การพัฒนาวิธีตรวจวัดดัชนีการตกผลึกแคลเซี่ยมออกซาเลตสำหรับประเมินศักยภาพการก่อนิ่ว ในปัสสาวะของผู้ป่วยโรคนิ่วไต

นายโบเหว่ย หยาง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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DEVELOPMENT OF CALCIUM OXALATE CRYSTALLIZATION INDEX FOR ESTIMATING LITHOGENIC POTENTIAL IN URINES OF PATIENTS WITH NEPHROLITHIASIS

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โบเหว่ย หยาง : การพัฒนาวิธีตรวจวัดดัชนีการตกผลึกแคลเซี่ยมออกซาเลตสำหรับประเมินศักยภาพการก่อนิ่วในปัสสาวะของผู้ป่วยโรคนิ่วไต (Development of calcium oxalate crystallization index for estimating lithogenic potential in urines of patients with nephrolithiasis) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.ชาญชัย บุญหล้า, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.ปิยะรัตน์ โตสุโขวงศ์

โรคนิ่วไตเป็นปัญหาสาธารณสุขที่สำคัญของไทย เป็นที่ทราบกันดีกันว่าก้อนนิ่วประกอบขึ้นจากผลึกที่พบในปัสสาวะและภาวะที่ก่อให้เกิดผลึกนิ่วในปัสสาวะ คือ การอิ่มตัวยวดยิ่งของปัสสาวะ ผลึกแคลเซี่ยมออกซาเลตเป็นผลึกนิ่วที่พบได้บ่อยที่สุด ในการศึกษานี้จึงพัฒนาวิธีการตรวจค่าการเกิดผลึกในปัสสาวะ เรียกว่า ดัชนีการตกผลึกแคลเซี่ยมออกซาเลต เพื่อใช้จำแนกผู้ป่วยโรคนิ่วไตออกจากคนปกติสุขภาพดี และศึกษาผลของปริมาตรปัสสาวะและสารในปัสสาวะได้แก่ ออกซาเลต ฟอสเฟต ซิเทรต โพแทสเซี่ยม และโซเดี่ยม ต่อค่าดัชนีการตกผลึกแคลเซี่ยมออกซาเลต การศึกษานี้วัดค่าดัชนีการตกผลึกแคลเซี่ยมออกซาเลตในตัวอย่างปัสสาวะ 24 ชั่วโมงของผู้ป่วยโรคนิ่วไต (72 ราย) และคนปกติสุขภาพดีที่มีอายุและเพศใกล้เคียงกับกลุ่มผู้ป่วย (71 ราย) แล้วประเมินค่าการวินิจฉัยของวิธีการตรวจวัดดัชนีการตกผลึกแคลเซี่ยมออกซาเลตในปัสสาวะ ผลการศึกษาพบว่าปริมาณออกซาเลตและฟอสเฟตในปัสสาวะทำให้ค่าดัชนีการตกผลึกแคลเซี่ยมออกซาเลตสูงขึ้น ขณะที่ปริมาตรปัสสาวะและปริมาณซิเทรตส่งผลให้ค่าดัชนีการตกผลึกแคลเซี่ยมออกซาเลตลดลง ค่าดัชนีการตกผลึกแคลเซี่ยมออกซาเลตในกลุ่มผู้ป่วยโรคนิ่วไตสูงกว่ากลุ่มคนปกติอย่างมีนัยสำคัญ และที่สำคัญผลการอัลตราซาวด์ในคนปกคนติสุขภาพดี 2 ราย ที่มีค่าดัชนีการตกผลึกแคลเซี่ยมออกซาเลตสูงมาก พบว่าทั้ง 2 ราย มีนิ่วในไตที่ยังไม่ก่ออาการ ผลการวิเคราะห์ receiver operating characteristic (ROC) พบว่าการตรวจวัดดัชนีการตกผลึกแคลเซี่ยมออกซาเลตในปัสสาวะมีค่าพื้นที่ใต้กราฟของ ROC เท่ากับ 0.9499 (95%CI: 0.9131-0.9868) ซึ่งบ่งชี้ว่าวิธีนี้มีอำนาจการจำแนกผู้ป่วยโรคนิ่วไตออกจากคนปกติอยู่ในระดับดีเยี่ยม เมื่อใช้ค่า cutoff ที่ 165 mg oxalate equivalence/day พบว่าวิธี้นี้ให้ค่าความไว ความจำเพาะ และความถูกต้อง เท่ากับ 83.33, 97.18 และ 90.21% ตามลำดับ สรุป การศึกษานี้พัฒนาวิธีตรวจวัดดัชนีการตกผลึกแคลเซี่ยมออกซาเลตในปัสสาวะได้สำเร็จ และค่าดัชนีการตกผลึกแคลเซี่ยมออกซาเลตที่วัดได้ขึ้นอยู่กับปริมาตรปัสสาวะ ปริมาณออกซาเลต ฟอสเฟต และซิเทรตในปัสสาวะ วิธีที่พัฒนาขึ้นนี้ให้ค่าความไวและความจำเพาะสูงเพียงพอสำหรับใช้จำแนกผู้ป่วยโรคนิ่วไตออกจากคนปกติ และยังเป็นวิธีที่ง่าย ราคาถูก เชื่อถือได้ และสามารถวิเคราะห์ได้ในห้องปฏิบัติการของโรงพยาบาล เพื่อใช้ตรวจกรองหาคนที่มีความเสี่ยงต่อการเกิดโรคนิ่วไต รวมถึงคนที่มีนิ่วในไตที่ยังไม่ก่อให้เกิดอาการหรือเป็นนิ่วระยะเริ่มแรก

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ลายมือชื่อนิสิต.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORD: KIDNEY STONE, NEPHROLITHIASIS, CALCIUM OXALATE CRYSTALLIZATION INDEX, COCI, CRYSTAL FORMATION, DIAGNOSIS
BOWEI YANG: DEVELOPMENT OF CALCIUM OXALATE CRYSTALLIZATION INDEX FOR ESTIMATING LITHOGENIC POTENTIAL IN URINES OF PATIENTS WITH NEPHROLITHIASIS
THESIS ADVISOR: ASSISTANT PROFESSOR CHANCHAI BOONLA Ph.D., THESIS CO-ADVISOR: PROFESSOR PIYARATANA TOSUKHOWONG

Nephrolithiasis is a significant health problem in Thailand. It is known that urinary crystals are building blocks of kidney calculi, and urinary supersaturation triggers crystal formation. The most common type of lithogenic crystals is calcium oxalate (CaOx). In this study, a new test, designated CaOx crystallization index (COCI), was developed to distinguish nephrolithiasis patients from healthy individuals based on the urinary crystallization capability. The effect of urine volume, oxalate, phosphate, citrate, potassium and sodium on COCI values was investigated. COCI values were determined in 24-hr urine obtained from nephrolithiasis patients (n = 72) and age- and sex-matched healthy controls (n = 71). The diagnostic potential of the urinary COCI test was evaluated. Increases in urine oxalate and phosphate caused increase in COCI values. In contrast, increases in urine volume and citrate caused decreased COCI values. Urinary COCI values of nephrolithiasis group were significantly higher than that of healthy group. Importantly, asymptomatic kidney calculi were detected by ultrasound imaging in two healthy subjects with elevated COCI values. Based on receiver operating characteristic (ROC) analysis, an area under ROC curve of the urinary COCI test was 0.9499 (95%CI: 0.9131-0.9868), indicated that this test had excellent discriminatory power to separate nephrolithiasis from healthy subjects. At the cutoff of 165 mg oxalate equivalence/day, the COCI test provided sensitivity, specificity and accuracy of 83.33, 97.18 and 90.21%, respectively. In conclusion, the COCI test was successfully established, and its values were primarily dependent on urine volume, oxalate, phosphate and citrate. The test provided high sensitivity and specificity for clinically discriminating the nephrolithiasis patients from healthy population. We suggest that the COCI test is a cheap, simple, non-invasive and reliable method that could be implemented in hospital laboratories to identify individuals at risk of kidney stone formation as well as those with asymptomatic urinary calculi or early stage of nephrolithiasis.

Field of study: Medical Sciences: Biochemistry

Student's Signature..... Advisor's signature.....

Academic Year 2012 Co-advisor's signature.....

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LIST OF ABRREVIATIONS

ANOVA	Analysis of variance
AUC	Area under curve
BRI	Bonn-risk index
CaOx	Calcium oxalate
CaP	Calcium phosphate
CE	Capillary electrophoresis
COP	Calcium oxalate precipitate
COCI	Calcium oxalate crystallization index
CT	X-ray computed tomography
COM	Calcium oxalate monohydrate
COD	Calcium oxalate dihydrate
$CaCl_2$	Calcium chloride
$C_6H_5Na_3O_7$	Trisodium citrate
CI	Confidence interval
FTIR	Fourier transform infrared spectroscopy
HPLC	High performance liquid chromatography
HCl	Hydrochloric acid
IVP	Intravenous pyelography
IQR	Interquartile range
KUB	Kidney-ureter-bladder

KCl	Potassium chloride
mg/L	Milligram per liter
mL	Milliliter
M	Molar
mM	Millimolar
$MgSO_4$	Magnesium sulfate
mmol. Ox equiv./L	Millimole oxalate equivalent per liter
mg. Ox equiv./L	Milligram oxalate equivalent per liter
mmol. Ox equiv./day	Millimole oxalate equivalent per day
mg. Ox equiv./day	Milligram oxalate equivalent per day
mg. Ox equiv./mg Cr	Milligram oxalate equivalent per milligram creatinine
NL	Nephrolithiasis
NaCl	Sodium chloride
$Na_2C_2O_4$	Sodium oxalate
nm	Manometer
OD	Optical density
pH	Potential of hydrogen
ROC	Receiver operating characteristic
rpm	Revolutions per minute
UV	Ultraviolet
µL	Microliter

XR	X-ray radiography
%	Percentage
^{o}C	Degree Celsius

CHAPTER I

INTRODUCTION

Kidney stone disease or nephrolithiasis (NL) has been a worldwide health problem. Overall prevalence of the disease varies among areas of the world: 1-5% in Asia, 5-9% in Europe, 13% in North America, and 20% in Saudi Arabia[1-3]. In Thailand, NL is endemic, particularly in the northeastern region with the male-to-female ratio of 2:1[4]. The type of kidney calculi is classified according to the major mineral composition. Calcium oxalate (CaOx) calculi (75%)[5] is the most common stone type found in all countries. Other renal calculi compositions include magnesium ammonium phosphate, ammonium urate, calcium phosphate, uric acid and cystine[6]. A study by Tosukhowong et al. in 2007 demonstrated that CaOx calculi was the most prevalence in all regions of Thailand[7].

The mechanism of kidney stone formation is complex and far from fully understood. However, it is well recognized that the initial driving force for renal calculi formation is the supersaturation of urine. Saturation is the state in which lithogenic free ions in the solution are in equilibrium with their respective salts. Increases in excretion of urinary lithogenic ions (also called calculi promoters) such as calcium, oxalate and phosphate and decreases in urine volume and calculi inhibitors such as citrate, potassium and magnesium will increase free ion activity and favor the formation and growth of

lithogenic crystals[8]. Low urine volume, hyperoxaluria, hypercalciuria, hypocitraturia, hyperuricosuria and hypomagnesiuria[9] are known risk factors for CaOx lithogenesis. Hyperoxaluria and hypercalciuria are principally found in the western countries. In contrast, the major urinary metabolic risk factors in Thai NL patients are hypocitraturia and hypokaliuria[10]. Hyperoxaluria is accounted for less than 10% and there is no significant difference in prevalence of hyperoxaluria between Thai NL patients and healthy individuals[10]. High urinary supersaturation for CaOx salt was demonstrated in Thai patients[11], and the CaOx crystals formed even in the low specific gravity urines[12]. Additionally, urinary citrate (a potent stone inhibitor) in Thai NL patients was significantly lower than healthy individuals[13, 14]. We hypothesize that despite the low urinary oxalate concentration, the CaOx crystal forming potential of the urine of Thai NL patients is higher than the healthy population due to low amounts of calculi inhibitors, especially citrate.

Currently, there are at least four established methods to estimate lithogenic potential and saturation of the urine. Oxalate tolerance test is used to determine the upper limit of metastability. The test changes the saturation by gradually adding sodium oxalate into urine to find the maximal amount of sodium oxalate that can be added without measurable crystallization[15]. In 1997, Grases et al. proposed a simpler test to evaluate the capacity of urine to crystallize calcium salts. They used a non-protected non-renewed surface to submerge in urine to allow supersaturated urinary substances to crystalize on the surface. This feature was used to evaluate the risk of urinary CaOx formation, and they showed that normal urine did not crystallize on the surface whereas lithogenic urine

induced the crystallization of calcium salts on the surface[16]. In 1998, Sriboonlue et al. found that oxalate was coprecipitated at pH 5 as calcium oxalate precipitate (COP) under excessive calcium ions and ethanol condition. They claimed that this was an indirect method to measure oxalate in urine, as the calcium concentration in COP was correlated well with the urinary oxalate concentration[17]. The Bonn-risk Index (BRI) developed by Laube et al. in 2000, is the most widely used method for estimating lithogenic potential in the unprepared native urine[18]. The BRI is calculated as ratio of urinary free calcium ions to the lowest amount of titrated ammonium oxalate that is required for initiating CaOx crystallization[19-21]. The BRI test has high diagnostic power to discriminate CaOx stone formers from healthy controls (sensitivity 69.7%, specificity 100%). However, sensitivity of the test is relatively low.

We hereby proposed a novel test designated as the calcium oxalate crystallization index (COCI) for estimating or reflecting the lithogenetic potential in the urine samples. The principle of the COCI test is to induce all of oxalate ions in urine to form CaOx crystals by addition of excess calcium. The CaOx crystals are then isolated, washed and re-dissolved. The absorbance at 215 nm (the maximum wavelength for oxalate) is measured using ultraviolet (UV) spectrophotometer. The COCI values depend on both levels of crystallizable oxalate and CaOx crystal-forming inhibitors in the urine. Therefore it can be used to estimate the CaOx lithogenic or crystal-forming potential of the urine sample.

In this study, we aimed to establish the proof of concept for the COCI method to estimate the lithogenic potential in urine samples of NL patients and healthy individuals.

The reference range for the urinary COCI test as well as the diagnostic thresholds of the test for distinguishing the NL patients from healthy controls was determined. The effect of urine volume, oxalate, phosphate, citrate, sodium and potassium on COCI values was investigated. We hope that in the near future the urinary COCI test will be clinically useful and suitable for use in the hospital laboratories as a cheap, simple and highly reproducible method for estimating the urinary risk for renal calculi formation[20].

CHAPTER II

LITERATURE REVIEW

The NL or kidney stone disease is one of major diseases in urology. In US, the prevalence of kidney stone disease is estimated to be approximately 12% of the population[22]. The prevalence of NL is higher in tropical and middle east countries[23]. In China, the overall prevalence of NL is 1-5%, however it can be found up to 10% in the southern region. In Thailand, the highest prevalence of NL is in the Northeast (16.9%)[4]. There are several probable reasons which may cause in rising prevalence of NL including: dietary lifestyle, comorbidity, drugs, age and sex and environment[22]. In addition, global warming has been predicted and suggested to have a significant impact on increasing prevalence of NL[24]. NL is highly recurrent causing difficulty of post-surgical management. Also, NL has a significant economic impact. In the US, the cost of stone management have been estimated at USD 2-5.3 billion/year[25]. Therefore, the NL introduces both health and economic problems across the world. Strategy applied to prevent the onset of this incurable disease is becoming more and more important. In addition, an effective screening test to detect early stage of NL is indeed required.

1. The etiology of nephrolithiasis

The etiology of NL is multifactorial. Basically, risk factors for stone formation can be classified as intrinsic and extrinsic factors. The intrinsic factors include demographic, anatomic and genetic factors[26], while the extrinsic factors include geographic predilection, climate, lifestyle and dietary habit. During the past few years, the prevalence of kidney stone in both males and females are markedly increased in industrialized countries[8]. This may be due to the changed lifestyle and dietary habit. Various studies show that the people with family history of kidney stones have approximately 3 times higher risk of kidney stone formation than the one without family history of kidney stone[27]. However, it should be noted that majority of NL patients have negative family history, which may imply that the influence of extrinsic factors superimposes on the intrinsic causes[28]. Kidney stone patients may have one or more metabolic risk factors for example: hyperoxaluria, hypercalciuria, hypocitraturia, hyperuricosuria, and hypomagnesiuria. Presence of these metabolic abnormalities can lead to the supersaturation of urine and cause crystal formation, which is the first pre-requisite step of kidney stone formation[29].

2. The pathophysiology of kidney stone formation

The pathophysiology of kidney stone formation is deduced majorly from the

experimental nephrolithic study. Three pathways have been proposed and demonstrated for kidney stone formation[30]. The first pathway is overgrowth on interstitial apatite plaque (interstitial nephrocalcinosis) at renal papilla as seen in idiopathic calcium oxalate stone formers. The second pathway, urinary crystals form, deposit and grow in the renal tubules, called intratubular nephrocalcinosis. The third pathway is free solution crystallization. However, evidences from human studies reveals two main pathways that is associated with the kidney stone formation: interstitial nephrocalcinosis and intratubular nephrocalcinosis[31].

The interstitial calcified materials in kidney stone patients was first described by Alexander Randall in the 1930s, so called Randall's plaques[32]. The plaque begins to form in basement membrane of Henle Loop, and it is abundantly found in renal papilla of NL patients regardless stone types[33]. Mineral analysis demonstrates that calcium phosphate (CaP, apatite form) is a principal constituent of the plaque[34]. Randall's plaques are also observed in non-stone forming kidneys, suggesting a normal defense mechanism for renal handling of excessive calcium and phosphate[35]. By undefined mechanism, the interstitial plaques move toward renal papilla and expose to urinary space of the renal calices. Stone grown on renal papilla plaque have been demonstrated by digital endoscopicphotography[36]. Intratubular calcification is believed to cause by precipitation and retention of crystals in the renal tubules[37]. Recent study in non-cancerous portion of nephrectomies shows that interstitial nephrocalcinosis is more common than tubular nephrocalcinosis[38]. Therefore, the capability of the urine to form calcium-containing crystals would be one of the determinants for kidney stone formation.

Since urinary oxalate play a key role in the CaOx kidney stone formation[9], and it has been believed that lower down the urinary oxalate concentration can reduce the likelihood for the formation of stone. Therefore, measuring oxalate or CaOx-forming potential in urine accurately and precisely is a very important issue to achieve in order to improve the NL diagnosis and management.

3. The diagnosis of nephrolithiasis

3.1 Clinical methods in diagnosing nephrolithiasis

In the clinical practice, diagnosis of NL is usually made by the plain film of kidney-ureter-bladder (KUB) and intravenous pyelography (IVP) or ultrasound examination as the primary examination for people who have typical or atypical renal colic. The X-ray computed tomography (CT) is considered to be the standard tool for diagnosing the kidney stone because the CT scan has higher accuracy and sensitivity relative to the KUB, IVP and ultrasound examination[39]. However, the unflavored side of CT is that it is more expensive and may cause more radioactive damage to the tested subjects.

Besides imaging methods, urine test is also a very important part for kidney stone diagnosis. For one part is to use the spot urine to test pH and count the white cell and red cell in urine, known as routine urinalysis. The other part is to collect 24-hr urine sample for measuring excretory amounts of stone promoters and inhibitors, such as calcium,

oxalate, citrate, potassium and so on, and this investigation, calls metabolic workup[40]. The disturbance of stone promoters and inhibitors leads to urinary supersaturation and subsequently initiates lithogenic crystallization. Therefore, if we can measure the crystal-forming potential in the urine well, we can diagnose the kidney stone in a very early stage or even before the onset of kidney calculi. That will make the treatment easier, and the patients suffer from much lesser pain.

3.2 Methods for measuring oxalate in urine

Three methods have been used for measuring oxalate in urine viz. high performance liquid chromatography (HPLC), oxalate oxidase enzymatic method and capillary electrophoresis (CE). Although, HPLC technique is fast and has high sensitivity and resolution, it hardly separates oxalate from the other components in urine sample and the obtained result may be not correct[41]. The colorimetric oxalate oxidase method has been widely used with high sensitivity, and it is commercially available as oxalate oxidase kit. However, the intra-class correlation coefficient for the enzymatic method is low, and this may cause some errors[41]. The CE method is rapid and the minimal detectable concentration (signal-to-noise ratio of 7) of oxalate in urine is 7 mg/L. The rapid analysis time, accuracy, and reproducibility make the CE procedure well suited for routine urinary oxalate and citrate determinations[42]. However, the sensitivity is lower than the other two methods. It has been suggested that using indirect fluorescence

detection may increase sensitivity of CE for determining urinary oxalate. An increase in sensitivity may enable the measurement of plasma oxalate by the CE

Nowadays, there are some new chemical methods for measuring oxalate in water or urine. Some of them use the UV method. Tang et al synthesized the specific ligand for oxlalate and proposed that this ligand can be used for specific detection of oxalic acid in water[43]. Zhai et al[44] found that the oxalic acid was able to react with dibromochloroarsenazo in zirconium (IV) and produced the hyperchromic complex, called dibromochloroarsenazo zirconium (IV) complex in hydrochloric acid medium.

3.3 Stone analysis by FTIR and light microscopy

FTIR stands for Fourier Transform Infrared, the preferred method of infrared spectroscopy. In infrared spectroscopy, infrared radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample, and some is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum. This makes infrared spectroscopy useful for several types of analysis.

In 2009, Iqbal Singh performed analysis of renal stones by FTIR Spectroscopy. He described the FTIR result for both calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD)[45]. Fig. 1 showed the standard COM (1a) and COD (1b) FTIR spectra[46]. We used this data as reference for identify the type of crystals we made from

COCI analysis.

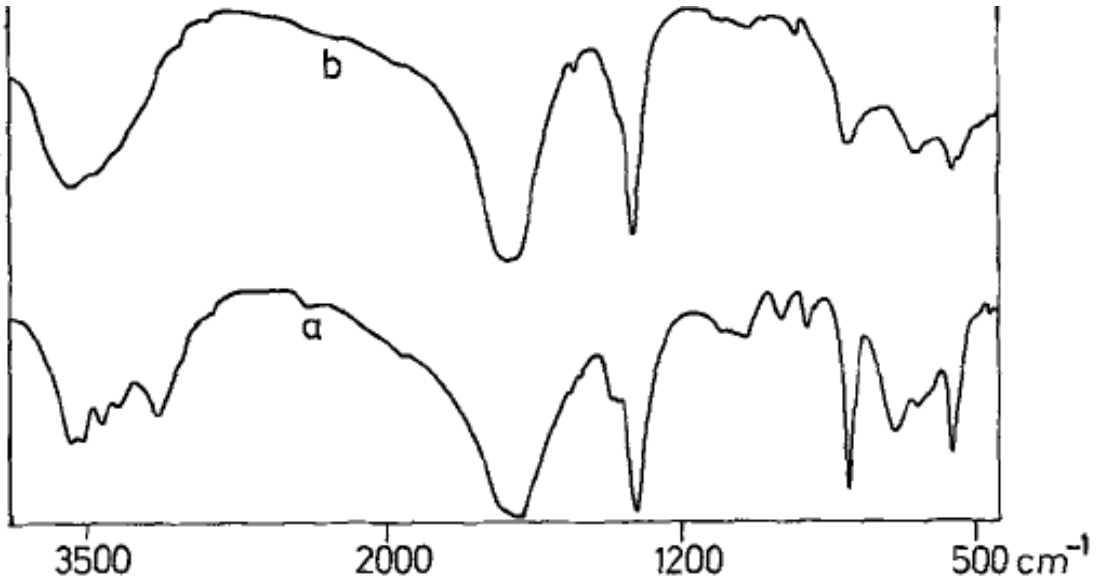

Fig. 1 Typical FTIR spectra of COM and COD generated from FTIR spectoscopy. a: FTIR result for COM, b: FTIR result for COD.

For the morphology of COM and COD, we compared our data with the data from Thongboonkerd et al. They clearly described the morphology of COM and COD under light microscopy[47]. Fig. 2 shows the morphology of COM and COD under light microscopy.

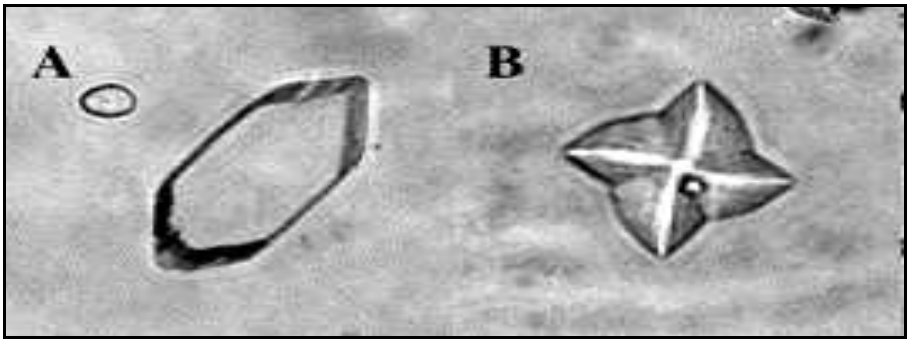

Fig. 2 Morphology of COM and COD under light microscopy. A: morphology for COM; B: morphology for COD

3.4 Methods for estimating lithogenic potential

There are four methods that focus on estimating lithogenic potential in urine. They all pay more attention on the degree of saturation of urine. Oxalate tolerance test is used to determine the upper limit of metastability[15]. The urinary saturation is increased by addition of increasing amounts of sodium oxalate in a constant volume of urine. The maximal amount of added sodium oxalate that causes no measurable crystallization is called permissible increment (PI) in oxalate. PI in oxalate in healthy people is higher than that in kidney stone patients, which means that the healthy people have higher oxalate tolerance (or resistance to CaOx crystallization) than kidney stone patients. In addition, urine dilution induced by water load reduces CaOx supersaturation, increases urine resistance to an oxalate load, maintains the inhibitory CaOx crystallization effect of urinary macromolecules, but does not alter the limit of metastability[15, 48, 49].

In 1997, Grases et al. developed a simple test to evaluate the risk of urinary calcium stone formation. They used a non-protected non-renewed surface to submerge in urine, sooner or later the supersaturated substances in urine crystallize on the surface. Normal urine does not crystallize whereas lithogenic urine induces the growth of calcium salts on the surface. Therefore, they use this feature to evaluate the risk of urinary CaOx formation. The test provided a good sensitivity and specificity of 92% and 73%, respectively. However, at least 40 mL of urine sample is needed for testing, and also determination of calcium is required[16].

In 1998, Sriboonlue et al. found that oxalate can be coprecipitated at pH 5 as calcium

oxalate precipitate (COP) under condition of excessive calcium ions and ethanol. The COP is washed to eliminate the interference (mainly calcium phosphate) by 0.1 M acetic acid equilibrated with calcium oxalate monohydrate crystal. The calcium content in the washed COP is measured using atomic absorption spectrometer, and it showed a high correlation with the original oxalate concentration both in artificial and real urine. Therefore, they suggest that this is an indirect method to measure oxalate in urine[17, 50].

The Bonn-risk Index (BRI) focuses on the in vitro CaOx crystallization behavior of unprepared native urine samples in order to establish a CaOx crystallization risk index. The BRI is the ratio of the concentration of ionized calcium and the amount of oxalate that must be added to 200 ml urine to initiate crystallization. A value of 1.0 has been set to be a cutoff value to distinguish stone formers from non-stone controls. The BRI is good to discriminate CaOx stone formers from healthy controls (sensitivity 69.7%, specificity 100%). However, the BRI assay requires measurements of calcium ions and the precise point that the first crystal is formed (to calculate the exact amount of titrated oxalate), This complicated procedure causes a limited use in only the research laboratories[19-21].

4. Diagnostic test evaluation

In order to evaluate the diagnostic potential of the new test, sensitivity, specificity and accuracy of the test must be calculated. Sensitivity is the proportion of "diseased" individuals who obtain scores above the cutoff point (positive result) of a screening test. Specificity reflects the capacity of an assessment instrument to yield a negative result for

a person without a diagnostic condition (non-diseased individuals)[51]. As shown in Table 1, sensitivity is calculated from the ratio of true positive to all diseased individuals. Specificity is the ratio of true negative to all non-diseased individuals. Accuracy is the ratio of true positive and negative to all.

Table 1 Diagnostic value calculation

Test result	**Diseased subjects**	**Non-diseased subjects**	**Total**
Positive	A (true positive)	B (false positive)	A + B
Negative	C (false negative)	D (true negative)	C + D
Total	A + C	B + D	A + B + C + D

$$Sesitivity = \frac{\mathbf{A}}{\mathbf{A + C}}; \quad Specificity = \frac{\mathbf{D}}{\mathbf{B + D}}; \quad Accuracy = \frac{\mathbf{A + D}}{\mathbf{A + B + C + D}}$$

CHAPTER III

MATERIALS AND METHODS

1. Materials

List of materials and equipments used in this study is shown in Table 2.

Table 2 Materials and equipments used in this study.

Materials and equipments	Product of
Calcium Chloride fused grain	MAY and BAKER LTD, ENGLAND
Centrifuge	KOKUSAN, TOKYO JAPAN
Citrate acid	SIGMA CHEMICHAL CO, GERMANY
Electrical analysis balance	SARTORIUS, GERMANY
Fourier transform infrared spectroscopy	Perkin Elmer, US
Hydrochloric acid fuming 37%	Merck KGaA, GERMANY
Oxalic acid	MALLINCKRODT, ST.LOUIS NY US
Optical Emission Spectrometer	Perkin Elmer, US
pH meter	METTLER TOLEDO, US
Phosphate buffer solution	SIGMA CHEMICHAL CO, GERMANY

Table 2 Cont.

Materials and equipments	Product of
Potassium chloride	SIGMA CHEMICHAL CO, GERMANY
Sodium chloride	USB Corporation Cleveland USA
Sodium phosphate	SIGMA CHEMICAL CO, GERMANY
UV-Visible Spectrophotometer	Thermo Scientific, US
Water Baths	GFL Gesellschaft Labortechnik, GERMANY

2. Calcium oxalate crystal preparation

2.1 Calcium oxalate monohydrate preparation

There are three types of CaOx crystals including calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD) and calcium oxalate trihydrate [52]. However, the main types found in the urines are COM and COD, and the COM is more injurious to renal cells than the COD. COM is easier to prepare in vitro than the COD, as it can be prepared in both water and buffer medium.

For COM preparation, we mixed 10 mM calcium chloride (1 mL) and 10 mM oxalate acid (1 mL), and then the mixture was incubated for 1 hour at 37 ^{o}C. The crystals were collected by centrifugation at 3,000 rpm for 10 min, dried and subjected to the FTIR for mineral analysis to ensure that the prepared crystals were COM. Alternatively, COM can

be prepared in the buffer. Briefly, 5 mM calcium chloride (1 mL) was mixed with 0.5 mM sodium oxalate (1 mL) in a buffer containing 90 mM Tris-Cl and 10 mM NaCl (pH 7.4). The solution was incubated at 25 ^{o}C overnight, and the crystals were harvested by centrifugation at 3,000 ×g for 5 min, dried at 60 ^{o}C and verified the COM spectra using FTIR [53]. Morphology of COM was observed under the light microscope.

2.2 Calcium oxalate dihydrate preparation

For the COD crystal, it could only be prepared in the buffer containing stone inhibitors. Briefly, COD was prepared in the tube using $CaCl_2 \cdot 2H_2O$ and $Na_2C_2O_4$ at a final concentration of 6.27 and 1.6 mM, respectively, in buffer containing 9.6 mM $C_6H_5Na_3O_7 \cdot 2\ H_2O$, 11.6 mM $MgSO_4 \cdot 7\ H_2O$ and 63.7 mM KCl (pH 6.5) [53]. Morphology of COD was observed under the light microscope.

3. Establishment of the COCI protocol

To make all oxalate ions in urine be crystallized, we use the method similar to the study by Sriboonlue et al. reported in 1998[17]. First we added 50 mM calcium chloride to the varied concentrations of oxalic acid solution (0, 1, 1.5, 2, 3 and 4 mM or 0, 90, 135, 180, 270 and 360 mg/L, respectively). The mixture then incubated at 37°C for one hour

and centrifuged at 4,000 rpm for 15 minutes. The supernatant was immediately discarded, and then the COM crystals were dried and re-dissolved in 8 N HCl (0.5 mL). The absorbance at 215 nm (maximum wavelength for oxalate) was measured. The graph for oxalate concentrations vs. OD_{215} was plotted to use as a standard oxalate curve (Fig. 3). The harvested crystals were verified to be COM by FTIR.

We had tried to measure the absorbance of the re-dissolved crystals at various wavelengths. Fig. 4a shows UV absorption spectra of the re-dissolved COCI crystals with various concentrations, and the maximum absorption was between 205 and 225 nm. Fig. 4b displays a comparison of slopes and R^2 values of standard lines of different wavelengths (205, 215 and 225 nm). We found that OD_{215} imparted the highest slope and R^2 value meaning that it had a least unexplainable error to reflect the oxalic acid concentration in the re-dissolved crystal solution. In other word, OD_{215} is the best absorbance that is proportional to the oxalic acid concentration.

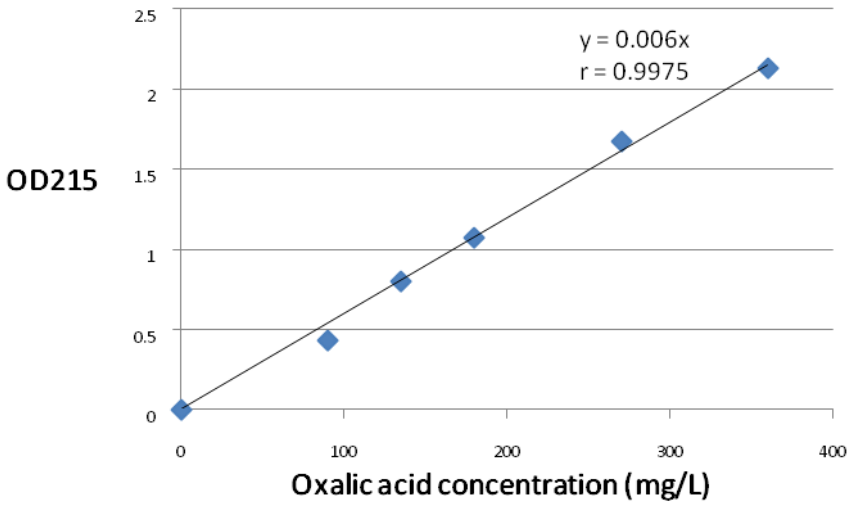

Fig. 3 COCI standard curve using UV absorbance at 215nm.

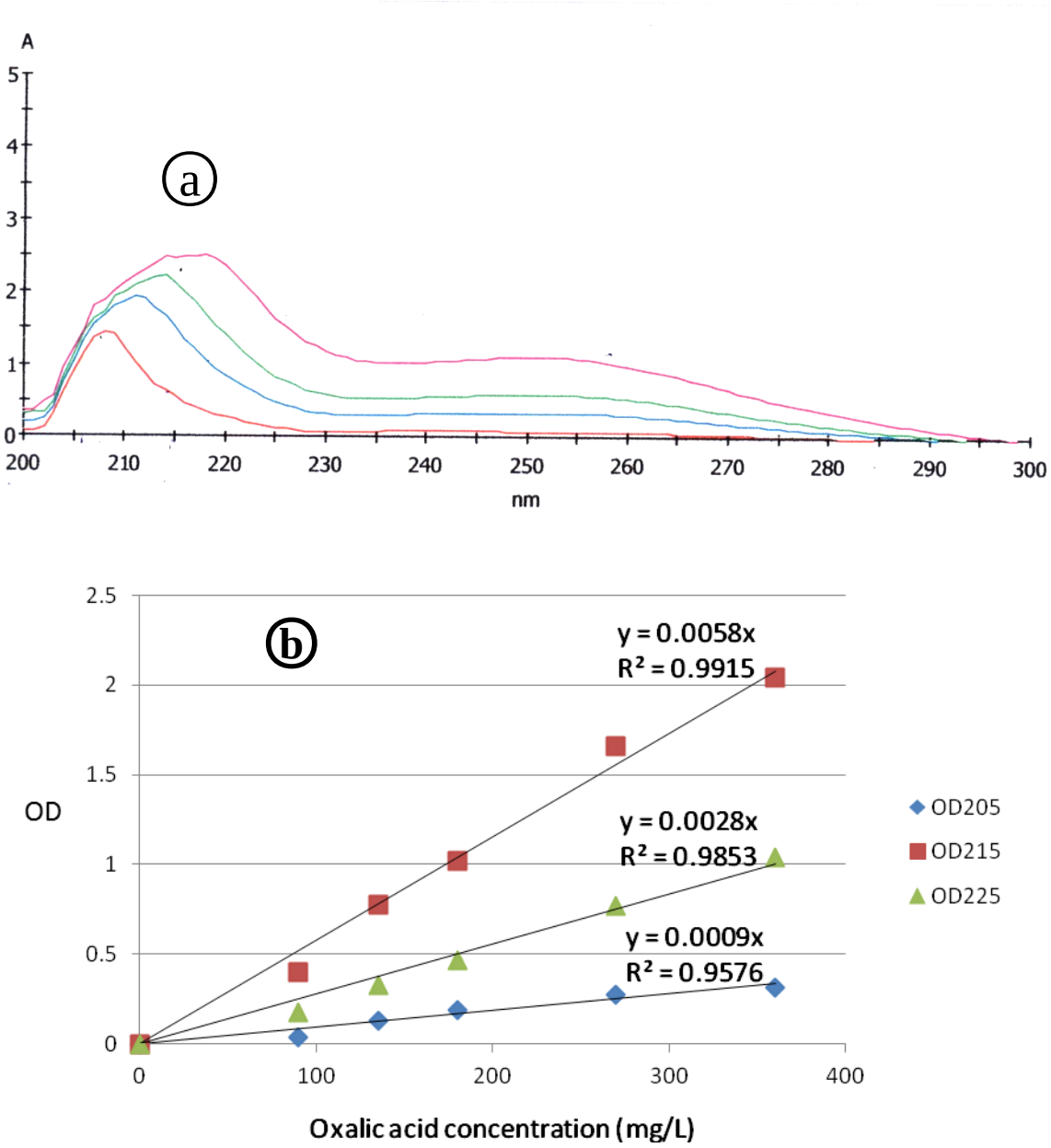

Fig. 4 The COCI standard curves at various wavelengths. a: UV spectrum of standard COCI crystals after re-dissolved in 8 N HCl (from 200nm to 300 nm). b: The absorbance at 205, 215, and 225 nm, used for setting standard curve of the COCI test.

5. Procedure for the determination of COCI in urine samples

The procedure of the COCI method is shown in Fig. 5. Varied concentrations of Ox solution (0, 1, 1.5, 2, 3 and 4 mM) were used for generating standard curve. Urine samples were filtered through 0.22 μm membrane filters for deproteination and decontamination. Since CaOx crystals in some urine samples, particularly healthy urines, were not visible after addition of excess $CaCl_2$ (50 mM), we spiked every urine sample (950 μL) with 50 μL of 80 mM oxalic acid solution to achieve the final concentration of 2 mM of spiked Ox. Subsequently, 1 mL of 100 mM $CaCl_2$ solution (final conc.: 50 mM) was added, and the tubes were incubated at 37 ^{o}C for one hour. The crystals were then harvested by centrifuge (immediately) at 4,000 rpm for 15 minutes, promptly washed once with 0.5 mL PBS (pH 6.8), and centrifuged again at 4,000 rpm for 15 minutes for crystal collection. The crystals were dried and re-dissolved in 8 N HCl (0.5 mL). The absorbance at 215 nm was measured. The standard curve of Ox concentrations vs. OD_{215} nm was plotted (Fig. 3). The actual OD_{215} of urine sample (OD_{urine}) was calculated from $OD_{Ox\text{-}spiked\ urine} - OD_{STD}$ 2mM Ox, and the COCI value of urine sample was the outcome of OD_{urine} divided by the slope of standard curve. The COCI values were expressed as mM Ox equivalence (equiv.) or mg Ox equiv./L (multiply by 90). COCI amount per day and normalized COCI (divided by urinary creatinine) were also calculated (Table 4). Within- and between-day coefficients of variation (%CV) for COCI were 2.16 and 5.44, respectively.

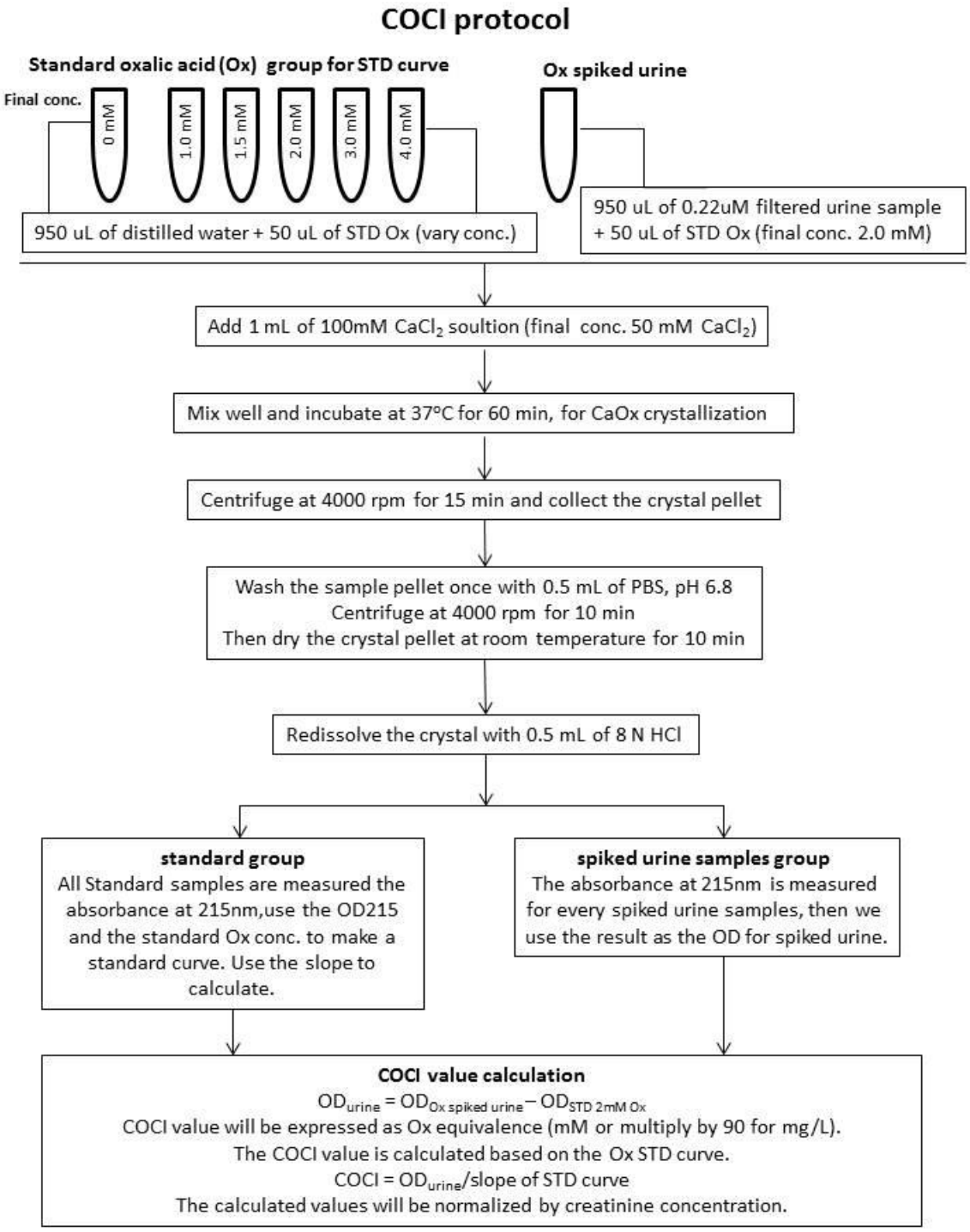

Fig. 5 The schematic diagram of the COCI procedure. The principle was to make all oxalate ions in urine sample to form CaOx crystals by addition of excess calcium, the crystals were then harvested, re-dissolved and measured absorbance at 215 nm.

6. Effects of urinary constituents on the COCI value

We investigated the effect of urinary volume, oxalate, phosphate, citrate, sodium and potassium on COCI values. For all of these analyses, 24-hr urine specimens collected from healthy volunteers were used. For the effect of urine volume, urine sample was diluted using deionized water to get different dilutions (undiluted, 60%, 80% and 90% dilution). To investigate the effect of oxalate, phosphate, citrate, sodium and potassium, different concentrations of these substances were added into urine samples prior to COCI analysis. The oxalic acid concentration varied from 0.5, 1, 2.5 to 5 mM, NaH_2PO_4 from 2.25, 4.5, 9.0 to 18 mM, citric acid from 2.5, 5, 10 to 20 mM, NaCl from 65, 130 to 260 mM, and KCl from 37.5, 75 to 150 mM. All treated urine samples were analyzed by the COCI method, and their COCI values were compared in each group. Each experiment was repeated 6 times.

7. Measurement of COCI in 24-hr urine samples

A total of 185 subjects were initially recruited for the study and divided into two groups, NL patients (n = 96) and healthy controls (n = 89). After matched by age and gender, the numbers of subjects in NL and healthy groups were reduced to 72 and 71, respectively (Table 3). The NL patients were recruited from the Sunpasit Prasong Hospital, Ubon Ratchathani province, Thailand. Their kidney calculi conditions were

confirmed by positive plain KUB film and/or IVP. Patients with anomalous kidney and other urinary tract diseases, i.e., horseshoe kidney, polycystic kidney, congenital vesicoureteral reflux, neurogenic bladder and any malignancies were excluded.

Table 3 Sex and age matched between nephrolithiasis patients and healthy volunteers.

	Healthy	Nephrolithiasis	P value
Numbers	71	72	-
Gender (male/female)	31/40	43/29	0.051
Age (years)	43.42 ± 12.86	47.87 ± 10.02	0.0506

The all recruited healthy controls (total = 89, aged 39.60 ± 15.38years, 45.5% male) were residents of rural communities in Ubon Ratchathani province, Thailand. The healthy condition was ensured from direct interview and/or previous medical evaluation reports. All healthy controls had no history and symptoms of NL.

The research protocol was reviewed and approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, and the Ethics Committee of the Sunpasit Prasong Hospital. Written-informed consents were received from all participants prior to specimen collection. Single 24-hr urine specimens were collected from all subjects using thymol as preservative. No restriction on oral intake was applied to the patients or control group prior to the collection of urine. The COCI values were determined in all urine specimens in duplicate manner. Urinary creatinine concentration was determined in all samples using Jaffe method.

8. Statistical analysis

Mean and standard deviation (SD) were used as representatives of the normal distributed data. For data with skewed distribution, median and interquartile range were used. Comparisons of means between two and three groups of subjects were performed using independent t-test and ANOVA test with Bonferroni post-hoc test, respectively. Also, Mann-Whitney and Kruskal-Wallis tests, the equivalent non-parametric tests of independent t-test and ANOVA test, respectively, were performed, and similar conclusive results were obtained.

An ROC curve analysis was used to evaluate how well the COCI test could discriminate the NL patients from healthy subjects. An area under curve (AUC) of 1.0 indicates that the test can perfectly separate the two groups, while an AUC of 0.5 indicates that the test lacks discriminatory power. An appropriate cutoff was chosen to calculate sensitivity, specificity and accuracy of the urinary COCI test. All statistical analyses were performed by STATA version 12 (StataCorp, TX) and graphs were created using GraphPad Prism 5 (GraphPad Software, CA). A two-sided $P < 0.05$ was considered statistically significant.

CHAPTER IV

RESULTS

1. Crystals Types Confirmed by FTIR and light microscope

To find the condition and methods for generating COM and COD crystals, we needed to analyze the types of COM and COD crystals that we prepared. Fig. 6 shows FTIR spectra and morphology of COM (6a) and COD (6b) crystals. According to various evidences in the literature[46, 53], these data ensured that our prepared crystals were veritably COM and COD.

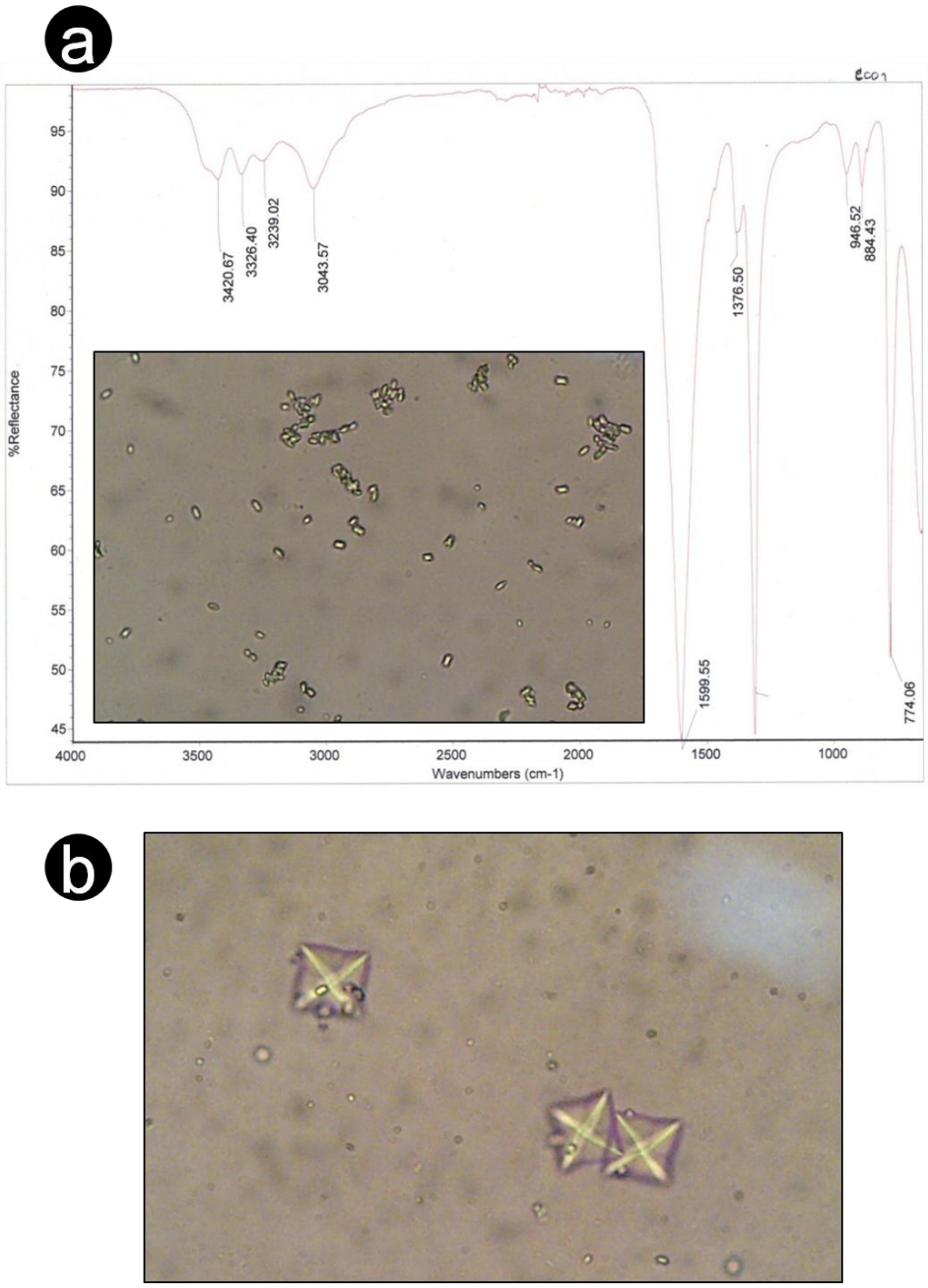

Fig. 6 Morphology and FTIR spectrum of COM and COD crystals. a: FTIR spectrum and light micrograph (inset: x100 magnification) of COM crystals. b: light micrograph of COD crystals (x400 magnification).

To verify the mineral constituent of crystals generated by COCI method, the harvested crystals from six selected cases (three NL and three healthy) were subjected to the FTIR

analysis. All COCI crystals primarily constituted with CaOx, however, in two cases (one NL and one healthy) calcium phosphate (CaP) was found as a minor component (Fig.7)

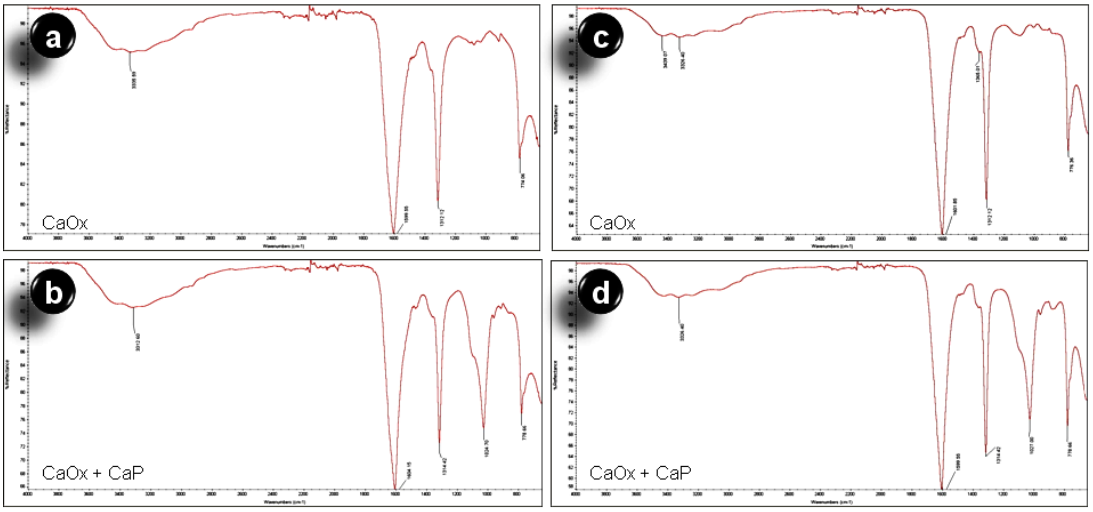

Fig. 7 The FTIR spectra of crystals harvested from the COCI procedure. CaOx was found to be primarily constituent in the COCI crystals (a-d). However, in some cases CaOx was mixed with CaP (b and d).

2. Effect of urinary constituents on COCI value

The effect of urine volume, oxalate, phosphate, citrate, sodium and potassium on COCI values was explored (Fig. 8). Increased urine volume (by gradually increasing dilution) caused decreased COCI values (Fig. 8a). Forty, 20 and 10% diluted urine (corresponded with 60, 80 and 90% water, respectively) had significantly lower COCI values than the undiluted urine (100% urine) ($P < 0.001$ for all). In contrast, increases in oxalate concentrations (0.5, 1, 2.5 and 5 mM) caused significantly increase in COCI values ($P <$

0.001 for all) (Fig. 8b). Also, increases in phosphate concentrations (2.25, 4.5, 9.0 and 18.0 mM) caused significantly increases in COCI values ($P < 0.001$ for all) (Fig. 8c). The COCI values trended to decrease after gradual addition of citric acid (2.5, 5, 10 and 20 mM) (Fig. 8d). At 20 mM citric acid, COCI value was significantly lower than the native urine control ($P < 0.05$ by Turkey's multiple comparison test). However, gradual additions of potassium (Fig. 8e) and sodium (Fig. 8f) had no significant influence on COCI values. Our data clearly demonstrated that urine oxalate and phosphate linearly, but urine volume and citrate reversely influenced COCI values.

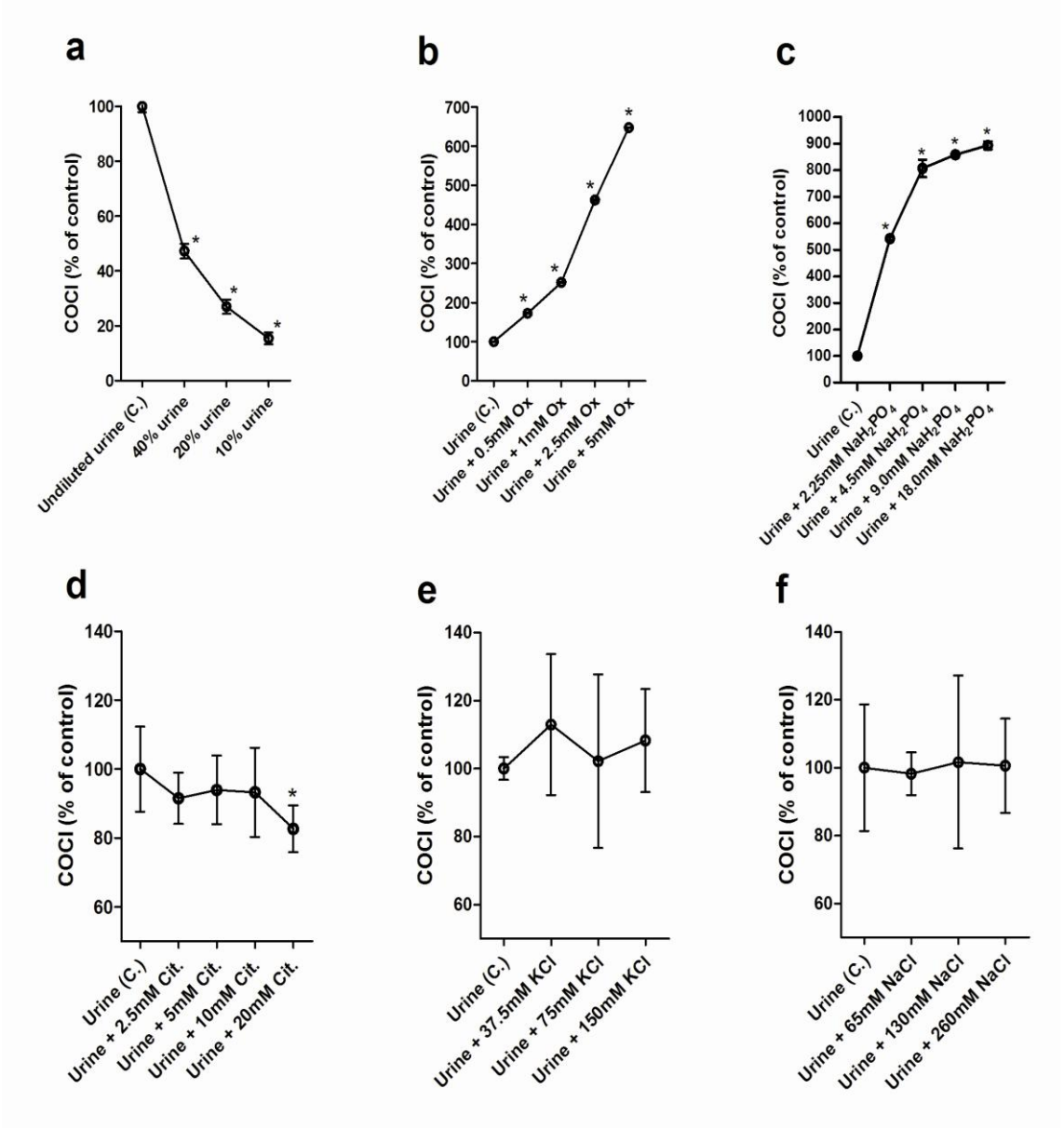

Fig. 8 The effect of urinary constituents on COCI value. a: effect of urine volume on COCI value. b: effect of various oxalate concentrations on COCI value. c: effect of various phosphate concentrations on COCI value. d: effect of various citric acid concentrations on COCI value. e: effect of various potassium concentrations on COCI value. f: effect of various sodium concentrations on COCI value. Increases in urine oxalate and phosphate, but decreases in urine volume and citric acid caused increase in COCI values.

3. Comparison of urinary COCI results between nephrolithiasis patients and healthy subjects.

We initially recruited 96 NL patients, 89 healthy volunteers for the study. In all cases, urinary COCI amount in NL group (456.50 ± 401.30 mg Ox equiv./day) was significantly higher than that in healthy group (74.86 ± 48.72 mg Ox equiv./day) (Fig.9b).

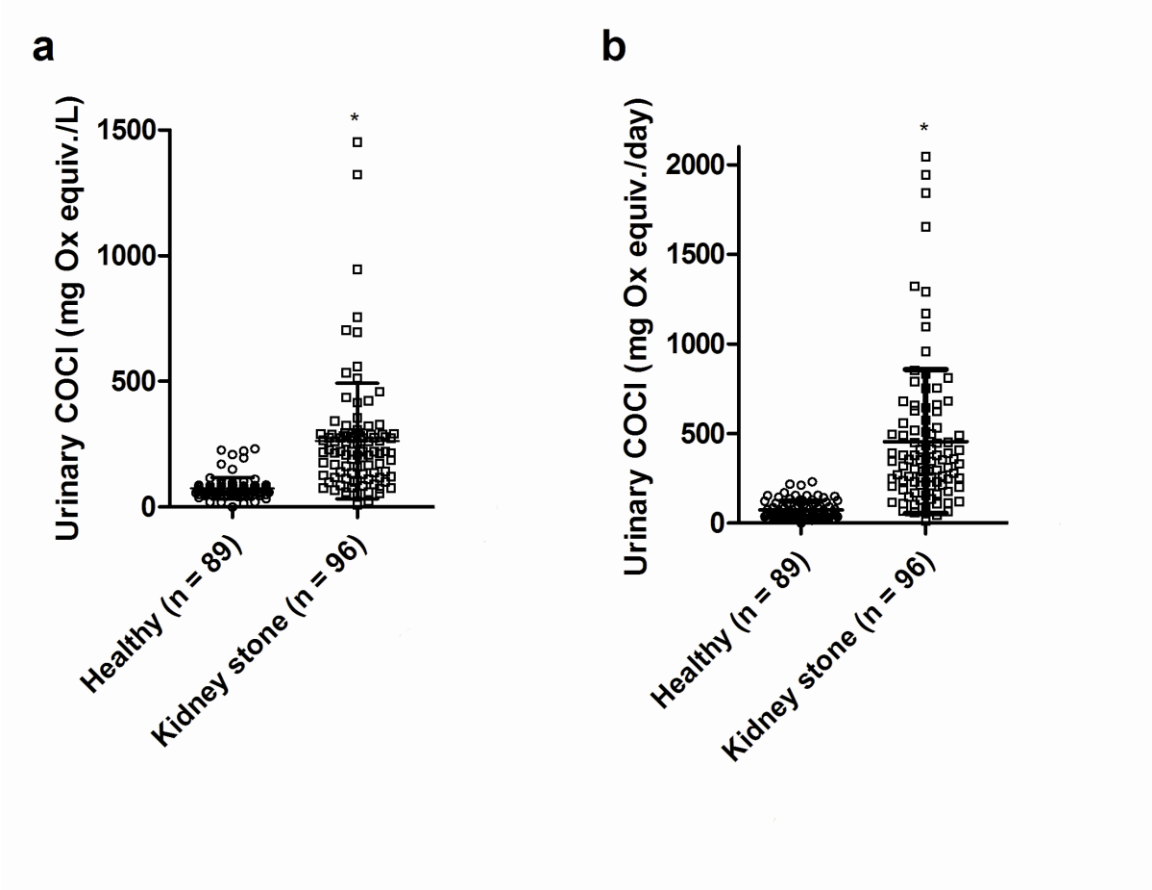

Fig. 9 Urinary COCI levels in all recruited subjects. Urinary COCI in nephrolithiasis group (n = 96) was significantly higher than that in healthy groups (n = 89), both expressed in mg Ox equiv./L (a) and mg Ox equiv./day (b). *P < 0.001 vs. healthy,

To minimize age and gender bias, age and sex of NL and healthy subjects were

matched. The matched subjects consisted of 71 healthy controls and 72 NL patients. Table 4 displays urinary COCI values between healthy controls and the matched NL patients. The mean of urinary COCI concentration in healthy subjects was significantly lower than the NL patients (67.83±29.83 mg Ox equiv./L vs. 270.3±205.8 mg Ox equiv./L, $P < 0.0001$). Likewise, the mean of urinary COCI amount in healthy group was significantly lower than the patient group (73.00±42.60 mg Ox equiv./day vs. 480.7±391.6 mg Ox equiv./day, $P < 0.0001$).

The urinary creatinine concentration between healthy controls and NL patients were also significantly different ($P = 0.008$). For normalized urinary COCI, the mean of urinary COCI in healthy controls and NL patients were significantly different (72.48±58.12 mg Ox equiv./g Cr vs. 333.0±238.6 mg Ox equiv./g Cr, $P < 0.0001$).

After we got the result from all sex – age matched health and NL samples, differences of COCI concentration and amount between the two groups were tested. Fig. 10 shows urinary COCI compared between NL patients (n = 72, aged 47.87 ± 10.02 years, 59.72% males), aged- and sex-matched healthy individuals (n = 71, aged 43.42 ± 12.86 years, 43.66% males). Patients with NL had significantly higher urinary COCI than healthy controls (Fig. 10).

Table 4 Comparison of urinary COCI between nephrolithiasis patients and healthy controls.

Variables	Healthy	Nephrolithiasis	P value
Number of subject	71	72	
Age (years)	43.42 ± 12.86	47.87 + 10.02	0.051
Male: Female (%)	43.66 : 56.34	59.72 : 40.28	0.051
Urinary Cr concentration			
Mean ± SD (g/L)	1.28 ± 0.70	0.93 ± 0.50	0.008
Median (IQR) (g/L)	1.10 (2.15)	0.79 (2.01)	0.008
Urinary COCI concentration			
Mean ± SD (mg Ox equiv. /L)	67.83 ± 29.83	270.30 ± 205.80	<0.0001
Median (IQR) (mg Ox equiv. /L)	64.44 (1.55)	260.60 (2.11)	<0.0001
Urinary COCI amount			
Mean ± SD (mg Ox equiv. /day)	73.00 ± 42.60	480.70 ± 391.60	<0.0001
Median (IQR) (mg Ox equiv. /day)	60.93 (2.39)	360.50 (2.75)	<0.0001
Urinary COCI normalized by Cr			
Mean ± SD (mg Ox equiv. /g Cr)	72.48 ± 58.12	333.00 ± 238.60	<0.0001
Median (IQR) (mg Ox equiv. /g Cr)	55.16 (2.37)	280.40 (2.42)	<0.0001

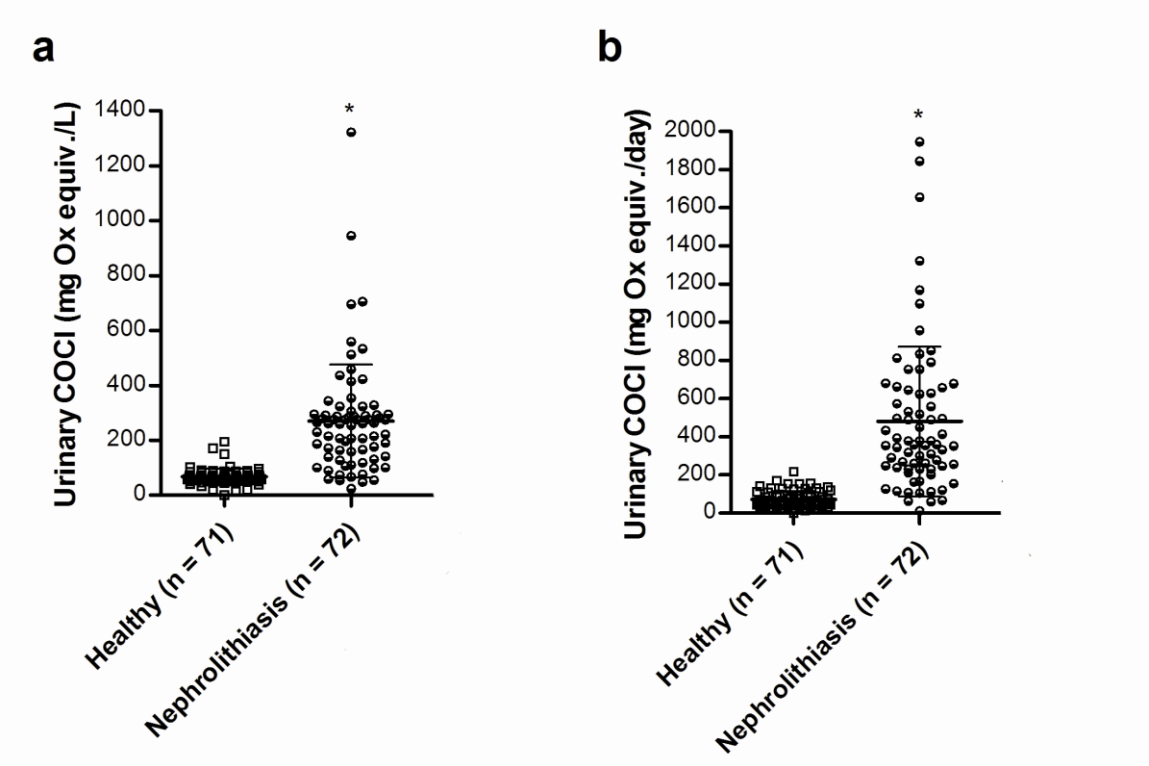

Fig. 10 Comparison of urinary COCI values between healthy controls and nephrolithiasis patients. *P < 0.0001 vs. healthy.

4. ROC analysis for urinary COCI test

Fig. 11 shows the ROC curve for the urinary COCI test for assessing how good the test can distinguish NL patients from healthy individuals. AUC of 0.9229 (95%CI: 0.8739 - 0.9720) and 0.9499 (95%CI: 0.9131 - 0.9868) were observed for the urinary COCI concentration (11a) and amount (11b), respectively. The AUC of normalized urinary COCI (11c) was 0.9259 (95%CI: 0.8838 - 0.9680).

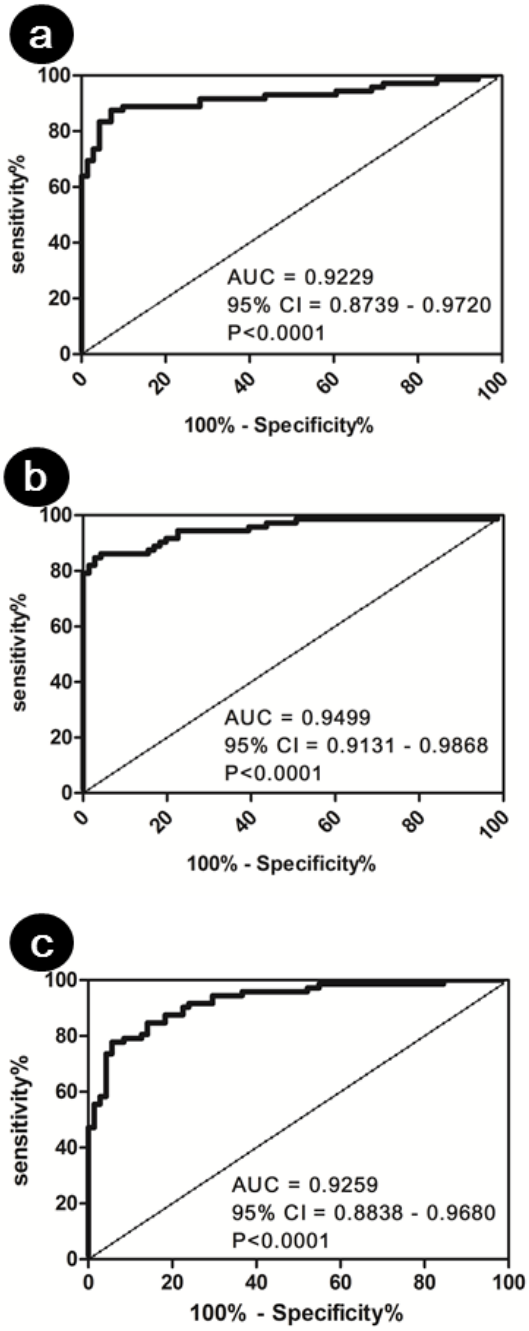

Fig. 11 ROC analysis of urinary COCI test. AUC of urinary COCI expressed as mg Ox equiv./L (a), mg Ox equiv./day (b) and mg Ox equiv./g creatinine (c) were 0.9229, 0.9499 and 0.9259, respectively. This data indicated that urinary COCI amount (mg Ox equiv./day) had the highest discriminating power to separate NL patients from healthy individuals.

5. Clinical value of COCI on diagnosing nephrolithiasis

The cutoff points of urinary COCI test for negative and positive results were chosen based on ROC analysis (Table 5). At the cutoff of 105 mg Ox equiv./L, the urinary COCI concentration had a sensitivity and specificity and accuracy of 83.33%, 95.77% and 89.51%, respectively. For urinary COCI amount at cutoff point of 165 mg Ox equiv./day, sensitivity, specificity and accuracy were 83.33%, 97.18% and 90.21%, respectively. For urinary COCI normalized by creatinine, at cutoff point of 36 mg Ox eqv /mg Cr, the test imparted sensitivity, specificity and accuracy of 72.22%, 95.77% and 83.92%, respectively (Table 5).

Table 5 The cutoff points of urinary COCI test and their diagnostic values

	COCI concentration (mg Ox equiv./L)	COCI amount (mg Ox equiv./day)	normalized COCI (mg Ox equiv./g Cr)
Cutoff	105	165	126
Specificity	95.77%	97.18%	95.77%
Sensitivity	83.33%	83.33%	72.22%
Accuracy	89.51%	90.21%	83.92%

6. Urinary COCI test for screening asymptomatic kidney calculi

Unfortunately, we found two subjects in the healthy groups who had very high urinary COCI values (> 200 for both concentration and amount units, subject #1: 227.22 mg Ox equiv./L and 231.77 mg Ox equiv./day, and subject #2: 208.84 mg Ox equiv./L and 213.02 mg Ox equiv./day). We suspected that these two subjects might have asymptomatic stones in their kidneys. We asked them to come to the urology clinic to perform an ultrasound imaging for detecting the presence of kidney calculi. Based on the ultrasound imaging (by Dr. Wattanachai Ungjaroenwathana), we found that both of them had kidney calculi (Fig. 12). We further invited them to come to the Sunpasit Prasong Hospital for CT scan imaging and surgical treatment, but they refused to participate. Based on the present ultrasound data, we concluded that the urinary COCI test was capable of detecting asymptomatic kidney calculi or an early stage of NL. Thus, it was clinically useful as a screening test for kidney stone disease.

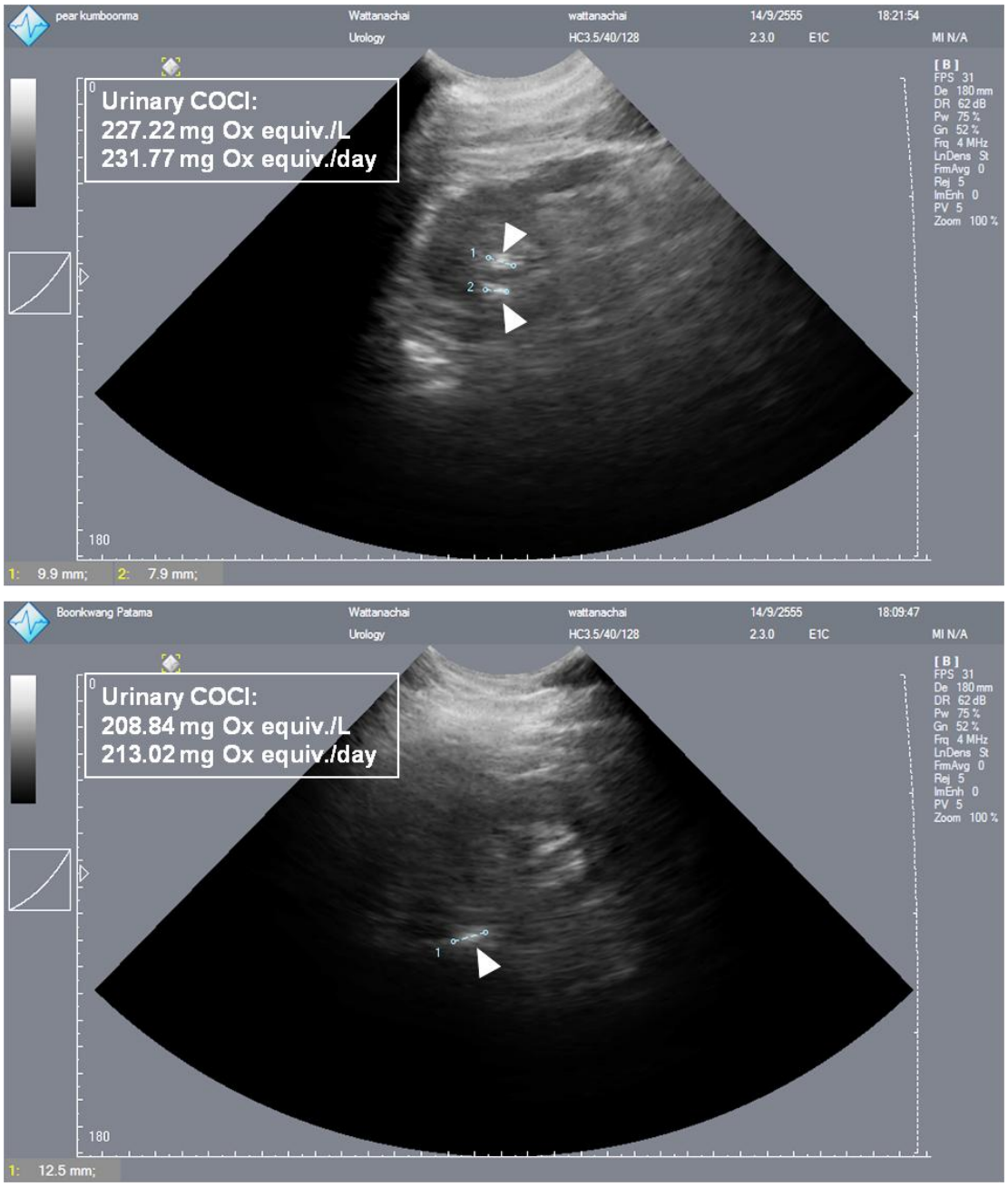

Fig. 12 Ultrasound imaging result from two subjects with elevated urinary COCI values. Arrows indicate the presence kidney calculi sized between 7.9 and 12.5 mm.

CHAPTER V

DISCUSSION AND CONCLUSION

The principle of the COCI test was to make all oxalate and its relevant ions in urine to form CaOx crystals by addition of excess calcium. The amount of yielded COCI crystals was measured using absorbance at 215 nm. Therefore, higher COCI value indicates the higher capability of urine to form the lithogenic crystals that manifests an increased propensity of stone formation. The test procedure was simple. It required only one and a half hours for the entire analysis. In term of equipment, only an ultraviolet spectrophotometer was required. We believe that this straightforward test could be implemented in the most laboratories in the hospitals. The urinary COCI value was expressed as mg Ox equiv./L or mg Ox equiv./day, because the COCI value was derived from a standard curve of varied concentrations of oxalic acid. Some limitation of the urinary COCI test should be mentioned. The stability of urine samples was a main concern. Urinary COCI values significantly changed in urine kept at 4^{o}C for more than 5 days. For the best result, urinary COCI had to be determined in the fresh urine samples. However, urine samples could be stored up to 5 days at 4 ^{o}C. Multiple freeze-thawed samples were not recommended.

Based on our FTIR data, CaP could be co-precipitated with CaOx in some cases (Fig.

7). Amount of harvested COCI crystals was measured based on absorbance at 215 nm (λ_{max} for oxalate). The other organic acids could also be absorbed at this wavelength. Therefore, other organic acids and ions including phosphate ions existed in the COCI crystals could increase the optical density. In other word, the obtained COCI values were indicative of total formation of the lithogenic calcium-containing crystals, primarily CaOx and CaP. In lithogenesis, it is well recognized that CaP or hydroxyapatite crystals is a main component of interstitial Randall's plaques that have been proved to be the origin of CaOx stones[30]. Therefore, measuring capability of urine to crystallize both CaOx and CaP would be a better approach to estimate the sole lithogenic potential of urine, and it could be more accurate to identify peoples at risk of kidney stone formation. However, the specific reaction to detect only CaOx should be further established, and asked if it could provide a higher power than the current approach to screen individuals at risk of CaOx stone formation.

Our data showed that urinary volume, oxalate and phosphate caused increased urinary COCI values, whereas higher urinary citrate content lowered down urinary COCI values. Basically, patients with NL may have one or more metabolic abnormalities such as: hyperoxaluria, hypercalciuria, hypocitraturia, hyperuricosuria, and hypomagnesiuria. The presence of these metabolic abnormalities leads to urinary supersaturation and causes crystal formation, which is the first pre-requisite step of kidney stone formation[29]. There are inhibitors which can prevent stone formation, such as citrate, potassium and magnesium. Citrate is the most potent urinary inhibitor that is able to reduce the growth of COM crystals at the millimolar concentrations [54]. Citrate complexes with calcium to

form a calcium citrate salt that is more soluble than calcium oxalate, and can therefore reduce calcium oxalate supersaturation. In other word, citrate reduces the urinary free calcium ion concentration without causing calcium citrate crystallization[55]. Even though citrate at low concentration had no significant inhibitory effect on the COCI value (Fig. 8d), there still was a downward trend in the COCI value. After addition of 20 mM citric acid into urine, significant decrease in COCI value was revealed. At high concentration of citrate, there were sufficient free citrate ions to compete oxalate ions for binding with excessive calcium ions (thus, lowering COCI value) while at low citrate concentration there still was free calcium ions left to combine with oxalate. This might be the explanation of the effect of citrate on COCI values. In sum, our current findings suggest that urinary COCI is a reflective of a balance between stone promoters (primarily oxalate and phosphate) and inhibitor (especially citrate).

The gold standard for determination of urinary crystal-forming potential is EQUIL2 calculation using 12 parameters including Na, K, Ca, Mg, NH_4, Cl, PO_4, SO_4, UA, Ox, citric acid and pH[56]. More convenient and cost-effective alternative methods have been developed, and the most cited method in the literatures is BRI[18]. However, the BRI procedure is complicated, and the test requires sophisticated equipment to kinetically detect the very first crystal nucleation. For COCI method, only an ultraviolet spectrophotometer was required for OD_{215} nm measurement. In addition, only 1 mL of urine was required for the COCI test, and sample pretreatment was not necessary except filtration through the 0.22 μm membrane. For the original BRI assay, 200 mL of unprepared native urine, determination of calcium ions and a sophisticated laser-probe

crystal system analyzer for kinetically detecting the onset of crystallization are required[18]. However, a micromethod of BRI is recently developed with the use of at least 1.5 mL urine[20]. In addition, to be more convenient a BRI-measuring device, called Urolizer®, is developed, and a clinical usefulness in metabolic monitoring patients with CaOx calculi is demonstrated[56]. In this study, we successfully developed a cheaper and simpler test tube-based endpoint method to estimate the CaOx crystal forming capability in 24-hr urine samples, and the test was clinically useful for distinguishing patients with kidney stone formation from those without.

In order to evaluate the clinical value of urinary COCI test, we compared the urinary COCI results from healthy and NL groups. There was a statistical difference in urinary COCI concentration and amount between the healthy and NL groups (Fig. 10). This suggested that the COCI test was able to distinguish NL patients from healthy peoples, and it efficiently reflected the lithogenic potential. For urinary creatinine, the healthy group had significantly higher than the NL group[57].

Basically, discriminatory power of the diagnostic test is classified regarding to an AUC as follows: excellent discrimination, AUC of ≥ 0.90; good discrimination, 0.80 - 0.90; fair discrimination, 0.70 - 0.80; and poor discrimination, AUC of ≤ 0.70[58-60]. Our data underlined the clinical usefulness of urinary COCI test for discriminating the stone forming patients from non-stone forming peoples, as an area under ROC curve was up to 0.9499 (95%CI: 0.9131 - 0.9868). The more important finding was that two subjects in the healthy group, who said they were healthy and had no history of kidney stone formation during a direct interview, had very high COCI values. The ultrasound imaging

showed that these two subjects had asymptomatic calculi in their kidneys (Fig. 12). This finding suggested that the urinary COCI test was a useful test to screen people at risk of kidney stone formation and to detect asymptomatic calculi. However, further study in large population needs to be conducted to verify this screening power.

As AUC of the urinary COCI test both for concentration and amount units were over 0.90, it was considered to have an excellent discriminatory power in separating NL from healthy individuals. The urinary COCI amount had a better specificity and sensitivity than the urinary COCI concentration. At the cutoff point of 165 mg Ox equiv./day, COCI amount showed the sensitivity, specificity and accuracy of 83.33%, 97.18% and 90.21%, respectively (Table 5). The BRI test was recently reported for separating children with CaOx stones (aged 5-18 years) from healthy children (aged 5 – 17 years) with sensitivity and specificity of 69.7% and 100%, respectively[21]. Although the diagnostic values of the COCI test are relatively high enough to be used in the clinics, validating studies in larger population as well as in pre-diagnostic urine specimens needs to be conducted prior to clinical implementation.

There are four commonly used radiological investigations in diagnosis of nephrolithiasis namely: X-ray radiography of the kidney-ureter-bladder (XR KUB), intravenous pyelography (IVP), ultrasound of the KUB, and computer tomography (CT) scan KUB. The sensitivity in XR KUB is relatively low (45%-58%)[61], and the sensitivity of the ultrasound is approximately 60% with a specificity of 90%. CT KUB has the highest sensitivity and highest specificity of 96% and 99% respectively[61]. From our current data, we suggest that although principles are different, our urinary COCI test

provides a much better diagnostic efficacy than the X-ray and ultrasound KUB. Despite the fact that the CT scan is more accurate than the urinary COCI test, there are more risks associated with an X-ray CT scanning.

In conclusion, a novel method for estimating the capacity of CaOx crystal formation in the urine, called COCI, was successfully developed. The primary determinants of urinary COCI values were urine volume, oxalate, phosphate and citrate. The urinary COCI in NL patients was significantly greater than healthy individuals. Self-reported healthy subjects with high urinary COCI values were confirmed to have asymptomatic kidney stones by ultrasound imaging. Based on ROC analysis, the urinary COCI test provided high diagnostic power for clinically discriminating NL patients from healthy controls. Therefore, the COCI test was a cheap, simple, non-invasive and reliable method that has a clinical potential to identify individuals at risk of kidney stone formation as well as those with asymptomatic urinary calculi. We hope that in the near future this test might be implemented as a useful screening test in the hospital laboratories to detect an early stage of kidney stone formation.

For the further study, urinary COCI measured in spot urine samples remains to be explored. Diurnal variation of urinary COCI values during day and nice in health peoples and NL patients should be investigated. Specific reaction to detect only CaOx COCI crystals needs to be developed. The other important further study is that parallel comparison of COCI method with other methods such as BRI and oxalate oxidase kit.

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APPENDICES

Reagents

1. **Standard oxalate acid solution**
    - Prepare 160 mM oxalate acid solution by mixing 16 mM oxalate acid in 100 mL deionized water
    - Use 160 mM oxalate acid solution to dilute to 120 mM
    - Use 160 mM oxalate acid solution to dilute to 80 mM
    - Use 120 mM oxalate acid solution to dilute to 60 mM
    - Use 80 mM oxalate acid solution to dilute to 40 mM

    For dilution, formula used was $N_1V_1 = N_2V_2$.

2. **100 mM calcium chloride solution preparation**
    - Use calcium chloride 1 M mixed with 1 L deionized water, and try to dissolve calcium chloride as much as possible
    - Use filter paper to filter all the solution 1 time in order to make the solution cleared
    - Test the concentration of this filtered solution by ICPS to determine the accurate calcium concentration in this solution (544 mM)
    - Use this solution to dilute to 100 mM calcium chloride solution

3. **Wash solution preparation**
    - Prepare 1×PBS similar to PBS used in cell cultivation
    - Adjust the pH to 6.8 with 12 N HCl

4. 8 N hydrochloric acid (HCl) preparation

- Use 12 N HCl to dilute to 8 N HCl

5. Blank preparation

Prepare blank for OD215 nm containing 8 N HCl and 10 mM calcium chloride (5 mL)

- add 0.09 mL of 544 mM calcium chloride solution (the one that I had filtered in reagent 2 had concentration of 544 mM)
- add 3.33 mL of 12 N HCl
- add 1.58 mL of deionized water
- Mixed well
- Total volume: 5 mL

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