>ow</sub> or LDM<sub>ow</sub> (HRD<sub>wo</sub>&RND<sub>ow</sub> or HRD<sub>wo</sub>&LDM<sub>ow</sub>) and mixtures consisting of HRD<sub>ow</sub> and RND<sub>ow</sub> or LDM<sub>ow</sub> (HRD<sub>ow</sub>&RND<sub>ow</sub> or HRD<sub>ow</sub>&LDM<sub>ow</sub>) (Table 2).

(Table 2)
2. Polarized microscopic images of mixtures

Polarized microscopic images of preparations are shown in Figures 3 and 4. The images confirmed dispersion of fine solid crystals of API in RND<sub>o</sub> and RND<sub>ow</sub> (Fig. 3bc, indicated by arrows). Images of the three layers of HRD<sub>wo</sub>&RND<sub>o</sub> revealed semi-solid crystals derived from the base distributed homogenously in the middle layer but not in the upper layer (Fig. 3d). Fewer semi-solid crystals were observed in the lower layer in comparison with the middle layer (Fig. 3d). Similar results were observed for HRD<sub>wo</sub>&KND<sub>o</sub> (data not shown).

In mixtures of HRD<sub>wo</sub> and steroidal creams (w/o&o/w), no significant differences in the areas were observed by polarized microscopy (Fig. 3e, for HRD<sub>wo</sub>&RND<sub>ow</sub> only).

(Fig. 3)

In mixtures of HRD<sub>ow</sub> and steroidal ointments (o/w&o) in which separation into three layers was observed, characteristic differences in the layers were noted, similar to those observed with HRD<sub>wo</sub>&RND<sub>o</sub> (Fig. 4d, for HRD<sub>ow</sub>&RND<sub>o</sub> only). In particular, non-uniformity of the base due to destabilization of the emulsion was observed immediately after mixing (Fig. 4a). In mixtures of HRD<sub>ow</sub> and steroidal creams (o/w&o/w), no significant differences in the areas were observed by polarized microscopy (Fig. 4e, for HRD<sub>ow</sub> &RND<sub>ow</sub> only).
3. NIR spectra

Peaks around 4300 and 5800 cm\(^{-1}\) derived from the first combination and first overtone of hydrocarbon\(^{(16)}\) were observed in NIR spectra for all preparations examined in this study. Moreover, gradual peaks around 5200 and 6800 cm\(^{-1}\) derived from the first combination and first overtone of the hydroxyl group\(^{(17)}\) were observed for the HRD\(_{wo}\) and HRD\(_{ow}\) preparations, indicating the presence of water. The original spectra were subjected to secondary derived processing to eliminate the influence of the baseline. A clear difference around 5200 cm\(^{-1}\) was observed between the ointments and creams; in other words, the peak in the negative direction was observed in the creams but not in the ointments (Fig. 5 and 6).

Each of the three layers of the mixtures of HRD\(_{wo}\) and steroidal ointments (w/o&o) (Table 2) were collected and analyzed by NIR spectroscopy. Only peaks derived from hydrocarbons (i.e., around 4300 and 5800 cm\(^{-1}\)) were observed in the upper and middle layers. In contrast, peaks derived from hydroxyl groups (i.e., around 5200 and 6800 cm\(^{-1}\)) were observed in the lower layer (Fig. 5a, for HRD\(_{wo}&RND_o\) only, Fig. S2 for HRD\(_{wo}&KND_o\). However, spectra of the upper and lower areas of HRD\(_{wo}&NRS_o\) (in which no visible separation occurred following centrifugation) were almost identical (Fig. 5b).
In mixtures of HRD<sub>ow</sub> and steroidal ointments (o/w&o) that separated into three layers following centrifugation, the NIR spectra of the upper, middle, and lower layers were similar to those of HRD<sub>wo</sub>&RND<sub>o</sub> and HRD<sub>wo</sub>&KND<sub>o</sub> (Fig. 6a for HRD<sub>ow</sub>&RND<sub>o</sub> only, Fig. S3 for HRD<sub>ow</sub>&KND<sub>o</sub>). In mixtures of HRD<sub>wo</sub> and steroidal creams (w/o&o/w, Fig. 6b for HRD<sub>wo</sub>&RND<sub>ow</sub> only) and HRD<sub>ow</sub> and steroidal creams (o/w&o/w, Fig. 6c for HRD<sub>ow</sub>&RND<sub>ow</sub> only) in which no separation was observed following centrifugation (Table 2), NIR spectra of the upper and lower areas of these mixtures were almost identical (Figs. S4 and S5 for HRD<sub>wo</sub>&LDM<sub>ow</sub> and HRD<sub>ow</sub>&LDM<sub>ow</sub>, respectively).

4. Influence of centrifugation time and temperature (HRD<sub>wo</sub>&KND<sub>o</sub> or HRD<sub>wo</sub>&RND<sub>o</sub>)

NIR spectra of the HRD<sub>wo</sub>&RND<sub>o</sub> and HRD<sub>wo</sub>&KND<sub>o</sub> (w/o&o) preparations immediately after mixing (i.e., prior to centrifugation) measured for samples taken from five random areas of the mixture were almost identical (Fig. S6). Detectable separation of these mixtures into layers depended upon the centrifugation time (30 s to 7 min) when examined at room temperature (Fig. 7a for HRD<sub>wo</sub>&RND<sub>o</sub> only). In contrast, no separation occurred when the
mixtures were centrifuged at 4 °C, irrespective of the centrifugation time (Fig. 7b for HRD\textsubscript{wo}&RND\textsubscript{o} only).

(Fig. 7)

Upon centrifugation (16500×g, 7 min) at 4 °C following the addition of 1 drop of MB aqueous solution (1.0 w/v\%) to HRD\textsubscript{wo}&RND\textsubscript{o} prior to mixing, the coloration tended to be darker toward the bottom of the sample. Upon addition of MB aqueous solution (1.0 w/v\%) or Sudan III liquid paraffin solution (1.0 w/v\%) to HRD\textsubscript{wo}&RND\textsubscript{o} at the same time prior mixing, a color gradation was observed from the top (colored red by Sudan III) to the bottom (colored blue by MB) of the mixtures (Fig. 8). When MB was diluted 10-fold (i.e. 0.1 w/v\%) for thinning the blue, leading to clarify the distribution of Sudan III, the gradation was observed more clearly (Fig. 8).

(Fig. 8)

The NIR peak around 5200 cm\textsuperscript{-1} derived from a hydroxyl group was observed more clearly in spectra proceeding from the top to the bottom of the HRD\textsubscript{wo}&RND\textsubscript{o} and HRD\textsubscript{wo}&KND\textsubscript{o} mixture samples after centrifugation at 4 °C (16500×g, 7 min) (Fig. 9).
Following secondary derived processing of the original spectra, the difference in the intensity of the peak in the negative direction around 5200 cm\(^{-1}\) between the upper and lower sites of the sample was greater for the HRD\(_{w/o}\&\text{RND}_o\) mixture than for the HRD\(_{w/o}\&\text{KND}_o\) mixture (Fig. 9).

5. Rheological testing of mixtures consisting of HRD\(_{w/o}\) and steroidal ointments

Figure 10 shows flow curves for mixtures of HRD\(_{w/o}\) and steroidal ointments. All flow curves exhibited a hysteresis loop, especially the curve for the HRD\(_{w/o}\&\text{KND}_o\) sample. Viscosity values at a shear rate of 9.58 s\(^{-1}\) for the HRD\(_{w/o}\&\text{RND}_o\), HRD\(_{w/o}\&\text{KND}_o\), and HRD\(_{w/o}\&\text{NRS}_o\) mixtures were 10.50, 14.56, and 20.38 Pas, respectively.

Discussion

In the present study, we evaluated the stability of mixtures using dye-based techniques in addition to polarized light microscopy and NIR spectroscopy. Mixtures of HRD\(_{w/o}\) and steroidal ointments (w/o&o) separated into three layers upon centrifugation. The separation
was more clearly observable using the dye-based analysis (Fig. 2 for HRD<sub>wo</sub>&RND<sub:o</sub> only) and indicated the presence of oily components in both the upper and middle layers and of water-soluble components in the lower layer. Polarized microscopic images and NIR spectra revealed characteristics specific for each layer (Figs. 3d and 5a for HRD<sub>wo</sub>&RND<sub:o</sub> only). The fact that only the hydrocarbon peak was observed in NIR spectra of the transparent upper layer suggests that this layer is composed of a hydrocarbon of relatively low density, such as liquid paraffin (Fig. 5a). The polarized microscopic image and NIR spectra obtained from middle layer of HRD<sub>wo</sub>&RND<sub:o</sub> after centrifugation suggest that the middle layer is composed primarily of white petrolatum, a high-density hydrocarbon, and that most of the water is localized in the lower layer (Fig. 3d and 5a). Similar results were obtained in a study using MRI<sup>10</sup>. Collectively, these results suggest that mixtures of an w/o-type compound and an oily base will likely be unstable. Of note, no change in appearance was observed when HRD<sub>wo</sub> was centrifuged alone at room temperature (16500×g, 7 min), and NIR spectra for subsamples taken from the top and bottom areas of the sample were consistent (data not shown). The proprietary formulation of HRD<sub>wo</sub> (an oily component, a water-soluble component, and a surfactant) thus appears to provide stability. However, our data indicate that destabilization of emulsions containing HRD<sub>wo</sub> is induced by mixing with steroidal ointments, resulting in a decrease in the uniformity and stability of such preparations. In previous report, separation to three layers was observed from both mixtures consisting of
HRD\textsubscript{wo} and KND\textsubscript{o} or white petrolatum after centrifugation, and the difference in the appearance of the phase separation was not observed each other\textsuperscript{(10)}. These results suggest that physicochemical properties of API of steroidal ointments may not affect the separation phenomenon. In contrast, no centrifugation-induced separation was observed for NRS\textsubscript{o} steroidal ointment preparations, even when mixed with HRD\textsubscript{wo} (Fig. 5b, Table 2). In terms of assessing stability, it is therefore important to consider the pharmaceutical characteristics of each preparation.

Centrifugation-induced separation into three layers was also observed for mixtures of HRD\textsubscript{ow} and steroidal ointments (o/w&o). In the case of these mixtures, destabilization of the emulsion was observed before centrifugation (Fig. 4b for HRD\textsubscript{ow} &RND\textsubscript{o} only). This result confirms the Chozai-shishin designation of o/w-type compounds and oily bases as “impossible” to mix (Table 1).

No separation was observed in mixtures of HRD\textsubscript{ow} and steroidal creams (o/w&o/w) after centrifugation (Table 2, Figs. 4e and 6c for HRD\textsubscript{ow} &RND\textsubscript{ow} only). The mixing of o/w-type components is defined as “mixing allowed in some cases” in the Chozai-shishin (Table 1), similar to the mixing of w/o-type compounds and oily bases. With respect to the stability of the base, our results suggest that mixtures of o/w-type and o/w-type compounds are better than w/o-type compound and oily base mixtures. Ohtani et al reported that water is easily separated in mixtures of o/w-type creams and ointments by centrifugation and that the
antimicrobial activity of paraben is reduced in the aqueous phase of such mixtures upon touching with a finger\textsuperscript{18}). Our results suggest that from a hygiene perspective, mixtures of o/w-type compounds are preferable.

Similar results to mixtures of HRD\textsubscript{ow} and steroideal creams were obtained in mixtures of HRD\textsubscript{wo} and steroideal creams (w/o&o/w) after centrifugation (Table 2, Figs. 3e and 6b for HRD\textsubscript{wo}&RND\textsubscript{ow} only). The mixing of w/o-type and o/w-type compounds is defined as “impossible” (Table 1). The stability of mixtures in which both bases contain an oily component, water-soluble component, and a surfactant is superior to that of mixtures of w/o-type compounds and an oily base. However, the skin penetration of the API is reportedly lower in mixtures prepared with this type of base\textsuperscript{19}). As mixing clearly affects the physico-chemical properties and API release of pharmaceutical preparations, it is important to verify the characteristics of a preparation under a variety of conditions. Solid crystal of the API were observed in each area examined of the HRD\textsubscript{wo}&RND\textsubscript{ow} (w/o-type and o/w-type, Fig. 3e) and HRD\textsubscript{ow}&RND\textsubscript{ow} (o/w-type and o/w-type, Fig. 4e) mixtures, suggesting that uniformity of API dispersion was maintained after centrifugation in these combinations. By the way, any mixing mass ratio of the mixture in the present study is 1:1. In the future, there is a need to evaluate the stability of mixtures mixed in clinical used ratio.

Non-uniformity of water means also non-uniformity the balance of content of water and oil overall. In other words, it can be considered that emulsification becomes easier.
disintegrated in a variety of sites, and then this phenomenon is affecting the stability of the formulation. NIR spectra collected for subsamples taken from five random sites of the HRD\textsubscript{wo}&RND\textsubscript{o} and HRD\textsubscript{wo}&KND\textsubscript{o} mixtures were consistent (Fig. S6), suggesting that both mixtures were homogenous before centrifugation. Centrifugation-induced separation into layers at room temperature progressed in a time-dependent manner beginning at 1 minute (Fig. 7a for HRD\textsubscript{wo}&RND\textsubscript{o} only). No separation was observed upon centrifugation at 4 °C, however (Fig. 7b, for HRD\textsubscript{wo}&RND\textsubscript{o} only). Because liquid paraffin, one of the additives, maintains a high viscosity at 4 °C, it is more difficult to induce separation at low temperatures. These results suggest that cold storage of HRD\textsubscript{wo}&RND\textsubscript{o} and HRD\textsubscript{wo}&KND\textsubscript{o} mixtures provides for superior stability compared with room temperature storage. However, the following points regarding our results must be considered: (i) bias was observed in the dye distribution (Fig. 8); (ii) the intensity of peaks of hydroxyl groups (around 5200 cm\textsuperscript{-1}) in NIR spectra increased with depth in the mixture samples (Fig. 9); and (iii) a clear difference between upper and lower areas of samples was observed in polarized microscopic images (Fig. S7), suggesting that water movement had occurred despite the apparent absence of separation into layers. Similar NIR spectra were obtained even if the centrifugation time was reduced to 30 s (Fig. S8), suggesting that movement of water is induced even under relatively mild conditions.

In secondary derived spectra, less variation was observed in values for the hydroxyl
group peak (around 5200 cm$^{-1}$) between the upper, middle, and lower areas of HRD$_{wo}$&RND$_o$ mixtures compared with HRD$_{wo}$&KND$_o$ mixtures (Fig. 9), which was attributed to differences in the rheological properties of RND$_o$ and KND$_o$. In other words, the white petrolatum used in KND$_o$ preparations exhibits higher viscosity and yield than that used in RND$_o$ preparations$^{20, 21}$. Furthermore, the viscosity of HRD$_{wo}$&KND$_o$ was higher than that of HRD$_{wo}$&RND$_o$ (Fig. 10). These results suggest that certain rheological properties of mixtures can inhibit water transfer. Of note, the yield of NRS$_o$ is higher than that of either RND$_o$ or KND$_o$$^{20}$, and the viscosity of HRD$_{wo}$&NRS$_o$ mixtures was higher than that of the other mixtures examined in the present study (Fig. 10). The absence of layer separation and changes in the NIR spectra of samples of the upper and lower areas of HRD$_{wo}$&NRS$_o$ mixtures upon centrifugation at room temperature strongly suggests that there is a relationship between the maintenance of uniform water distribution and the viscosity of the base. Thixotrophy was more notable in HRD$_{wo}$&KND$_o$ mixtures compared with HRD$_{wo}$&RND$_o$ and HRD$_{wo}$&NRS$_o$ mixtures (Fig. 10). KND$_o$ alone is also reportedly highly thixotrophic$^{21}$. The thixotropic properties of HRD$_{wo}$&KND$_o$ mixtures is thus a reflection of the rheological properties of KND$_o$.

As factors that determine the stability and uniformity of the mixed formulation, we consider the balance of content of oleaginous base such as white petrolatum, water and surfactant is important. Moreover, rheological property of mixtures is also important factor as
shown in the present study. These factors are thought to be involved in a complex manner.

**Conclusion**

The present study examined the stability of moisturizer and external-application dermatologic preparation mixtures prepared with combinations of typical bases. NIR spectroscopy and dye-based analyses were used to characterize the movement of water in the mixtures prior to layer separation induced by centrifugation. Although external-application preparations are often evaluated based on changes in appearance over several months after mixing, the NIR spectroscopy–based approach described here would be useful for estimating the stability of external-application preparations immediately after mixing.
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Fig. 1 Definition of each part when layer separation was observed (a) or not observed (b) after centrifugation.

Fig. 2 Appearance of the mixture consisting of HRD_{w0} and RND_{o} after centrifugation. One drop of methylene blue (MB) aqueous solution (1.0 w/v%) or Sudan III liquid paraffin solution (1.0 w/v%) was added prior to mixing.

Fig. 3 Polarized microscopic images of the upper, middle, and lower layers or areas after centrifugation (16500×g, 7 min) of HRD_{w0}&RND_{o} and HRD_{w0}&RND_{ow} mixtures. (a) HRD_{w0}, (b) RND_{o} and mixture (HRD_{w0}&RND_{o}), (c) RND_{ow} and mixture (HRD_{w0}&RND_{ow}), (d) each layer of HRD_{w0}&RND_{o} mixture after centrifugation, (e) each area of HRD_{w0}&RND_{ow} mixture after centrifugation. Arrows indicate API crystals.

Fig. 4 Polarized microscopic images of the upper, middle, and lower layers or areas after centrifugation (16500×g, 7 min) of HRD_{ow}&RND_{o} and HRD_{ow}&RND_{ow} mixtures. (a) HRD_{ow}, (b) RND_{o} and mixture (HRD_{ow}&RND_{o}), (c) RND_{ow} and mixture (HRD_{ow}&RND_{ow}), (d) each layer of HRD_{ow}&RND_{o} mixture after centrifugation, (e) each area of HRD_{ow}&RND_{ow} mixture after centrifugation. Arrows indicate API crystals.
Fig. 5  NIR spectra of mixtures of HRD$_{wo}$ and steroidal ointments ((a) RND$_o$ and (b) NRS$_o$).  

*Left:* original spectra (4000–8000 cm$^{-1}$), *Right:* secondary derivative spectra (5000–5600 cm$^{-1}$). Upper, middle, and lower layers or areas after centrifugation (16500×g, 7 min).

Fig. 6  NIR spectra of mixtures of moisturizers and steroidal preparations (4000–8000 cm$^{-1}$).  
(a) HRD$_{ow}$ and RND$_o$, (b) HRD$_{wo}$ and RND$_{ow}$, (c) HRD$_{ow}$ and RND$_{ow}$. Upper, middle, and lower layers after centrifugation (16500×g, 7 min).

Fig. 7  Appearance of the HRD$_{wo}$$&$RND$_o$ mixture after centrifugation (16500×g, 0.5, 1, 3, 5, and 7 min) at room temperature (RT) or 4 °C.

Fig. 8  Appearance of the HRD$_{wo}$$&$RND$_o$ mixture after centrifugation at 4 °C (16500×g, 7 min). Methylene blue (MB) aqueous solution alone or both MB aqueous solution and Sudan III liquid paraffin solution (1.0 w/v%) was added prior to mixing.

Fig. 9  NIR spectra of mixtures of HRD$_{wo}$ and steroidal ointments ((a) KND$_o$ and (b) RND$_o$), 7 min after centrifugation at 4 °C (16500×g). *Left:* Original spectra (4000–8000 cm$^{-1}$), *Right:* secondary derivative spectra (5000–5600 cm$^{-1}$). Upper, middle, and lower areas after
centrifugation.

Fig. 10  Flow curves of mixtures of HRD\textsubscript{wo} and steroidal ointments. ○: HRD\textsubscript{wo} & RND\textsubscript{o}, ●: HRD\textsubscript{wo} & KND\textsubscript{o}, △: HRD\textsubscript{wo} & NRS\textsubscript{o}. 
Table 1. Mixing possibilities for external-application skin preparations listed in Chozai-shishin dispensing guide.

<table>
<thead>
<tr>
<th>Type of base</th>
<th>o</th>
<th>w</th>
<th>o/w</th>
<th>w/o</th>
<th>gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oily base (o)</td>
<td>☐</td>
<td>×</td>
<td>×</td>
<td>△</td>
<td>×</td>
</tr>
<tr>
<td>Water-soluble base (w)</td>
<td>×</td>
<td>☐</td>
<td>△</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>Oil in water (o/w)</td>
<td>×</td>
<td>△</td>
<td>△</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>Water in oil (w/o)</td>
<td>△</td>
<td>×</td>
<td>×</td>
<td>△</td>
<td>×</td>
</tr>
<tr>
<td>Gel base (gel)</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
</tbody>
</table>

☐: Mixing allowed  
△: Mixing allowed in some cases  
×: Mixing impossible
Table 2. Appearance of mixtures consisting of moisturizing creams and steroidal preparations after centrifugation (16500×g, 7 min).

<table>
<thead>
<tr>
<th>Moisturizer</th>
<th>Steroidal ointment (o)</th>
<th>Steroidal cream (o/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNDₜₒ</td>
<td>KNDₜₒ</td>
</tr>
<tr>
<td>HRDₜₙₒ</td>
<td>θ</td>
<td>θ</td>
</tr>
<tr>
<td>HRDₜₜₒ</td>
<td>θ</td>
<td>θ</td>
</tr>
</tbody>
</table>

θ : Separated into three layers
○ : Not separated
− : Not examined
Fig. 1 Definition of each part when the layer separation was observed (a) or not observed (b) after centrifugation.

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Fig. 2 Appearance of the mixture consisting of HRD<sub>wo</sub> and RND<sub>o</sub> after centrifugation. One drop of methylene blue (MB) aqueous solution (1.0 w/v%) or Sudan III liquid paraffin solution (1.0 w/v%) was added prior to mixing.
Fig. 3  Polarized microscopic images of upper, middle and lower layer or site after centrifugation (16500  g, 7min) of HRD$_{wo}$ & RND$_o$ and HRD$_{wo}$ & RND$_{ow}$. (a) HRD$_{wo}$, (b) RND$_o$ and mixture (HRD$_{wo}$ & RND$_o$), (c) RND$_{ow}$ and mixture (HRD$_{wo}$ & RND$_{ow}$), (d) each layer of HRD$_{wo}$ & RND$_o$ after centrifugation, (e) each site of HRD$_{wo}$ & RND$_{ow}$ after centrifugation. Arrows indicate the crystal of API.
Fig. 4  Polarized microscopic images of upper, middle and lower layer or site after centrifugation (16500 RND, 7 min) of HRD<sub>ow</sub>&RND<sub>ow</sub> and HRD<sub>ow</sub>&RND<sub>ow</sub>. (a) HRD<sub>ow</sub>, (b) RND<sub>ow</sub> and mixture (HRD<sub>ow</sub>& RND<sub>ow</sub>), (c) RND<sub>ow</sub> and mixture (HRD<sub>ow</sub>&RND<sub>ow</sub>), (d) each layer of HRD<sub>ow</sub>&RND<sub>ow</sub> after centrifugation, (e) each site of HRD<sub>ow</sub>&RND<sub>ow</sub> after centrifugation. Arrows indicate the crystal of API.
Fig. 5  NIR-spectra of mixtures of HRD\textsubscript{wo} and steroidal ointments ((a) RND\textsubscript{o} and (b) NRS\textsubscript{o}). \textit{Left}; Original spectra (4000-8000 cm\textsuperscript{-1}). \textit{Right}; second derivative spectra (5000-5600 cm\textsuperscript{-1}). Upper, middle and lower; each layer or site (after centrifugation) (16500 \textsuperscript{\textcircled{g}}, 7 min).

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Fig. 6 NIR-spectra of mixtures of moisturizer and steroidal preparations (4000-8000 cm⁻¹). (a) HRDₜₒ and RNDₜₒ, (b) HRDₜₒ and RNDₜₒ, (c) HRDₜₒ and RNDₜₒ. Upper, middle and lower; each layer after centrifugation (16500 g, 7 min).
Fig. 7 Appearance of the HRD<sub>w0</sub>&RND<sub>0</sub> after centrifugation (16500 g, 0.5, 1, 3, 5 and 7 min) at room temperature (RT) or 4 °C.

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Fig. 8  Appearance of the HRD_{w,o}&RND_{o} after centrifugation at 4 °C (16500 g, 7 min). Methylene blue (MB) aqueous solution alone or both of MB aqueous solution and Sudan III liquid paraffin solution (1.0 w/v%) were added prior to mixing.
Fig. 9  NIR-spectra of mixture of \( \text{HRD}_{\text{wo}} \) and steroidal ointments ((a) KND\( _o \) and (b) RND\( _o \)), 7 min after centrifugation at 4 °C (16500 \( \ddot{\text{g}} \)).

*Left:* Original spectra (4000-8000 cm\(^{-1}\)), *Right:* second derivative spectra (5000-5600 cm\(^{-1}\)). Upper, middle and lower; each site after centrifugation.
Fig. 10 Flow curves of mixtures of HRD<sub>wo</sub> and steroidal ointments. □ : HRD<sub>wo</sub>&RND<sub:o</sub>, □ : HRD<sub>wo</sub>&KND<sub:o</sub>, □ : HRD<sub>wo</sub>&NRS<sub:o</sub>