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Quality control of hospital preparations: Establishment of a simple and rapid method for quantifying ulinastatin in vaginal suppositories

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Ulinastatin vaginal suppositories, used to prevent threatened premature delivery, are frequently used in hospitals. However, there is no established method for quantifying ulinastatin contained in suppositories. Therefore, we investigated a simple and efficient method for quantifying ulinastatin contained in suppositories. Our analytical method involved removal of the base; optimising the enzyme inhibition reaction time and enzyme reaction time; and measuring the absorbance. The modified method was reproducible, operation time was significantly shortened, and cost was reduced to approximately 1/17 of that of the previously reported method. This simple and rapid quantitative method could contribute to the improvement of quality control of ulinastatin vaginal suppositories as an extemporaneous hospital preparation.

1. Introduction

Ulinastatin, a glycoprotein obtained by the separation and purification of human urine, inhibits various proteolytic enzymes (trypsin, elastase, among others), and is clinically used for treating acute pancreatitis, acute exacerbation phase of chronic recurrent pancreatitis, and acute circulatory insufficiency due to haemorrhagic and bacterial shock (Kanayama et al. 1995). Because it is known to exhibit an anti-inflammatory effect by inhibiting elastase and cytokine activity (Kanayama et al. 1999), ulinastatin vaginal suppositories are considered to be effective for the treatment of threatened premature delivery in obstetrics (Kanayama et al. 1995; Kanayama et al. 1996), and it is frequently used as a hospital preparation in Japan. Our hospital also prepares ulinastatin vaginal suppositories and evaluates ulinastatin content in the prepared suppository. The Japanese Pharmacopoeia has described a method for quantifying ulinastatin, the principle of which is based on the trypsin inhibitory activity of ulinastatin (The Japanese Pharmacopoeia, 17th ed., 2016). However, there is no clarification on the method of quantifying ulinastatin contained in suppositories. The previously reported quantitative method (hereinafter referred to as the existing method) is time-consuming and the operation is complicated. Therefore, in this study, we investigated an analytical method involving removal of the base followed by measuring the absorbance. This small-scale, simple, and rapid quantitative method was examined to shorten the operation time, reduce the quantity of reagents, and increase the convenience to evaluate the quality of hospital preparations.

2. Investigations and results

2.1. Base removal method

The examination results of the method for removing the base material are shown in Fig. 1A. When the base was removed with organic solvents, hexane and chloroform, the correlation coefficients were -0.9971 and -0.9970, respectively, in the concentration range of 0–300 Units/mL, indicating good linearity, and the absorbance values were similar. However, when compared with that of the sample, which was measured without adding the base component, the absorbance value was high (about 0.02). In addition, the linearity of the calibration curve and the absorbance value were similar to those of the sample from which the base was removed with an aqueous membrane filter (pore size 0.22 μm). Therefore, in either of the methods using an organic solvent, such as hexane and chloroform, the base material was removed in the same manner as in the case of using an aqueous membrane filter (pore size 0.22 μm).

2.2. Enzyme inhibition reaction time and enzyme reaction time

Enzyme inhibition reaction time and enzyme reaction time are shown in Fig. 1B and Fig. 1C, respectively. When enzyme inhibition was performed at reaction times of 1, 2, and 4 min, the correlation coefficients were -0.9980, -0.9950, and -0.9958, respectively, in the concentration range of 0–300 Units/mL, indicating good linearity, and the absorbance values were similar (Fig. 1B). However, the highest absorbance was obtained when the enzyme reaction time was 4 min (Fig. 1C).

2.3. Method for measuring absorbance

The examination results of the method for measuring absorbance are shown in Fig. 1D. The absorbance measured using the spectrophotometer was corrected by the optical path length, and it was adjusted to the absorbance measured using the microplate photometer. When using a spectrophotometer and a microplate photometer, the correlation coefficients were -0.9977 and -0.9957, respectively, in the concentration range of 0–300 Units/mL, indicating good linearity, and absorbance values were similar.

2.4. Reproducibility of calibration curve using the modified method

Regarding the calibration curve using the modified method, the average value, standard deviation, and coefficient of variation for the absorbance of the standard solution at 0–300 Units/mL are shown in Table 1 (n=6). The calibration curve was in accordance with Beer's law, with less dispersion of absorbance and good reproducibility.

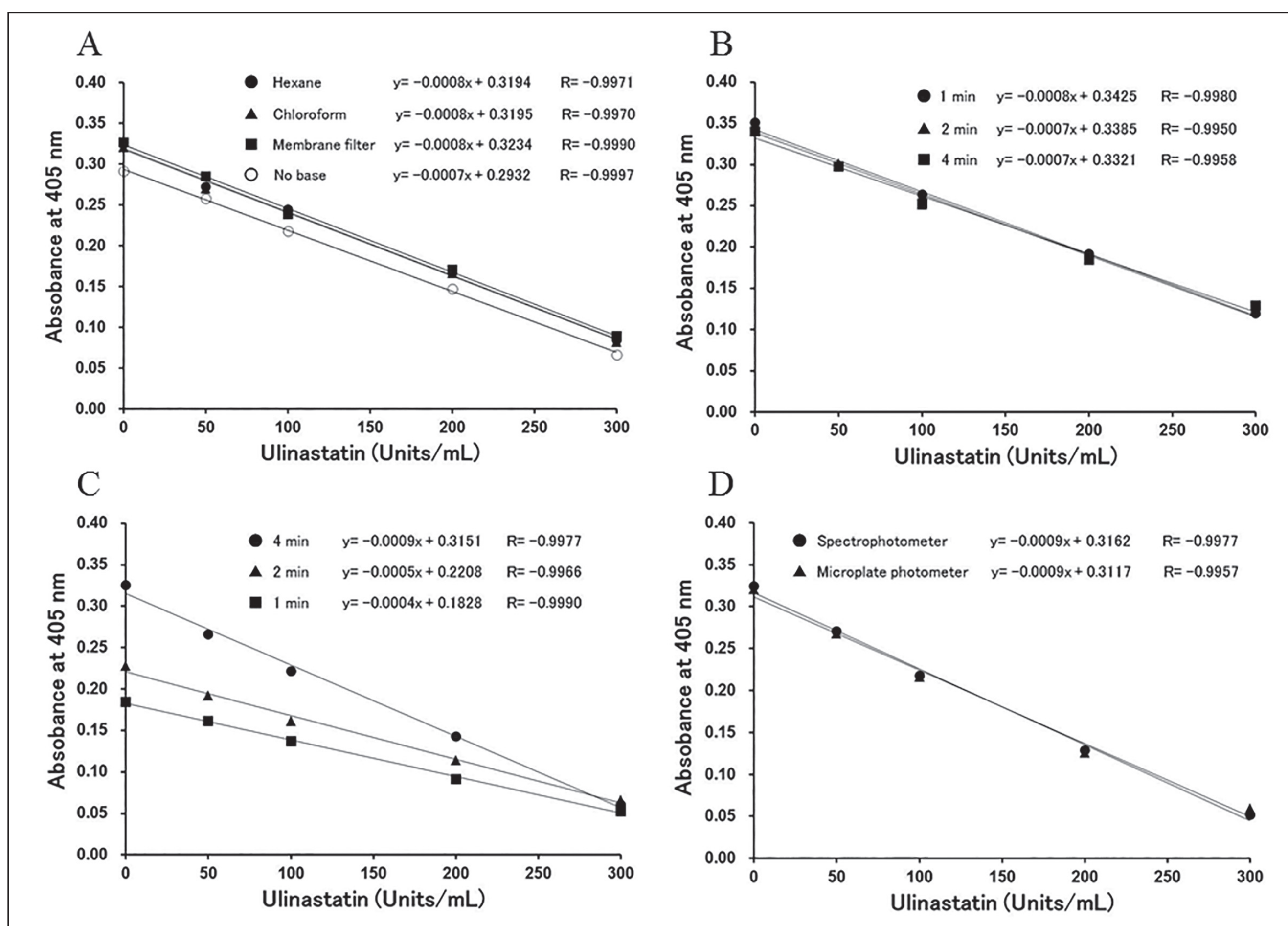

Fig. 1: Various conditions examined for establishing the modified method.
A: Base removal method, B: enzyme inhibition reaction time, C: enzyme reaction time, D: measurement of absorbance.
Vertical axis: Absorbance at 405 nm, Horizontal axis: Ulinastatin concentration (Units/mL)

Table 1: Reproducibility of calibration curve for the modified method

Ulinastatin concentration (Units/mL)	Average value of absorbance	Standard deviation	Coefficient of variation
0	0.322	0.003	0.009
50	0.269	0.008	0.030
100	0.208	0.007	0.034
200	0.111	0.005	0.045
300	0.055	0.004	0.073

2.5. *Content uniformity test*

Results of the content uniformity test are shown in Table 2. When measurement was performed using the existing or modified method for 10 suppository samples prepared in our hospital, the average content was 102.2% or 101.6% respectively, and the standard deviation was 5.2 or 5.1% respectively. Substituting these values into formula 1 of the judgement value, judgement values of 14.7 % and 13.7 % were obtained respectively, which conformed to the content uniformity test of the Japanese Pharmacopoeia, 17th revision.

2.6. *Time and cost required for measurement*

Using the existing and modified methods shown in Fig. 2, the time that five pharmacists, engaged in the preparation of hospital preparations, required for the quantification of 25 samples (10 samples of ulinastatin vaginal suppositories prepared and 15 samples necessary for preparing the calibration curve [five concentration points in triplicate]) was compared. In comparison with that of the existing method, the measurement time of the modified method significantly decreased (Table 3A). Moreover, with the modified method, the cost for measuring one sample decreased to about 1/17 of that reported for the existing method (Table 3B).

3. Discussion

When preparing hospital preparations, it is important to improve facilities, system, formulation technology, and quality assurance to ensure efficacy and safety. In our hospital, the preparation procedure manual (checklist) is prepared for all hospital preparations

Table 2: Content uniformity of ulinastatin suppositories

	Sample No.	1	2	3	4	5	6	7	8	9	10	Average	Standard deviation	Judgement value
Existing method	%Estimate	106.5	96.3	107.5	105.8	95.2	104.5	97.2	107.6	105.1	96.6	102.2	5.2	14.7
Modified method	%Estimate	107.7	95.5	106.3	103.7	97.3	101.3	94.3	105.5	106.6	97.5	101.6	5.1	13.7

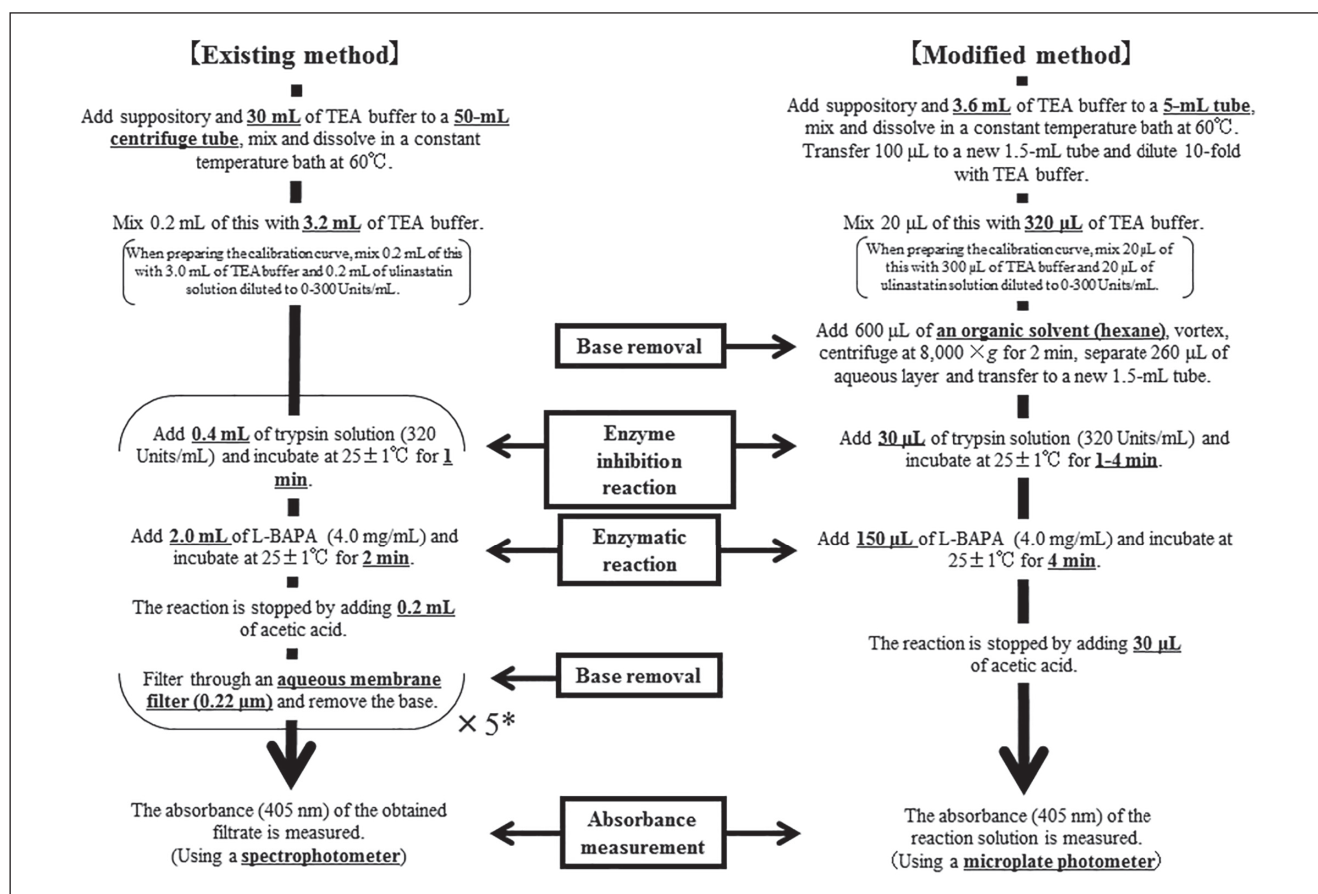

Fig. 2: Details of existing (left) and modified (right) methods. In the modified method, an organic solvent (hexane) was used for base removal, the total reagent volumes were scaled down to about 1/10, and the absorbance was measured using a microplate photometer, enabling simple and rapid quantification. *Performed using five samples at a time. The same steps were repeated five times for 25 samples.

based on the procedure manual; hospital preparations are prepared by two or more pharmacists while systematically checking and recording the preparation process, and striving to ensure quality.

In the quality evaluation after ulinastatin vaginal suppository preparation, we measured the ulinastatin content in suppositories using a procedure already reported. However, the existing method is time-consuming and the operation is complicated. The Japanese Pharmacopoeia has described a method for quantifying ulinastatin, the principle of which is based on the trypsin inhibitory activity of ulinastatin (Fig. 3). In this method, when trypsin acts on a colourless L-BAPA solution, *p*-nitroaniline is released and the reaction solution turns yellow. However, when ulinastatin that inhibits trypsin activity is added to this reaction system, the release of *p*-nitroaniline is suppressed and the absorbance decreases. By measuring the absorbance of this yellow coloured substance, *p*-nitroaniline, the concentration of ulinastatin is determined indirectly. In this study, we aimed to establish a modified small-scale, simple, and rapid quantitative method. To set the conditions of the modified method, the capacity of the sample was scaled down to 1/10 of that of the existing method. Firstly, conditions were examined for the following four items: base removal method, enzyme inhibition reaction time, enzyme reaction time, and absorbance measurement method (a spectrophotometer or a microplate photometer).

To measure the content of ulinastatin contained in the suppository, it is necessary to remove Witepsol W-35® constituting the suppository base. Regarding base removal method in the existing method, the suppository base is removed using an aqueous membrane filter (pore size 0.22 μm). However, because one filter is required for each sample, the method is complicated and time-consuming. Moreover, the membrane filters bear a very high cost (Table 3B). Therefore, we considered separating the suppository base by using an organic solvent. By removing the base using an organic solvent (hexane or chloroform), the linearity of the calibration curve was maintained within the concentration range of 0–300 Units/mL (Fig. 1A). Compared with that reported for the method without mixing the base material, the absorbance was high (about 0.02), probably because the absorbance increased due to the presence of some residual base. However, because the linearity of the calibration curve was maintained when measuring the absorbance by mixing the same amount of base as that in the suppository for preparing the calibration curve and uniformly influencing the base in each sample, the method was considered to be sufficiently suitable. In addition, the linearity and absorbance values of the calibration curve obtained by separating and removing the base with hexane or chloroform were similar to those obtained with the sample from which the base was removed with an aqueous membrane filter (pore size 0.22 μm) (Fig. 1A). Therefore, we decided to carry out the measurement by removing the base using an organic solvent, which is easier to perform. We decided to use hexane (to which user exposure is less and hexane is less contaminating to the environment) because similar calibration curves were obtained with hexane and chloroform. Organic solvents often precipitate proteins. However, in this study, the same absorbance was observed with organic solvent (hexane or chloroform) and without organic solvent (membrane filter) (Fig. 1A). Therefore, we suggested that there was no effect of protein precipitation.

Investigation of the enzyme inhibition reaction time showed that the linearity of the calibration curve and the absorbance values were the same regardless of any inhibition time of 1, 2, or 4 min, and the difference in enzyme inhibition reaction time did not affect the measurement results between 1 and 4 min (Fig. 1B). Because enzyme inhibition reaction time was set to 1 min in the existing method, rapid operation was required, and it was difficult to handle many samples at the same time. For example, in the enzyme inhibition and enzymatic reactions, only five samples at a time could be handled. We had to repeat the same steps five times for 25 samples

Table 3: Comparison of approximate estimates of time required for measurement and cost per sample

A. Time estimates				
Quantitative method	n	Average (min)	Standard deviation	*P*
Existing method	5	110.5	9.6	*<0.001
Modified method	5	64.3	12.5	

B. Cost estimates			
Reagents/Consumables	Unit price (yen)	Existing method (yen/required quantity)	Modified method (yen/required quantity)
0.1 M TEA buffer solution (mL)	0.03	1.0/33	0.13/4.3
Trypsin solution (mL)	2.0	0.8/0.4	0.06/0.03
L-BAPA solution (mL)	40	80/2.0	6.0/0.15
Acetate (mL)	2.0	0.4/0.2	0.2/0.1
10-mL Syringe (piece)	4.5	4.5/1	
15-mL Centrifuge tube (piece)	24	48/2	
50-mL Centrifuge tube (piece)	30	30/1	
0.22-μm Aqueous membrane filter (piece)	500	500/1	
Hexane (mL)	2.0		1.2/0.6
1.5-mL Micro tube (piece)	4.0		12/3
5-mL Micro tube (piece)	18		18/1
Total (yen)		664.7	37.6

(Fig. 2, left *). However, in the modified method, the enzyme inhibition reaction time was set to 1–4 min. This allowed handling of a large number of samples at the same time (Fig. 2, right).

Analysis of the enzyme reaction time revealed that the linearity of the calibration curve was maintained regardless of any enzyme reaction time of 1, 2, or 4 min, and the longer the enzyme reaction time was, the higher was the absorbance obtained (Fig. 1C). However, the linearity of the calibration curve was not maintained over 6 min (data not shown). If the linearity of the calibration curve is maintained, the measurement sensitivity should improve as a high absorbance is obtained. Therefore, we set the enzyme reaction time to 4 min, which was the upper limit value where there was no problem within the examined range.

When comparing the results of measuring the absorbance using a spectrophotometer with those using a microplate photometer, regardless of which method was used for measurement, the linearity of the calibration curves and absorbance values were similar (Fig. 1D). Therefore, we decided to measure absorbance with a microplate photometer, which is easier and quicker.

Furthermore, when comparing the time required for measurement by each method, the time required for the measurement by the modified method was significantly shortened compared with that reported for the existing method (Table 3A). Because measurements were performed using a 1.5-mL microtube or a 96-well microplate owing to the reduced reagent amount (to about 1/10), the base was not removed with a membrane filter but separated and removed by an organic solvent (hexane), and the absorbance was measured with a microplate photometer, many samples can be analysed easily and quickly (Fig. 2, right). In addition, the cost of measuring one sample was reduced to about 1/17 (Table 3B)

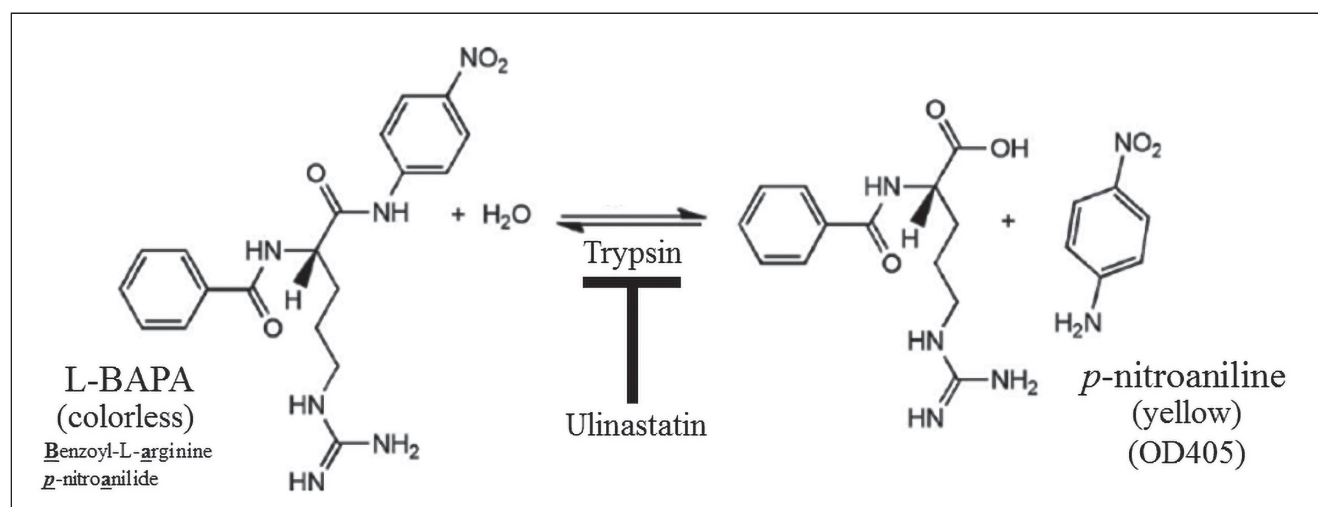

Fig. 3: Schematic of ulinastatin quantification principle. When trypsin acts on a colourless L-BAPA solution, *p*-nitroaniline is released and the reaction solution turns yellow (Reichelt et al. 2011). However, when ulinastatin that inhibits trypsin activity is added to this reaction system, the release of *p*-nitroaniline is suppressed and the absorbance (OD405) decreases.

because of the decrease mostly in the cost for membrane filter and reduced reagent capacity used for measurement. As the number of samples increases, the modified method becomes more powerful, thereby significantly reducing time and cost. In addition to ulinastatin vaginal suppositories, other suppositories in the hospital are also prepared using oleaginous bases, and this modified method can be applied to other hospital preparations.
As a result of examining the method for the quantitative determination of ulinastatin vaginal suppository, we could establish a useful, convenient, and inexpensive quantitative method as shown in Fig. 2, which led to a reduction in workload. The quantitative method established was reproducible, easy, quick, and inexpensive; it is considered to be useful in improving the quality control of hospital preparations and allowing cost reduction in hospitals. In the future, we propose to establish quality evaluation methods for other hospital preparations, and improve formulations considering patient background, and perform clinical evaluation.

4. Experimental

4.1. Materials

We used 50,000 Units of Miraclid® injection solution (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) as a raw material for ulinastatin vaginal suppository, and Witepsol W-35® (Watahan Trading Co., Ltd., Tokyo, Japan) as a suppository base. 2, 2′, 2′′-Titrilotriethanol (Triethanolamine; TEA) buffer (special grade) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used adjusted at pH 7.8. Hexane, chloroform, and acetic acid (special grade) were purchased from the Nacalai Tesque (Kyoto, Japan). Trypsin was derived from porcine pancreas (Wako). N-α-benzoyl-L-arginine-*p*-nitroanilide (L-BAPA) was purchased from Peptide Laboratories (Osaka, Japan).

4.2. Preparation of ulinastatin vaginal suppositories (5,000 Units)

An oleaginous suppository base (Witepsol W35®) was melted by heating at a constant temperature of 60 °C, and 24 mL of the base was emulsified with 3 mL of Miraclid injection solution® using a Polytron homogeniser PT3100 (Central Scientific Commerce, Inc., Tokyo, Japan). This emulsion was injected into a 0.9-mL suppository container (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan), allowed to solidify by cooling, and further solidified at 4 °C.

4.3. Modified method for the determination of ulinastatin contained in the suppositories

The ulinastatin vaginal suppository (0.9-mL) prepared using the method described in the preceding paragraph (or 0.9-mL suppository base only to create calibration curve) and 3.6-mL of TEA buffer were added to a 5-mL tube, mixed, and melted by heating at a constant temperature at 60 °C. Then, 100 µL of this suspension was transferred to a new 1.5-mL tube, diluted 10-fold with TEA buffer at 60 °C, and mixed. Then, 20 µL of this was added to a new 1.5-mL tube containing 320 µL of TEA buffer and mixed. Concurrently, 20 µL of this suppository base-only suspension was added to a new 1.5-mL tube containing 300 µL of TEA buffer and 20 µL of ulinastatin solution diluted to 0-300 Units/mL for the calibration curve.
To these were added 600 µL of an organic solvent (hexane) at room temperature to remove the oleaginous base, vortexed, centrifuged at 8,000 ×g for 2 min (to separate the oleaginous layer from the aqueous layer), and 260 µL of aqueous layer (layer in which ulinastatin is dissolved) was transferred to a new 1.5-mL tube. To this was added 30 µL of trypsin solution (320 Units/mL) for enzyme inhibition reaction and the mixture was incubated at 25±1 °C for 1-4 min. Then, to this was added 150 µL of L-BAPA solution (4.0 mg/mL) to give a coloured product (yellow) for enzymatic reaction and the mixture incubated at 25±1 °C for 4 min. The colour reaction was stopped by adding 30 µL of acetic acid, and the absorbance of the reaction solution at 405 nm was measured using a microplate photometer. The existing method was based on the method of Satake et al. (2011) and Kamimura et al. (2007).

4.4. Test items

4.4.1. Removal of the base

From the dissolved and diluted ulinastatin vaginal suppository, the base components were separated and removed using either hexane or chloroform, and the process was monitored using a colorimetric reaction. Calibration curves were prepared in the concentration range of 0–300 Units/mL in the base removal method with hexane and chloroform, and separation efficiencies using the two separation solvents were compared and examined. In addition, as a control, according to the existing method, a solution from which the base was removed by an aqueous membrane filter (pore size 0.22 µm) (Millex®, Merck, Tokyo, Japan) and a solution prepared without the addition of a base component were similarly analysed to prepare the calibration curves, and the most appropriate base removal method in combination was comprehensively examined. All the steps, except base removal, were carried out based on the modified method shown in Fig. 2.

4.4.2. Enzyme inhibition reaction time and enzyme reaction time

Trypsin solution and L-BAPA were added to the solution in which ulinastatin vaginal suppository was dissolved and diluted, and the trypsin enzyme inhibition reaction time and the enzyme reaction time were set at 1, 2, and 4 min each, and calibration curves were prepared in the concentration range of 0–300 Units/mL for each reaction time, and the optimal enzyme inhibition reaction time and enzyme reaction time were compared and examined. All steps other than enzyme inhibition reaction and enzyme reaction were carried out based on the modified method shown in Fig. 2.

4.4.3. Method for measuring absorbance

A calibration curve was prepared by measuring the absorbance at 405 nm using a spectrophotometer (Biochrom 4060, Pharmacia LKB Biochrom Ltd., Cambridge, England) and a microplate photometer (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA, USA). Then, measurement results of the spectrophotometer and the microplate photometer were compared. All steps, other than the absorbance measurement, were carried out based on the modified method shown in Fig. 2.

4.4.4. Reproducibility of calibration curve using the modified method

Regarding the calibration curve using the modified method, the average value, standard deviation, and coefficient of variation for the absorbance of the standard solution at 0–300 Units/mL were calculated (n=6) and reproducibility was evaluated.

4.4.5 Content uniformity test

We randomly selected 10 out of 30 suppositories prepared at our hospital and ulinastatin content was measured using the modified method. Then, based on the judgement formula (Eq. 1) of content uniformity test described in the Japanese Pharmacopoeia, 17th revision, judgement value was calculated by judgement coefficient k=2.4 prescribed in the Japanese Pharmacopoeia and the standard deviation of the main drug content in the preparation. When the judgement value does not exceed 15.0%, it was set as conformity.

Judgement value : $|M-X|+ks$ (1)

M: Reference value (100.0%), X: Average value of main drug content in preparation (%), k (Judgement coefficient): 2.4 (when n=10), s: Standard deviation of main ingredient content in formulation

4.4.6. Time and cost required for measurement

For pharmacists engaged in preparing hospital preparations, the time required for the quantitative determination of ulinastatin contained in suppositories was measured and compared using the existing and modified methods shown in Fig. 2. Statistical analysis was carried out by unpaired *t* test, and $p < 0.05$ was judged as significant difference. In addition, the cost per sample when quantitating ulinastatin contained in suppositories was estimated for both methods and compared.

Conflict of interest: All authors of this paper have no conflicts of interest to be disclosed.

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