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FORMATION AND GROWTH INHIBITION OF CALCIUM OXALATE CRYSTALS BY TAKUSHA (*ALISMATIS RHIZOMA*)

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Abstract

The aim of this study was to examine the effect of Takusha (*Alismatis rhizoma*), a Kampou medicine, on the formation, growth and aggregation of calcium oxalate (CaOx) crystals by a seed crystal system, an undiluted urine system and a continuous flow crystallizer system.

In the seed crystal method using a metastable solution, the inhibitory effect on aggregation and growth of CaOx crystals was calculated using a Coulter counter. Takusha had a strong inhibitory effect on the aggregation and growth when the concentration was above 10 mg/ml. In measuring the metastable limit by the microplate method, Takusha had a mild inhibitory effect on the formation of crystals above the concentration of 1000 mg/ml. In the undiluted urine system, the formation and growth of CaOx crystals precipitated in response to a load of sodium oxalate were measured. Takusha had a strong inhibitory effect at concentrations of 100 to 1000 mg/ml. In the continuous flow crystallizer system, nucleation rate, growth rate and crystal mass were decreased in proportion to the increase of added Takusha.

These results suggest that Takusha strongly suppressed each step of crystal formation, growth and aggregation of CaOx crystals, and therefore, may be a useful drug for preventing CaOx urolithiasis.

Key Words: Takusha (*Alismatis rhizoma*), calcium oxalate crystals, crystal aggregation, undiluted urine system, continuous flow crystallizer system.

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Introduction

Although extracorporeal shock wave lithotripsy has revolutionized the treatment of renal stones, many problems remain, such as the long-term renal damage, hypertension and symptomatic recurrences [4, 5]. Stone fragments that are retained after treatment may serve as nidi for the formation of new stones. A prophylactic approach that has not been extensively investigated is the use of chemical treatment to prevent or reduce the rate of stone recurrence in individuals subjected to lithotripsy. In recent years, a variety of prophylactic agents have been used to reduce recurrences in patients suffering from calcium nephrolithiasis, including hydrochlorothiazide, orthophosphate, alkali-citrates and magnesium. Some inhibitors have been chosen and used for clinical treatment. Although a number of studies have clarified that some macromolecules and low molecular substances are inhibitors of calcium oxalate (CaOx) crystallization, the clinical use of these inhibitors to prevent the formation of CaOx stones has been limited except for citrate, magnesium and orthophosphate [8, 9]. It is well known that glycosaminoglycans (GAGs) as well as urinary proteins are present in the matrices of urinary stones and show strong inhibition against CaOx crystals [10, 19, 20, 21]. But these macromolecules, even though they exerted powerful inhibition on CaOx crystals, are not expected to increase in the urine probably because of their high molecular weight.

Takusha (*Alismatis rhizoma*) is a kind of Chinese drug that is contained in some Kampou drugs in Japan. Kampou means a traditional Japanese herbal therapeutic system that originated in China. For many years, Choreito that contains Takusha has been used for the treatment of pollakisuria, thirst or urinary stone disease. Little is known about its components, and few reports are available about Takusha in the field of stone research. Koide *et al.* [6] reported that Takusha had strong inhibition using a seed crystal system *in vitro* and *in vivo*. The role of Takusha in an undiluted urine system as well as in a continuous flow crystallizer system has not been reported in the literature. Thus, the aim of this study was to evaluate the efficacy of Takusha in preventing the nucleation and growth *in vitro* of CaOx crystals in a seed crystal system, an undiluted urine

system, a continuous flow crystallizer system and scanning electron microscopy (SEM).

Materials and Methods

Materials

Takusha was obtained from Tsumura & Co. (Tokyo, Japan). CaOx monohydrate crystals were purchased from Nacarai Tesque Co. (Kyoto, Japan). Other reagents were of analytical grade and purchased from Sigma Chemical Co. (Tokyo, Japan).

Preparation of Takusha

As Takusha contains insoluble components, one gram of Takusha was dissolved in 50 ml of doubly distilled water at room temperature for 48 hours, then centrifuged. Its supernatant was freeze dried and weighed. The test solution was prepared at the concentration of 10 mg per ml.

Preparation of the solution for seed crystal method [11, 13]

Calcium chloride solution (1 mM) was added to sodium oxalate solution (0.2 mM) with 0.15 M sodium chloride and adjusted to pH 6.0 with 10 mM sodium cacodylate, and filtered through a 0.45 μm Millipore filter (Millipore Co., Tokyo, Japan). CaOx monohydrate crystals were added to the mixed solution and the number of the crystals was counter using a Coulter counter (Model TA II, fitted with an NEC personal computer, aperture size 100 μm), (Coulter Co., Tokyo, Japan). The crystal size distribution was counted and compared before and after 1 hour incubation [11]. Then, the inhibitory activity of the aggregation (Ia) and growth (Ig) was calculated from the relative change of the crystal number and volume, respectively, according to the method of Ryall *et al*. [13]. In this metastable solution, both crystal aggregation and growth proceed without new crystal nucleation. One hundred percent of Ia or Ig means complete inhibition and 0% means nil inhibition.

Preparation of undiluted urine method [14, 17]

Twenty-four hour urine specimens were collected from 5 healthy men aged 30-43 years old, and pooled. None showed any sign of blood by chemical test. Urine specimens were centrifuged at 6,000 X*g* for 60 minutes (RS-206, TOMY, Tokyo, Japan) and filtered through 8 μm followed by 0.22 μm Millipore GV filters, being used as SF (spun and filtered urine), then ultrafiltered using a hollow fiber bundle (AIP-1010, Asahikasei KK, Tokyo, Japan) with a normal molecular cut-off of 10 kDa (UF; ultrafiltered urine). Each 100 ml urine was prepared for a further experiment. The method of inducing CaOx crystallization in undiluted urine has been described elsewhere [14, 17]. Briefly, the minimum amount of oxalate required to produce crystals in 200 μl of urine by adding 2 μl of a graded concentration series of sodium oxalate was used to determine the metastable limit by microplate and inverted microscopy [17]. Once the metastable limit had been measured, an amount of oxalate, 0.3 mmol/L (final concentration) in excess of this limit, was added to urine specimens dropwisely. A Coulter counter was used to monitor the crystal particle size every ten minutes during a 60 minute incubation period in a 37°C shaking water bath. After 60 minute incubation, samples for SEM were prepared. Takusha was added into UF specimens. Urine samples for SEM were prepared at 60 minute incubation. Each 500 μl urine was filtered on a 0.22 μm (10 mm) Millipore filter. Crystals on the filter were dried in a dessicater at room temperature, then mounted on a stub and coated for SEM. The stubs were examined with a JEOL-JSM 840 (Tokyo, Japan) using a 15 kV accelerating voltage, a 6×10^{-9} A probe current and a working distance of 15 mm.

Preparation of the solutions for continuous flow crystallizer method [1, 2, 3, 7, 12, 16]

A continuous flow crystallizer system, incorporating a 75 ml beaker with an internal chamber volume of 50 ml was used, which was almost the same as reported previously [18]. The composition of the two feed solutions was based on those used by Robertson and Scurr [12]. The final concentrations of calcium and oxalate were 6 mmol/l and 0.6 mmol/l respectively, while those of Takusha were 0, 1, 10, 100 and 1000 μg/ml. The pH was maintained at 5.8. Equal volumes of the two feed solutions were pumped into the chamber using a multi-pump PA-42 (Yamato Co., Tokyo, Japan) at the rate of 12 ml/min. The 50 ml volume of the chamber was maintained by removing the overflow with a vacuum pump. The term, nucleation rate (B^0), growth rate (G) and crystal mass (M_T), was used according to the references [1, 2, 3, 7, 16], and then calculated by the formula described in previous reports. Nucleation rate was calculated from the intercept with the y-axis and growth rate from the slope of the population density distribution curve.

Statistical analysis

Experiments were repeated to give a total of 6 values at each concentration of Takusha. Data were analyzed using the Wilcoxon rank sum test.

Results

In a seed crystal system, Takusha showed strong Ia and Ig according to the concentration as shown in Figure 1. Potent Ia was observed above the concentration of 10 μg/ml. High concentration at 100 or 1000 μg/ml suggested dissolution or disaggregation of seeded crystals. Potent Ig

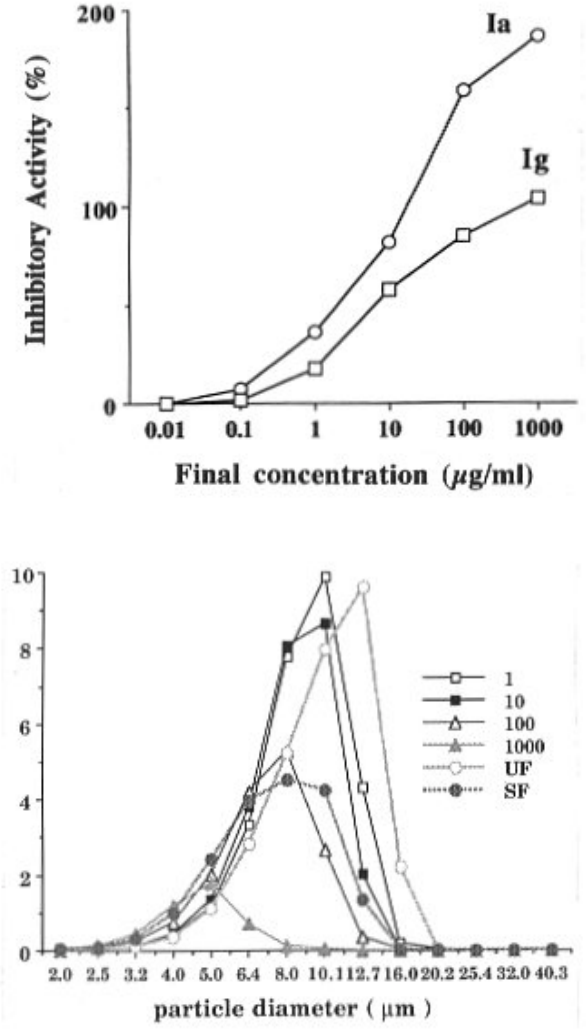

Figure 1. Inhibitory activity of CaOx crystals of Takusha in the seed crystal system. Mean value was presented. Zero percent means no effect on crystals and 100% means complete inhibition of aggregation or growth. A value over 100% means disaggregation or dissolution of the crystals in the metastable solution. Ia: Inhibitory activity of crystal aggregation, Ig: Inhibitory activity of crystal growth.

Figure 2. Crystal size distribution in undiluted urine system using various concentrations of Takusha. Numbers 1, 10, 100 and 1000 mean the final concentration of Takusha (mg/ml) UF: Ultrafiltered urine. SF: Spun and filtered urine.

could also be observed over the concentration of 100 μg/ml.

The volume of additional oxalate required to form crystals was the same in each solution except 1000 μg/ml of Takusha plus UF. The final concentration of 1000 μg/ml of Takusha affected crystal formation and changed the metastable limit. The same amount of sodium oxalate was added to each solution and the results were compared. In the undiluted urine system, while the crystal volume of UF was significantly increased with time, and at 60 minutes, the crystals formed in UF showed large crystals, SF urine showed a significantly lower volume compared with UF or UF + Takusha. Figure 2 shows the crystal size-volume distribution at 60 minute incubation. In UF, crystal size and volume were larger than in UF + Takusha and SF. The mean crystal size (standard deviation: SD) of UF, UF + 1, UF + 10, UF + 100 , UF + 1000 μg/ml of Takusha and SF were 12.5 (1.2), 10.1 (0.8), 9.2 (0.4), 7.6 (0.5), 4.8 (0.3) and 8.3 (0.2) μm respectively. Significant differences were observed between UF and other solution. Scanning electron micrographs were obtained at each experiment (Fig. 3). In all samples, CaOx dihydrate crystals were precipitated. In UF, CaOx dihydrate crystals were formed and most of them were aggregated, which was compatible with the data shown in Figure 2. With increasing concentrations of Takusha, the crystals were not aggregated. Addition of Takusha at the concentration above 10 μg/ml to UF showed minimal aggregation that is thought to be almost the same as SF. In SF, most of the crystals were single and somewhat aggregated. At 1000 μg/ml of Takusha showed small crystals, in which dissolution of the crystals was not observed.

As the solutions of the continuous crystallizer system, we selected previously reported solutions, originally reported by Robertson and Scurr [12]. Figure 4 is a sample of the population density distribution of the crystals formed in control solution. A good correlation between number density and crystal diameter was observed. When added to the oxalate solution Takusha, at concentrations above 10 μg/ml, inhibited the nucleation rate (Fig. 5) and crystal mass (Fig. 6), significantly. Takusha inhibited the growth rate at concentrations above 100 μg/ml (Fig. 7).

Discussion

Chinese herbal medicines have been used safely for more than 2000 years and recently have gained a better reputation because of their efficacy and rareity of side effects as compared to Western medicines. Takusha is a kind of Chinese drug that has been used for many years in China as well as Japan. Some components in Takusha have been identified including: 1) Alismol A, 2) Alismoxide, 3) Alisol C monoacetate, 4) 25-O-Methylalisol A, 5) Alisol B, 6) Alisol A, and 7) Alisol B monoacetate. Major effects include: 1) diuretic, 2) anti-hypertensive, 3) anti-hypercholesterolemic, 4) anti-coagulant and 5) anti-stone formation. For many years Choreito that contains Takusha

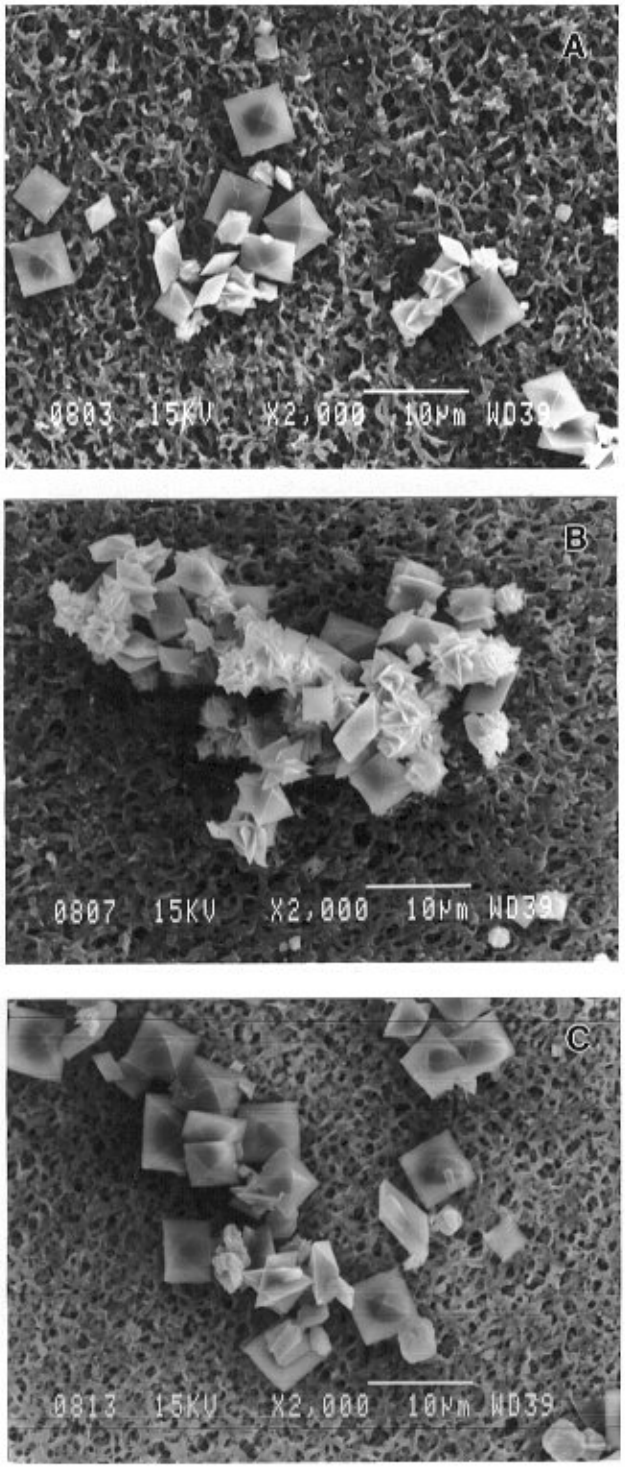

Figure 3. Scanning electron micrographs of the crystals formed in undiluted urine system. (**a**) Spun and filtered urine (SF): (**b**) ultrafiltered urine (UF): and (**c**) 10 μg/ml of Takusha + UF.

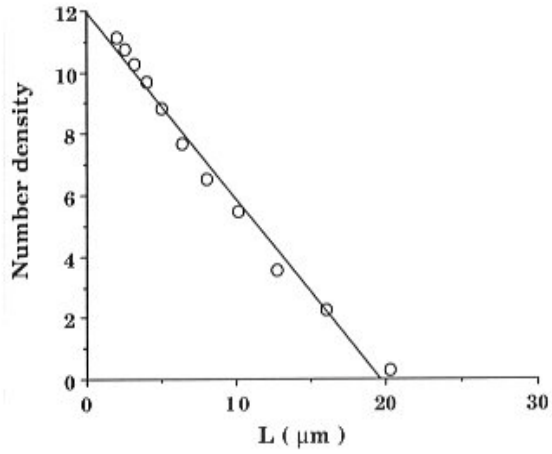

Figure 4. Sample of population density distribution of crystals formed in continuous flow crystallizer system obtained from control solution. L: Crystal size. Nucleation rate is calculated from the intercept with the y-axis and growth rate from the slope of the population density distribution curve. The number density (y-axis) is given in a logarithmic scale. Open circles represent data obtained from the test solution and the slope was calculated with the publlished formulae [1, 2, 3, 7, 12, 16].

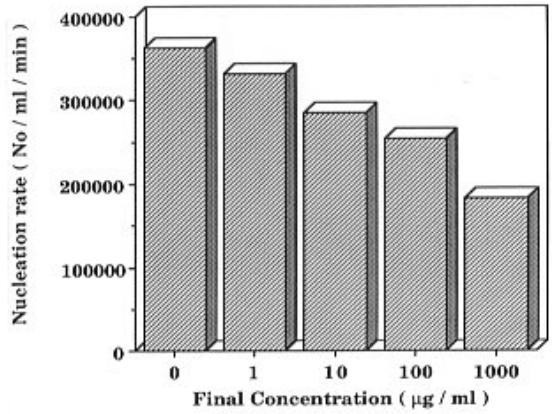

Figure 5. Nucleation rate of Takusha in the continuous flow crystallizer system. Over the concentration of 10 mg/ml of Takusha significantly suppressed the nucleation rate.

has been used for the treatment of pollakisuria or thirst. Very few studies are available on the relationship between Chinese herbal medicines and urinary stones.

The present study showed potent inhibition by Takusha of CaOx crystal formation, aggregation and growth

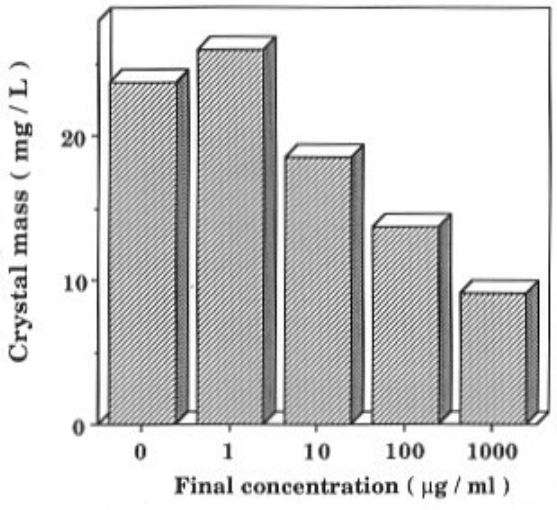

Figure 6. Crystal mass of Takusha in the continuous flow crystallizer system. Takusha inhibited the crystal mass production at concentrations over 10 mg/ml.

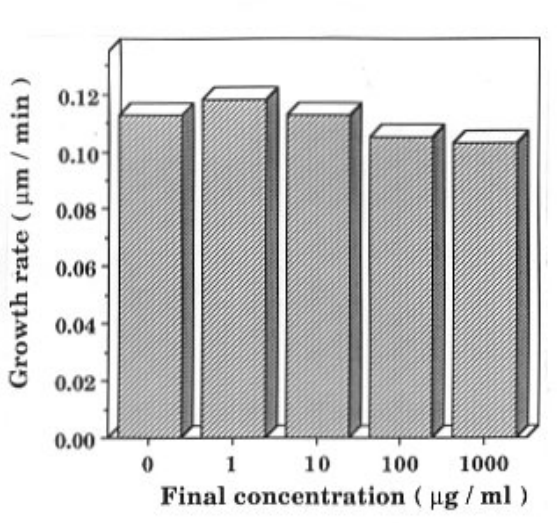

Figure 7. Growth rate of Takusha in continuous flow crystallizer system. Takusha inhibited the growth rate at concentrations over 100 mg/ml.

in vitro. We do not know which component exerts an effect on the stone disease. At present, in the field of stone research, there are few reports about Takusha. Koide *et al*. [7] screened 16 Kampou drugs concerning inhibitory activity on CaOx crystals using a seed crystal method and selected Takusha and Kagosou as potential Kampou drugs for stone prophylaxis. They reported using a seed crystal system *in vitro* that Takusha exerted strong inhibition and that a significant protective effect was seen in stone forming rats *in vivo*. Since their screening method was the seed crystal method, which sometimes overestimates inhibitory activity, we reexamined Takusha using the seed crystal method, undiluted urine method and continuous flow crystallizer method.

An undiluted urine as well as seed crystal system seemed to offer good systems to evaluate the inhibitory potency on CaOx crystals. Though the seed crystal system is a well controlled and reproducible system for determining and screening inhibitors, it also has the weak point of overestimating the inhibitory activity. Chondroitin sulfate (ChS), the most abundant glycosaminoglycan in normal urine, is reported to inhibit crystal aggregation and growth when used in seeded crystal system, but it showed little or no inhibition against CaOx crystals in an undiluted urine system [15]. The undiluted urine method offers the best batch system in precisely determining the inhibitory potency. Continuous flow crystallizer, or mixed suspension mixed product removal (MSMPR), systems have found wide application in research related to kidney stone disease [1, 2, 3, 7, 12], since they permit the study of factors affecting the crystallization of stone minerals at levels of supersaturation comparable to those found in human urine.

In this study, we could demonstrate a powerful inhibition on CaOx crystals, although Takusha did not dissolve CaOx crystals in the undiluted urine system or continuous flow crystallizer system. However, these data suggested that this drug may be useful in the prevention or reduction of the number of calcium stones. As stated by Koide, the long use of Kampou drugs has proven that it has fewer side-effects when compared with Western drugs and, therefore, it seems suitable for long-term treatment. There still exists an important problem, in that, in general, the prescription of Chinese herbal medicine has often been prepared using a mixture of several Chinese herbs empirically combined. And such a mixture of Chinese herbal medicines appears to be much more potent in the treatment of various diseases than any of the individual chemical components of those Chinese herbs. Therefore, it might be important to examine the pharmacological properties of this Chinese herbal medicine using the entire mixture.

In summary, this study indicates that Takusha is a potent inhibitor of CaOx crystal formation, aggregation and growth. Further study is needed to identify the pure component that regulates the inhibitory activity of Takusha. Moreover, the role of insoluble component as well as pharmacological kinetics in humans has to be examined.

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Discussion with Reviewers

T. Koide: The authors showed almost same results of Takusha that we previously presented in the literature. We extracted Takusha by boiling and filtration. Is your extraction method the same?

Authors: Takusha, already extracted in boiling method, was presented from Tsumura & Co. One gram of Takusha was dissolved in 50 ml of doubly distilled water at room temperature for 48 hours, then centrifuged. Its supernatant was freeze dried and used as a test substance at the concentration of 10 mg per ml.

M.I. Resnick: It is unfortunate that little is mentioned about the agent itself. It would be helpful if the authors describe more of what is known regarding Takusha and also it would be helpful if they could hypothesize as to what agents they feel may have a role in its effect on calcium oxalate crystallization. This would help in possibly theorizing as to its clinical benefit. Additionally, since most readers are not familiar with the drug it would be valuable if information was given as to how the agent is prepared and administered.

Authors: Unfortunately, the exact composition of Takusha has not known yet. We will continue to clarify the true agent showing efficient inhibitory activity against calcium oxalate crystals.

J.M. Baumann: They studied calcium oxalate precipitation with and without the addition of Takusha in ultrafiltered urine of healthy men. However, this approach brings no more information than that one gained from tests in artificial solutions, because ultrafiltration removes all the promoting and inhibiting macro-molecules thought to be important in stone disease. Therefore, in this paper, results of precipitation tests which are obtained in whole urine after addition of Takusha should also be presented.
Authors: Ultrafiltered, undiluted urine method is thought to be good screening test system, especially in evaluating the inhibitory potency of the macromolecules. Since Takusha contained macromolecular substances, this test system was not omitted. Whole urine plus Takusha *in vitro*, as well as *in vivo* examination is necessary. Further separation of Takusha according to each molecular weight is now on work.

H-G Tiselius: The major concern with the findings presented in this paper is that although the drug extract doubtlessly influences the crystallization process, there is no evidence that the active constituents of Takusha are excreted in urine. Such information is fundamental for all further conclusions and work with this drug. Since the authors have advanced possibilities to measure crystallization properties, it would be a great advantage and an improvement of the paper if they could include at least a few measurements on urine properties in patients before and during treatment with Takusha.
Authors: In this experiment, Takusha was shown to have strong inhibitory effect against calcium oxalate crystallization in *in vitro* system. We are very interested in what the most powerful inhibitory portion in Takusha is. Moreover, we now prepare a clinical investigation as next experiment.