Preliminaries Methods for Detecting Urine Crystalline by Nanoparticle Size Analyzer

Yuni Warty1*, Freddy Haryanto1, Leni Aziyus Fitri1, Tri Sunu Agung Nugroho2, Herman3

1Nuclear Physics and Biophysics Research Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung Jl. Ganesha 10 Bandung 40132, Indonesia
2Department of Urology, Faculty of medicine, Padjajaran University, Hasan Sadikin Hospital and Faculty of Medicine, Syiah Kuala University
3Magnetic and Photonic Physics Research Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung Jl. Ganesha 10 Bandung 40132, Indonesia

Email: yuniwarty@s.itb.ac.id

Abstract. Urine crystals can be found in healthy urine and unhealthy urine (urolithiasis). Size analysis, in both with the nanoparticle size analyzer (PSA) is very useful for distinguishing urine crystallites under 1000 nm. The choice of methods and materials that are appropriate at the preparation stage before measurement will determine the accuracy of the data. The purpose of this study was to establish the right methods and materials for detecting crystals by PSA. PSA was used to compare intensity-autocorrelation curves (mean diameter) and polydispersity index of techniques and materials reviewed. Those: solution concentration; protein-coagulating; distilled water volume; micropore diameter; centrifugation value; and measurement angle. The best processing methods for urine crystallites detection was found. Antiseptics and protein-coagulation with NaNO3 (2%) and formaldehyde (2.5%) were added to the urine, respectively. Urine was diluted with 50 ml distilled water then filtered through a 3 µm paper filter to remove the macromolecule. The supernatant was obtained by centrifuging the filtrate at 4000 rpm for 15 minutes. The angle (90°) applied to the measurement with Rayleigh scattering. These processing methods can remove biological cells and macromolecules in the urine. This preparation stage in the next research was applied to detect various types of urine crystals.

1. Introduction
Urolithiasis is a common urological disease. Several studies report that developing countries tend to increase in the number of patients with urolithiasis (1-5). Changes in environmental factors, including dietary habits, fluid intake, and obesity, are factors increase the incidence of urolithiasis (6). Urolithiasis has been indicated to be more common among men with women with a ratio of three to two (7). The prevalence was 1-5% in Asia, 5-9% in Europe, 13% in North America, and 20% in Saudi Arabia (8). Percutaneous nephrolithotomy, retrograde ureteroscopy, and shock wave lithotripsy are treated to remove urinary stones, but the recurrence rate was > 50% at a mean followup time of 10 years (9, 10).
Urine examination was one method for diagnosing clinical urological disease (11). Various crystals can be found in the urine of healthy urine or urine of urolithiasis. Some of the crystals most often found are Oksalat, Phosphate, and Uric Acid. The existence of these crystals was possible for the formation of urinary stones (12). The first formation of urinary stones is the nucleation of minerals due to the supersaturation of urine with a size of less than 10 nm. Nucleus develops to form micrometer-sized aggregates. If the crystal settles and it stored in the urinary tract, for a certain time, a urinary stone will form 0.1 cm to several cm (13).

Qualitative and quantitative analysis of urine crystals and urinary stones can improve the information causing urolithiasis. Analysis of kidney stones and urine crystals in micro sizes using various methods has been widely reported. Such as normal urinary particle size distribution and urine urolithiasis using Transmission Electron Microscope was 100 nm to 350 nm and 10 to 1000 nm, respectively. Urinary nanocrystals information is more accurate to explain the mechanism of urinary stone formation from urine crystals, but the analysis of nanocrystallites in urine is still limited. Also, the results of nanocrystalline research are more scientific because of the nature of its nanosized crystals. For example, the specific surface area is much higher in nanosized crystals that will be stronger to form aggregates than crystals the size of a micrometer.

However, precise measurement and distribution of urine crystallites are not easy. Some factors that must be considered are the surface energy that makes nanoparticles easily aggregated, and the urine excreted from the human body always contains large amounts of protein and cell fragments. The results presented in this paper suggest, The best processing method for urine crystallite detection with nanoparticle size analyzer.

2. Materials and Methods

2.1. Reagent
Nanosphere ™ 3100A 100 nm ± 3 nm, sodium chloride, Sodium Azide, Absolute Ethanol, Formaldehyde, Aquabides, healthy urine samples.

2.2. Instruments
The size distribution of crystallites in urine was founded by the nanoparticle analyzer SZ-100 (HORIBA). The experimental conditions were as followings; DPSS laser light source, 532 nm, the incident angles of 90° and 173°, and the temperature 25°C.

2.3. Sample Analysis Process
2.3.1. Standardization of tools. The sample of Nanosphere ™ 3100A was dissolved with NaCl solution which had different concentrations (5%, 0.5%, 0.05%, 0.025%, 0.001%). The effect of the angle difference and the Neutral density filter difference on the detection result determined with the appropriate concentration from the previous step.

2.3.2. Processing method of sample urine. Samples of fresh morning urine were collected. The pH and concentration of these urine samples were detected and then added 2% NaN3 (weight/volume ratio) solution as antiseptic. This sample was characterized by a particle size analyzer with comparing intensity-autocorrelation curves (mean diameter), and polydispersity index of techniques and materials reviewed. Several parameters were reviewed; the effect of dilution, the effect of Protein Removal, the effect of filtration, and centrifugation.

3. Results and discussion

3.1. Standardization of tools
Nanosphere ™ samples which have a particle size of 100 ± 3 nm were dissolved with NaCl (5%, 0.5%, 0.05%, 0.025%, 0.001%). Figure 1 (a) Showed the particle diameter curve of the Nanosphere ™
sample vs. the concentration of NaCl solvent. Obviously, it was better to process the Nanosphere™ with a solvent concentration of NaCl 0.05% than with the other for the detection of Nanosphere™ particle diameter, mostly in the size of the following aspect of particle. The size of the Nanosphere™ in 0.05% NaCl solution was best suited to the actual size (101.9 nm) than that concentration. Some other concentrations were 94.9 nm, 104.9 nm, 128.9 nm, 1634.5 nm, respectively.

Nanosphere™ dissolved with 0.05% NaCl was characterized by PSA by applying angles of 90° and 173°. The difference in intensity of the autocorrelation curve can represent the stability of the sample, and the rate of the curve can illustrate the size of the particle in the solution (14, 15). However, the intensity autocorrelation curve is different in the same sample at two different angles, as shown in figure 1 (b).

Neutral density filters are operated to adjust the best-detected scattering light intensity of the incoming light sample. This parameter can be determined manually or automatically. In the Nanosphere™ samples, a 100% ND filter was best suited for measurements of Nanosphere™ samples. The best ND filters can be seen in the particle size in the ND variations applied.
3.2 Processing Method of sample urine
Proteins were removed from urine with the addition of formaldehyde and absolute ethanol reagents. The result was investigated by comparing the intensity autocorrelation curve. A high decay rate of the intensity autocorrelation curve and a short decay time indicate small particles. Conversely, a low autocorrelation curve decay rate and a long decay time indicate larger particles. From the results of examining a urine sample with the same preparation process and different reagent bleeds, the autocorrelation curve shown in Figure 2 (a). The figure shows that formaldehyde has a low decay rate and a shorter decay time and a smoother intensity autocorrelation curve. It can be concluded that with formaldehyde, in the sample was no sedimentation of large particles, so this sample was stable. Meanwhile, the addition of ethanol reagent to the sample results in a curve with a long decay time so that the particles inside are larger. From the picture, it can also be seen that the graph is more fluctuating, which indicates the solution system in the sample was unstable because of the large particle sedimentation and particle accumulation.

Figure 2. (a) Intensity-autocorrelation curves of urine crystallites after the addition of formaldehyde and ethanol. (b) The effect of filtration and centrifugation for 15 min on urine samples

Macromolecular substances and proteins in urine are removed through the process of filtration and centrifugation. This is done to avoid their influence and contribution in detecting results. Figure 2 (b) shows the distribution curve of the particle size of urine by applying different centrifugations (4000, 6000, 8000, 10000, 13,500) rpm. Before centrifugation, for 15 min, the sample is first filtered through a filter membrane with a 3.0 µm pore diameter. The particle size in samples with the application of five different centrifugations was 54.3 nm, 67.1 nm, 94.9 nm, 220.5 nm, 843.5 nm, respectively. These results indicate an increase in particle size on the addition of large centrifugations. The higher the centrifugation, the greater the particle size detected. It can be concluded that the greater the centrifugation, the more unstable the sample, and particle sedimentation will occur.

The sample needs to be diluted using distilled water to prevent the increase in supersaturation. Table 1 shows the effect of dilution on particle size and polydispersity index (PDI). Urine sample was tested to 50%, 37.5%, 25%, 10%, 5%, respectively. The sample was detected three times with parallel processing, like the data in the table. The most appropriate dilution of urine was 50%, and this was indicated by the shortest decay time and lowest PDI value.
Table 1. The effect of dilution on the particle size, average diameter, polydispersity index, and average PDI

| Parameters                  | 50%     | 37.5%    | 25%     | 10%    | 5%     |
|-----------------------------|---------|----------|---------|--------|--------|
| Mean diameter (nm)          | 106.1105.9 | 105.9 | 106.1106.9 | 106.7 | 127    | 122    | 123.5  | 127    | 113.2  | 123.7  | 128.8  | 115    | 107    |
| Average diameter (nm)       | 106±0.1 | 107±0.4  | 124±3   | 121±2  | 117±11 |
| Polydispersity index (PDI)  | 0.049   | 0.014    | 0.049   | 0.051  | 0.06   | 0.028   | 0.035   | 0.03   | 0.008  | 0.161  | 0.104  | 0.018  | 0.023  |
| Average PDI                 | 0.025   | 0.053    | 0.031   | 0.059  | 0.051  |

4. Conclusions
Formaldehyde is more suitable to be used as a reagent for removing proteins to detect results at an angle of 90°. In addition, the urine was dissolved with 50% distilled water and distilled with a 3 µm microporous membrane pore diameter and centrifuge at 4000 rpm. The higher the centrifugation, the greater the particle size detected. It can be concluded that the greater the centrifugation, the more unstable the sample, and particle sedimentation will occur. Detection results on several aspects of this preparation were applied to detect various types of urine crystallites in the next research.

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References
[1] Jing Z, GuoZeng W, Ning J, JiaWei Y, Yan G and Fang Y 2010 Urol Res 38 1111-1115.
[2] Penniston KL, McLaren ID, Greenlee RT and Nakada SY 2011 J Urol 185 1731-1736.
[3] B. Grohe, J. O’Young, D. A. Ionescu, G. Lajoie, K. A. Rogers, M. Karttunen, H. A. Goldberg, and G. K. Hunter 2007 J. Am. Chem. Soc. 129 14946
[4] C. Escobar, K. J. Byer, and S. R. Khan 2007 BJU Int. 100 891.
[5] M. Tsujihata 2008 Int. J. Urol 15 115.
[6] Habbig S, Beck BB and Hoppe B 2011 Kidney Int 80 1278-1291.
[7] Scales CD Jr, Curtis LH, Norris RD, Springhart WP, Sur RL, Schulman KA and Preminger GM 2007 J Urol 177 979-982.
[8] Jing Z, GuoZeng W, Ning J, JiaWei Y, Yan G and Fang Y 2010 Urol Res 38 1111-1115.
[9] Ozturk U, Sener NC, Goktug HN, Nalbant L, Gucuk A and Imamoglu MA 2013 Urol Int 91 345-349.
[10] Lao M, Kogan BA, White MD and Feustel PJ 2014 J Urol 191 440-444.
[11] Z. Q. Ye, Y. L. Deng, and C. Dong 2003, ch. 3 31
[12] R. Dyer and B. E. Nordin 1967 Nature 215 751–752.
[13] N. Laube, B. Mohr, and A. Hesse 2001 J. Cryst. Growth 233 367–374.
[14] T. Okubo and K. Kiriyama 1997 J. Mol. Liq. 72 347
[15] C. Rühlmann, M. Thieme, and M. Helmstedt 2001 Chem. Phys. Lipids 110 173.