Quality Testing of Tacrolimus Ointment Mixed with Various Type of Ointments or Creams

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Received January 25, 2018; accepted August 9, 2018

Tacrolimus ointment is used worldwide to treat atopic dermatitis. Although tacrolimus ointment is not suitable for clinical admixtures, it is often mixed with various ointments or creams, such as corticosteroids, antibacterial agents, and moisturizing agents. There is only one report of quality testing of admixtures of tacrolimus ointment with adaparene gel (Differin® Gel). In this study, we used HPLC to evaluate the pharmaceutical stability of tacrolimus mixed with eight different dermatologic ointments or creams. No decrease in the tacrolimus content was observed in any of the mixtures after 4 weeks of storage at room temperature, indicating that tacrolimus admixtures are stable.

Key words tacrolimus ointment; admixture; quality testing; atopic dermatitis; HPLC

In general, commercially available pharmaceutical ointments and creams are designed to be used alone. However, steroidal external agents are frequently mixed by clinical pharmacists based on medical prescriptions by physicians with the aim of improving patient compliance, minimizing the potential for steroid-associated adverse drug effects, and inducing additive and multiplier effects from mixture with moisturizing agents. However, admixtures of ointments or creams can be affected by separation and changes in quality, potentially resulting in effects such as contact dermatitis or contamination of ointment pots with methicillin-resistant Staphylococcus aureus.

Atopic dermatitis associated with dermatologic ointment and cream use is linked to multiple etiologic factors. The symptoms and complications associated with atopic dermatitis can be reduced by suppressing the inflammation via medical treatment. Tacrolimus ointment (Protopic® Ointment: ProO) is an external anti-inflammatory agent used worldwide for the clinical treatment of atopic dermatitis. ProO is a first-line agent for treating atopic dermatitis and provides effective cure even of cases resistant to external steroid medicines.

ProO contains 0.1% (or 0.03% for pediatric use) tacrolimus hydrate (Fig. 1), which is a macrolide immunosuppressant produced by Streptomyces tsukubaensis. As tacrolimus strongly inhibits the production of cytokines by T cells, its mode of action is thought to involve suppression of inflammatory cell activity. However, ProO is not well suited for use in admixtures due to the special pharmaceutical technology. Mixing several topical medicines into one formulation has been shown to improve patient compliance due to ease of medication management. Although many reports have described changes in the appearance or stability of active components of steroidal ointment or cream mixtures, only one report has been published describing evaluation of the stability of ProO mixed with adaparene gel (Differin® Gel).

In this study, we used HPLC to evaluate the stability of tacrolimus in ProO mixed with other dermatologic ointments or creams used to treat atopic dermatitis. We found that the active ingredient did not change appreciably after extended storage at room temperature. Mixtures of eight different ointments or creams with ProO were evaluated, as shown below:

**Fig. 1. Structure of Tacrolimus Hydrate**
novel evidence was off-label. The results of the present study should be encouraging to atopic dermatitis patients and their physicians and pharmacists.

MATERIALS AND METHODS

**Ointments and Creams**  Drugs used in this study were as follows: atopic dermatitis remedy, tacrolimus hydrate (Protopic® Ointment, Astellas Pharma Inc., Tokyo, Japan); adrenal cortical hormone mixture, betamethasone valerate and gentamicin sulfate (Dermosol® Ointment, Iwaki Seiyaku Co., Ltd., Tokyo, Japan); moisturizing agent, urea (PASTARON® CREAM, Sato Pharmaceutical Co., Ltd., Tokyo, Japan); antifungal agent, ketoconazole (Nizoral® Cream, Janssen Pharmaceutical K.K., Tokyo, Japan); adrenal cortical hormones, clobetasone butyrate (KINDARON® OINTMENT, Maeda Pharmaceutical Industry Co., Ltd., Toyama, Japan) and diflorasone acetate (Diacort® Ointment, Pfizer Inc., N.Y., U.S.A.); antibacterial agent, nadifloxacin (Acuatim® cream, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan); active form of vitamin D3 external agent, maxacalcitol (Oxarol® Ointment, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan).

**Tacrolimus Hydrate Used for Calibration Curve**  Tacrolimus hydrate was also purchased from the Pharmaceutical and Medical Device Regulatory Science Society of Japan as a standard.

**Solvents**  Chloroform, ethanol, ethyl acetate, methanol, and tetrahydrofuran (THF) for elution of tacrolimus from ProO were purchased from KANTO CHEMICAL CO., INC. of guaranteed reagents. 2-Propanol, THF, and distilled water for HPLC were purchased from KANTO CHEMICAL CO., INC. of HPLC grade.

**Sample Preparation**  A total of 5.0 g of ointment or cream was added to 5.0 g of ProO and mixed thoroughly by hand using spatula. Mixed samples were placed in 10 g bottles, which were then covered with caps and stored at room temperature for 1, 2, and 4 weeks.

The mixtures (400 mg) were dissolved in THF (10 mL) by heating at 60°C and stored at 4°C for 24 h for the purpose of precipitating part of the base. After the liquid was filtered (φ=0.45 µm) using a membrane filter, each filtrate was analyzed quantitatively by HPLC (Fig. 3).

**Quantitative Analysis**  Quantitative analysis was carried out by HPLC using a Unifinepak C18 column (φ=4.6 mm×250 mm, particle size: 5 µm (JASCO, Tokyo, Japan) and system equipped with a UV detector (λ=220 nm, UV-2075 plus, JASCO). The flow rate was 1.0 mL/min. The temperature of the column oven was 50.0°C. The mobile phase was composed of water, isopropanol, and THF at a volumetric ratio of 7:2:1. Data were processed using an LC-Net II/ADC chromatography data system (JASCO).
RESULTS AND DISCUSSION

Pharmacists often prepare mixtures of ointments or creams in response to orders by dermatologists. At community pharmacies that do not own specialized mixing machines, pharmacists typically mix various ointments or creams by hand using spatula. Samples used in this study were prepared by hand mixing to reflect the conditions in a typical pharmacy in Japan.

We first examined the elution of tacrolimus from samples for quantitative HPLC analysis. We attempted to elute tacrolimus from ProO using ethanol or methanol due to its high solubility in these solvents, which are commonly used to elute ingredients from ointments. However, we observed that a lump of the ointment remained in both solvents. The same results were obtained after heating the samples at 60°C (Table 1). These results indicated that not all of the tacrolimus was eluted in ethanol or methanol. We then examined other solvents for elution, including chloroform, ethyl acetate, and THF, as shown in Table 2. ProO dissolved in each of these solvents upon heating at 60°C, and precipitated base was then obtained by storing the samples at 4°C.

Using chloroform as the solvent, ProO was mixed with DerO, DiaO, KinO, AcuO, and OxaO and dissolved by heating at 60°C, with subsequent precipitation of the base by storage at 4°C. In the case of AcuC, NizC, and PasC, the mixtures did not dissolve. All samples dissolved in ethyl acetate and THF by heating at 60°C, except for PasC mixed with ProO, and base precipitate was obtained after storage at 4°C. Because we planned to use THF as a solvent in the mobile phase for the measurement of tacrolimus by HPLC, we selected it as the solvent for elution. We then examined other solvents for elution, including chloroform, ethyl acetate, and THF, as shown in Table 2. ProO dissolved in each of these solvents upon heating at 60°C, and precipitated base was then obtained by storing the samples at 4°C.

Table 1. Results for Various Solvents Used for Elution of Tacrolimus from ProO

| Solvent  | Elution of tacrolimus |
|----------|-----------------------|
| Ethanol  | Not good              |
| Methanol | Not good              |
| Chloroform | Good                  |
| Ethyl acetate | Very good |
| THF     | Very good             |

Table 2. Elution of Tacrolimus from Samples Using Chloroform, Ethyl Acetate, and THF

| Chloroform | Ethyl acetate | THF |
|------------|---------------|-----|
| ProO       | ○             | ○   |
| ProO DerO  | ○             | ○   |
| ProO DiaO  | ○             | ○   |
| ProO KinO  | ○             | ○   |
| ProO AcuC  | ×             | ○   |
| ProO NizC  | ×             | ○   |
| ProO PasC  | ×             | ×   |
| ProO AcuO  | ○             | ○   |
| ProO OxaO  | ○             | ○   |

○: Soluble by heating at 60°C and a part of base was precipitated by stored at 4°C. ×: Insoluble.

To improve separation of the tacrolimus and clobetasone butyrate peaks, we examined the effect of changing the solvent ratios to 6 : 2 : 2 and 7 : 2 : 2. We obtained a single peak with overlap of the tacrolimus and clobetasone butyrate peaks in analyses using the solvent ratio of 6 : 2 : 2, with each compound exhibiting the same retention time (15.7 min). Using a solvent ratio of 7 : 2 : 2, we obtained two separate peaks, with retention times of 24.7 and 26.3 min for clobetasone butyrate and tacrolimus, respectively (Table 3). Based on these results, the pretreatment procedure was determined, as shown in Fig. 3. The details refer to “Sample preparation” described above.

ProO was dissolved completely under the experimental condition used in this study. The amount of tacrolimus in ProO agreed with the amount in the interview form of ProO. For these reasons, all tacrolimus in ProO was measured under the experimental condition used in this study.

A tacrolimus calibration curve was prepared using the standard reagent. Good linearity and correlation ($R^2=0.999$) were obtained over the concentration range examined (Fig. 4). On the chromatograph, the peak assigned to tacrolimus exhibited good symmetry and separation from the other peaks. These results suggest that any observed decrease in the area of the peak assigned to tacrolimus would be due to a decrease in the concentration of the drug.
tacrolimus content. Changes in the physical characteristics of tacrolimus as a result of mixing with other compounds would be manifested as a change in retention time.

In measurements conducted just after preparation, none of the samples exhibited a change in the retention time of tacrolimus. Assuming perfect dispensation of tacrolimus in the samples, the ratio of the measured amount to the theoretical amount would be 1.0. As such, this ratio was also evaluated as an indicator of the accuracy of dispensation. Preparation by hand mixing fulfilled the criteria for dispensation, as all samples were within the limits (target value ±10%). All samples were measured three times by HPLC at 0, 1, 2, and 4 weeks, and the ratio between the average measured values and theoretical values are shown in Table 4. The theoretical values were calculated with an equation below. Because when we weighted samples for HPLC, measured weight was different every time, so we calculated the theoretical value and then assessed the ratio between the average measured values and theoretical values.

\[
\text{Theoretical value} = \frac{\text{Average of three times weight of sample}}{(\text{Average of three times weight of ProO}) \times 2} \times \text{tacrolimus in ProO}
\]

It shows that the denominator is average of three times weight (mg) of samples for HPLC and the numerator is double value that average of weight three times weight (mg) of ProO for HPLC. Tacrolimus in ProO means that measured concentration of tacrolimus in ProO by HPLC.

If the ideal weighing was carried out, the content of tacrolimus in ProO should be the same value as the content of tacrolimus in the mixed sample since the volume of the THF solvent used was same.

As amount of weighed mixed sample is larger than 0.4 g, the value of tacrolimus obtained will be larger. The marginal inexact in weighing may change the result since the weighing

Table 4. Results of Measured Three Times Values of Tacrolimus by HPLC, and the Ratio between the Average Measured Values and Theoretical Values

|       | 0 week | 1 week | 2 week | 4 week |
|-------|--------|--------|--------|--------|
|       | Val. (Weight) | Ave. | Theo. | R<sup>6</sup> | Val. (Weight) | Ave. | Theo. | R<sup>6</sup> | Val. (Weight) | Ave. | Theo. | R<sup>6</sup> | Val. (Weight) | Ave. | Theo. | R<sup>6</sup> |
| ProO  | 21.2 (197.44) | 22.1 | — | — | 25.1 (204.64) | 25.0 | — | — | 24.1 (203.50) | 25.2 | — | — | 21.7 (202.34) | 22.5 | — | — | 27.1 (403.19) |
| DerO + ProO | 22.6 (400.26) | 22.8 | 22.1 | 1.0 | 22.8 (401.58) | 22.0 | 19.6 | 1.1 | 25.8 (401.85) | 24.5 | 25.3 | 1.0 | 23.8 (402.63) | 22.7 | 19.1 | 1.1 | 24.7 (398.51) |
| DiaO + ProO | 23.4 (399.33) | 23.1 | 22.2 | 1.0 | 22.0 (402.70) | 20.3 | 19.3 | 1.1 | 25.0 (401.05) | 25.8 | 25.3 | 1.0 | 23.8 (402.63) | 21.8 | 25.3 | 0.9 | 22.7 (398.63) |
| KinO + ProO | 23.4 (401.91) | 23.1 | 22.2 | 1.0 | 20.9 (403.95) | 21.4 | 19.6 | 1.1 | 25.1 (401.05) | 25.5 | 25.1 | 1.0 | 23.4 (401.29) | 21.8 | 25.4 | 0.9 | 25.9 (399.21) |
| AcuC + ProO | 21.7 (399.11) | 22.2 | 22.2 | 1.0 | 20.3 (401.94) | 20.0 | 19.6 | 1.0 | 26.6 (401.05) | 25.9 | 25.4 | 0.9 | 22.7 (407.40) | 21.8 | 25.3 | 0.9 | 25.8 (398.51) |
| NizC + ProO | 22.5 (405.32) | 23.2 | 22.1 | 1.1 | 21.5 (406.94) | 21.1 | 19.6 | 1.1 | 25.6 (401.05) | 26.0 | 25.1 | 1.0 | 24.5 (400.96) | 21.2 | 25.4 | 0.9 | 24.4 (403.96) |
| PasC + ProO | 25.0 (401.17) | 24.5 | 22.1 | 1.1 | 19.9 (405.44) | 20.3 | 19.7 | 1.0 | 25.6 (401.05) | 26.0 | 25.1 | 1.0 | 22.7 (401.34) | 20.7 | 25.5 | 0.9 | 26.0 (401.05) |
| AcuO + ProO | 21.0 (403.03) | 22.5 | 22.1 | 1.0 | 20.8 (402.55) | 21.2 | 19.6 | 1.1 | 25.2 (403.95) | 25.1 | 25.5 | 0.9 | 21.7 (404.32) | 22.1 | 25.4 | 0.9 | 22.9 (405.16) |
| OxaO + ProO | 23.6 (405.43) | 22.7 | 22.2 | 1.0 | 20.8 (402.63) | 18.2 | 401.49 | 19.9 | 19.6 | 1.0 | 24.6 (402.84) | 24.6 | 25.6 | 1.0 | 24.6 (402.84) |

Fig. 4. Tacrolimus Calibration Curve

Table 4. Results of Measured Three Times Values of Tacrolimus by HPLC, and the Ratio between the Average Measured Values and Theoretical Values

- Val: measured value of tacrolimus by HPLC. Figure in parentheses are measured weight of ProO or samples, and unit of weight is mg.
- Ave.: average measured values.
- Theo.: theoretical values.
- R: ratio between average measured values and theoretical values.
amount of 0.4 g is very small. In the case of 0.2 g weighing, the same situation may be investigated.

The theoretical value was determined with the consideration of the correction in the weighing process. The weighing amount of the mixed sample was two times of the weighing amount of ProO, since the mixed sample was made by mixing of the same amount of ProO and another ointment or cream.

To obtain the precise value, same operator proceeded the all weighing.

The ratio indexes \( R \) of all samples in our study were \( \geq 0.9 \) and \( \leq 1.1 \).

We also examined the effect of differences in the base, as ProO contains a special ‘droplet dispersion’ type base. When DerO, DiaO, and KinO, which contain an oleaginous base, were mixed with ProO, the ratios fluctuated between 1.0 and 1.1 from week 0 to 4 weeks of storage (Figs. 5(a)–(c)). A ratio of 1.1 was observed for DerO, DiaO, and KinO at week 1 and for DerO and KinO after 2 weeks of storage, which was within an acceptable range of error. In the case of mixtures of ProO with AcuC, NizC, and PasC, which consist of an oil/water type emulsion base, the ratio slowly decreased from 1.0 and 1.1 to 0.90 and 0.90 for tacrolimus mixed with AcuC and PasC, respectively, by 4 weeks of storage (Figs. 5(d), (f)). However, the ratio did not fall below 0.9, which was within the acceptable range of error. By contrast, the ratio for tacrolimus mixed with NizC almost remained at 1.0 from week 0 through 4 weeks of storage (Fig. 5(e)).

Collectively, these results revealed no change in the quality of tacrolimus in mixtures of ProO and eight different ointments or creams stored for 4 weeks at room temperature. Hand mixing of the preparations had no adverse effect on the quality of tacrolimus in samples analyzed after storage for 4 weeks. Our results also confirm the quality of ointment mixtures prepared by common process at community pharmacies.

In the present study, we examined the stability of tacrolimus mixed with five different ointments or three different creams but did not examine potential changes in the base itself resulting from mixing. This issue will be addressed in future studies. Our results provide broad support for evidence-based medicine performed by dermatologists and pharmacists.

**Conflict of Interest** The authors declare no conflict of interest.

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