HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF URINARY CITRIC ACID CYCLE INTERMEDIATES IN NORMAL AND DISEASES

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ชื่อวิทยานิพนธ์ การวิเคราะห์หาสารอินเตอร์มีเดียตในวัฏจักรเครบส์จากปัสสาวะโดยวิธีโครมาโตกราฟฟีประสิทธิภาพสูง

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บทคัดย่อ

สารอินเตอร์มีเดียตในวัฏจักรเครบส์ได้แก่ ซิเตรท, ซัคซิเนท, มาเลท, ฟูมาเรทและไอโซซิเตรทจากปัสสาวะ 24 ชั่วโมงของคนปกติในกรุงเทพ 30 คน คนปกติภาคตะวันออกเฉียงเหนือ 15 คน กลุ่มผู้ที่ไม่สามารถทำให้ปัสสาวะเป็นกรด 15 คนและกลุ่มผู้ที่มีนิ่วในไต 15 คนซึ่งวิเคราะห์ด้วยวิธีโครมาโตกราฟีประสิทธิภาพสูง พบว่าคนปกติในกรุงเทพและภาคตะวันออกเฉียงเหนือมีระดับการขับถ่ายซิเตรทสูง (0.48 ± 0.35 และ 0.51 ± 0.48 มิลลิโมลาร์) เมื่อเทียบกับการขับถ่ายในกลุ่มผู้ที่ไม่สามารถทำให้ปัสสาวะเป็นกรดและกลุ่มที่มีนิ่วในไต (0.10 ± 0.19 และ 0.20 ± 0.32 มิลลิโมลาร์) ในขณะที่ระดับการขับถ่ายซัคซิเนทของคนปกติในกรุงเทพสูง (0.49 ± 0.21 มิลลิโมลาร์) เมื่อเทียบกับกลุ่มอื่นๆทุกกลุ่ม (0.27 ± 0.08, 0.24 ± 0.06 และ 0.25 ± 0.13 ในคนปกติภาคตะวันออกเฉียงเหนือกลุ่มผู้ที่ไม่สามารถทำให้ปัสสาวะเป็นกรดและกลุ่มผู้ที่มีนิ่วในไตตามลำดับ) ระดับการขับถ่ายมาเลท, ฟูมาเรทและไอโซซิเตรทในทั้ง 4 กลุ่มไม่มีความแตกต่างกัน ผลจากการศึกษาครั้งนี้จึงสนับสนุนการใช้ระดับการขับถ่ายซิเตรทในปัสสาวะเป็นตัวบ่งชี้ที่ดีในการวินิจฉัยแยกโรคภาวะที่ไตไม่สามารถทำให้ปัสสาวะเป็นกรด

Thesis Title High performance liquid chromatographic determination of urinary citric acid cycle intermediates in normal and diseases

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ABSTRACT

Incidence of distal renal tubular acidosis (dRTA) and urinary stone disease in Thailand is rather high, especially in the northeast of Thailand. Urinary citrate was a screening test for detecting dRTA cases. In this study, citrate and other urinary citric acid cycle intermediates such as succinate, malate, fumarate, and isocitrate were simultaneously determined in 24 hr urine specimens from 30 healthy subjects in Bangkok, 15 healthy subjects from the northeast of Thailand, 15 villagers with acidification defect, and 15 villagers with renal stone by a reversed-phase high performance liquid chromatographic method, employs a one-step esterification procedure with phenacyl bromide as esterification reagent. This simple and rapid analysis, is performed

at 254 nm with a simple eluent water-methanol mixture and gives high enough recovery to determine the urinary citric acid cyele intermediates with a high accuracy and precision. The excretion levels of citrate in both normal groups from Bangkok and Northeast, 0.48±0.35 and 0.51±0.48 mM, respect tively, show significantly higher than acidification defect (0.10±0.19 mM) and renal stone (0.20±0.32 mM). Urinary succinate excretion of normal from Bangkok (0.49±0.21 mM) shows significantly higher than in any other group; 0.27±0.08, 0.24±0.06, and 0.25±0.13 mM for normal from northeast, acidification defect and renal stone, respectively. The urinary malate, fumarate, and isocitrate excretion show no significantly different between normal and diseases. This study also supported the using of urinary citrate excretion as a screening test for detecting dRTA cases.

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LIST OF ABBREVIATIONS

HPLC	=	High performance liquid chromatography
dRTA	=	distal renal tubular acidosis
TCA	=	tricarboxylic acid
ml	=	millilitre
mM	=	millimolar
umol	=	micromole
mg	=	milligram
Fig	=	figure
nm	=	nanometre
et al	=	et alii
h	=	hour
L	=	liter
g	=	gram
M	=	molar

CHAPTER I

INTRODUCTION

The tricarboxylic acid cycle (TCA cycle) or Kreb's cycle or citric acid cycle has been accepted as the final common pathway for the aerobic oxidation of carbohydrates, fats and proteins in most biological systems. This cycle also plays an important role in the biosynthesis of cell material in anaerobes grown on an organic compound of low molecular weight as their sole carbon source, e.g. acetate. A study of this cycle metabolism requires the simultaneous determination of its intermediates. The lack of rapid and accurate analytical methods for these compounds, however, has remained a problem. The enzymatic, colorimetric and fluorimetric techniques developed for the determination of these acids are laborious and timeconsuming, and are subject to considerable error. In addition, they do not themselves to a simultaneous determination of all acids.

Various attempts have been made to determine simultaneously these citric acid cycle intermediates by means of chromatographic techniques. Paper and thin-layer chromatography provide only a qualitative assessment of the balance of the individual acids. Several papers have been published on the separation procedures applicable to high-performance liquid chromatography (HPLC) for the analysis of carboxylic acids (1). Four main methods have commonly been used, namely ion-exchange and ion-exclusion separation, solvophobic chro-

matography, ion-pair chromatography and reversed-phase chromatography of derivatized products.

Ion-exchange and ion-exclusion separations are generally performed on silica-based ion exchangers and on exchangers with a styrene-divinyl benzene copolymer structure (2). In solvophobic chromatography, the addition of acids or acidic buffers to the mobile phase lowers the pH and suppresses the dissociation of the carboxylic groups of the solutes. Under these conditions, hydrophobic interaction of the organic structure of the solutes with reversed-phase stationary phases is induced and in this instance a solvophobic chromatographic separation may be obtained (3).

Ion-pair chromatography has also been applied to the separation of acids, using silica gel and cellulose coated with a reagent capable of forming ion pairs and a non-polar mobile phase (4). Non-polar counter ions have also been used in reversed-phase ion-pair chromatography. However, none of these methods meets the requirements of simplicity, rapidity and, mostly, reliability.

Recently, Mentasti et al (5) described an HPLC method for the determination of organic acids in wine samples, which couples their conversion into phenacyl esters without recourse to problematic solvent extraction with the ease of application of reversed-phase liquid chromatography. Caccamo et al (6) described an improved HPLC procedure for the assay of organic acids in wine, which involves substan-

tial modifications of the Mentasti's method. The main modification is that of buffering the reaction mixture which results in a better yield from the derivatization reaction, thus eliminating a source of error in the determination of polycarboxylic acids, especially "citric acid".

Derivatizing agents have been used in liquid chromatography for the reversed-phase separation of organic acids. The products mainly investigated are differently substituted phenacyl, naphthacyl, p-nitrophenyl, and p-nitrobenzyl esters.

DERIVATIZATION

The derivatization of acid by phenacyl bromide:

$$RCOOH + BrCH_2COC_6H_5 \text{ -----> } RCOOCH_2COC_6H_5 + HBr$$

take place with satisfactory yields only in the presence of a catalyst. Among those previously suggested, they have tested triethylamine (7), fluoride ions (8) and 18-crown-6 (9). Both triethylamine and fluoride allow activation of the $RCOO^-$ anion by hydrogen bonding, but preliminary measurements showed that triethylamine gives very low yields with acid such as citric and malic acid. In contrast, fluoride is an effective catalyst for the derivatization but its strong interaction with dissociable protons makes it poorly selective.

18-Crown-6 proved to be very effective on promoting the derivatization of carboxylic acids previously converted into potassium salts. Complexation of K^+ by the ether

allows dissociation of the carboxylate salts in a suitable solvent. Preliminary neutralization to pH 7-8 with KOH or $KHCO_3$ ensures the absence of interference from phenols and esters (under these conditions neither neutralization nor hydrolysis occurs). The solvent that gave the best derivatization yields was water-acetone (1:3). At higher water contents the derivatization was less effective, and at higher acetone contents the reaction became very slow. A heating period of 40 minutes at 100 C was satisfactory for all the acids investigated.

Buckalew et al (10) reported urinary citrate excretion in distal renal tubular acidosis (dRTA) was extremly low. The high prevalence of dRTA in the northeast region of Thailand is reported by Nilwarangkur (11) with the female to male ratio was 3:1. All patients with generalized muscle weakness and the peak of admission was in the middle of summer. The etiologies or risk factors remained unknown. Doctors working in the northeast all agree that they frequently have to care for the overt cases of primary renal tubular acidosis. The disease in not mild, some of the clincal manifestations especially muscle weakness which may lead to respiratory failure are life threatening and its sequelae may lead to nephrocalcinosis and chronic irreversible renal failure osteomalacia and body deformities. Early recognition of the disease and the supplement with sodium bicarbonate might abort its sequelae and serious complications. This is one of the public health problems in the northeast of Thailand.

The short ammonium chloride acid load is both the most effective screening procedure and a definitive diagnostic test for dRTA, but it requires the administration of a potentially noxious agent, multiple timed blood and urine specimens, and in most instances, at least 24 hours of hospitalization. In children, acidloading test is hard to tolerate and unacceptable to their parents when the child is asymptomatic.

Several previous studies in complete and incomplete dRTA have documented hypocitraturia (10), thought to result from enhanced proximal tubular reabsorption of filtered citrate in response to intracellular acidemia (12). Urinary citrate has been reported previously as a screening test in familial RTA by Norman et al (13). They measured urinary citrate excretion in the healthy, asymptomatic children of parents with complete familial dRTA and reported that hypocitraturia was the first clue that suggested the presence of dRTA and separated patients from their normal siblings. They beleive that 24-hour urine citrate excretion represents a simple, accurate screening test for the early diagnosis of familial dRTA in children at risk. The diagnosis should subsequently be confirmed by appropriate blood studies and measurement of acid excretion during an acid load. In Thailand, Nilwarangkur (11) has reported the use of urinary citrate as a screening test for detecting dRTA cases (urinary citrate < 0.3 mmol/l had 93.4% sensitivity and 12% specificity).

The present study was untertaken to obtain additional data on the urinary excretion of citrate and other citric acid cycle intermediates which never reported before.

Harrison and his coworkers proposed that hypocitraturia in patients with dRTA was related to the "fundamental metabolic abnormality of the renal tubule cell" Because the major part of citrate metabolism is intimately connected to energy production in the cell through the Krebs cycle, investigators began to connect the close relationship between citrate excretion and acid-base balance to pH dependemt enzyme activity in the Krebs cycle. The first of these hypotheses was made by Milne and his co-workers (12). They started that it was through the condensing enzyme of Ochoa, which was more active at high pH, that alkalosis increased the excretion of citrate. Edwards, Mody and Crawford (15) suggested a different mechanism, namely, that citrate from the circulation was utilized by kidney tubular cells for energy production needed for active transport mechanisms, one of which was active hydrogen ion transport. According to this hypothesis, the reduced citrate excretion in acidosis was the result of incresed the utilization rather than a reduced production. Dedmond and Wrong (16) put forward a slightly different hypothesis, namely, that in patients with renal tubular acidosis, the defect, inherited in a proportion of patients, was in one of the Krebs cycle enzymes, leading to

both the reduced citrate excretion as well as to the inability to maintain a normal hydrogen ion gradient across the tubular membrane. An enzyme defect has often been suspected, and it has been suggested that carbonic anhydrase activity is defective (17); however, redcells (18) and material obtained by renal biopsy (19) both showed normal levels of this enzyme. A deficiency of some enzyme essential for the normal function of the Krebs cycle might be responsible both for the tubular failure to secrete hydrogen ion against a concentration gradient as well as the reduced excretion of citrate and α-ketoglutarate. Dedmond and Wrong observation furnished no direct evidence of an enzyme deficiency in the tricarboxylic acid cycle. Yaffe et al (19) have examined several enzymes, including a number concerned in the Krebs cycle, in renal biopsy tissue from a 4 year old child with renal tubular acidosis. No defects were discovered except for TPN diaphorase in the histochemical assay but this deficiency was not corroborated by direct biochemical assay. The nature of the presumed enzyme defect in renal tubular acidosis, therefore remains a mystery and at present there is no direct evidence that the reduced excretion of citrate in primarily due to enzyme defect.

The quantity of citrate excreted in the urine of man and animals varies under different physiologic conditions. Like other small inorganic and organic ions, the excretion of citrate is determined in part by transport processes in the membranes of tubular epithelial cells. In addition,

citrate represented a group of substances in which specific metabolic processes in the kidney participate in the regulation of excretion. By studying the mechanisms by which renal citrate excretion is altered, we can gain insight into fundamental aspects of intracellular metabolism in the kidney.

GENERAL PROPERTIES OF RENAL CITRATE HANDLING

Citrate level in the plasma of humans and other mammals range between 0.05 and 0.3 mM. At physiologic pH nearly all the plasma citrate is in the form of triply charged citrate ion that circulated unbound to larger molecules and is freely filtered in the glomerulus. Citrate is extensively reabsorbed in the nephron. This reabsorption occurs predominantly in the proximal segments of the tubule (20).

The levels of citrate in renal venous blood are lower than those in the arterial blood entering the kidney. Studies in humans (21) and other species have shown that more citrate disappears into the kidney than can be accounted for by the metabolism of all the citrate reabsorbed in the tubules. The citrate that disappears into the kidney is metabolized chiefly by the abundant mitochondria of the cells of the proximal convoluted tubules. CO_2 and glucose are the major end products of citrate metabolism in the kidney, but the proportions of these and other products formed from citrate are not known

with certainly. Nieth and Schoollmeyer (21) estimated that citrate provides upto 10% of the energy needs of the kidney in humans. This estimate, however, was made with the assumption that all of the citrate used by the kidney was completely oxidized to CO_2 and water, an assumption that is probably invalid. Baruch et al (22) measured renal CO_2 production from infused ^{14}[C]citrate in the dog and concluded that about 15% of the CO_2 formed in the kidney arises from citrate oxidation. Whatever the exact figures, it is clear that one-third to one-fifth of the citrate supplied to the kidney in the arterial blood is metabolized and makes a small but definite contribution to the energy supply of that organ.

Figure 1 provides an overall picture of the typical renal handling of citrate using values obtained by Nieth and Schoollmeyer (21) in humans. Forty-four micromoles of citrate enter the kidney each minute in the arterial plasma. Each minute 8.8 umol are filtered, of which 75% or 6.6 umol, are reabsorbed and 25%, or 2.2 umol, are excreted. About 1.5 umol of citrate are removed from the peritubular blood, so that a total of 8.1 umol of citrate are taken up and metabolized by the kidney each minute. Qualitatively similar results for the renal handling of citrate have been obtained in the dog and rat.

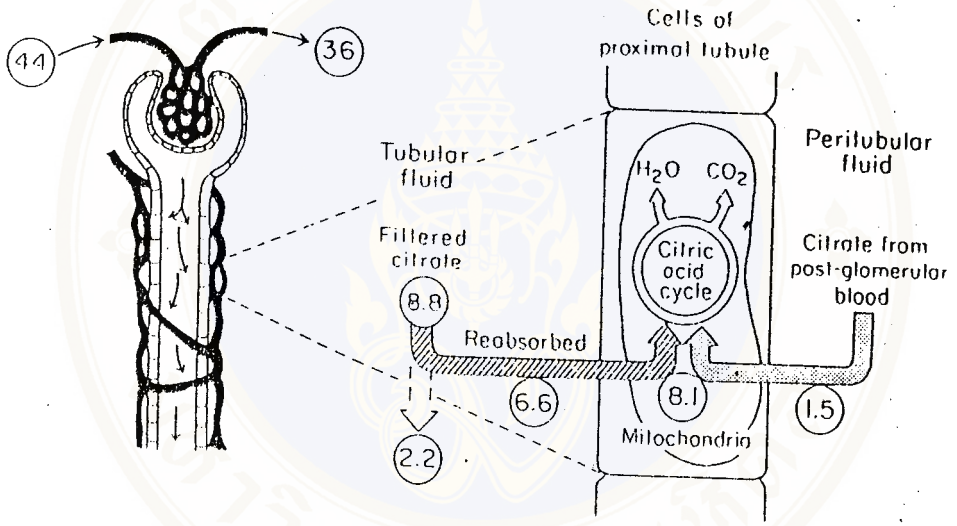


FIG.1. Diagram of characteristics of citrate handling by nephron. Circled numbers are representative of values obtained in humans in micromoles per minute. Citrate is removed from both tubular fluid and postglomerular blood and is metabolized in in tubular cells. Data are from Nieth and Schollmeyer (21).

CAUSES OF ALTERED CITRATE EXCRETION

1.VARIATIONS IN ACID-BASE BALANCE

As early as 1931 metabolic alkalosis was noted to increase citrate excretion in humans, an observation subsequently confirmed in other mammalian species. In humans, as as much as 60% of filtered citrate may appear in the urine urine during alkalosis (23).

The already low levels of citrate in the urine of most normal experimental animals make it difficult to measure decrease in citrate excretion as reliably as increases. Consequently, the effect of acidosis on citrate excretionhas not been studied as extensively as the effect of alkalosis. Nevertheless, the studies that have been done show that metabolic acidosis causes an acute in citrate excretion. In humans, within a few hours after ammonium chrolide administration, citrate excretion fell by 20-50% (24). These changes in citrate clearance induced by alkalosis or acidosis are accompanied by concomitant changes in citrate concentration in the renal cortex. Alter et al (25), shows that acute metabolic alkalosis incresed citrate concentration by over twofold and an acute acidosis reduced it by over half.

As noted previously, under normal acid-base conditions net peritubular uptake of citrate occurs. Data on the effect of alkalosis or acidosis on peritubular citrate are not available except during citrate infusion.

Under these conditions, Baruch et al (26) found that in the dog during sodium bicarbonate administration the arteriovenous difference for citrate became slightly negative, indicating peritubular release rather than uptake of citrate. A small net renal utilization of citrate was still present. This result indicates that it is likely that with normal plasma citrate levels metabolic alkalosis inhibits peritubular uptake as well as reabsorption of citrate, although more data are need on citrate utilization at normal plasma citrate concentration in both acidosis and alkalosis.

Acetazolamide causes the excretion of an alkaline urine and, systemically, a metabolic acidosis. The excretion of citrate after acetazolamide administration rises slightly after the first hour and then falls sharply to level as low as half of the levels in the control period (24). The persistent hypocitraturia produced by acetazolamide suggests that the cells of the proximal tubule are responding to the systemic metabolic acidosis with a fall in cytoplasmic pH. Further evidence that intracellular pH becomes acidotic after acetazolamide administration is the finding that citrate levels as well as citrate excretion fall after this agent is given (27). The effect of acetazolamide in diminishing citrate excretion can be prevented by concomitant administration of sodium bicarbonate, citrate excretion increasing steadily as plasma bicarbonate concentration increases, just as it does in the absence of acetazolamide (28). Thus, the effect of acetazolamide appears related to

the system and intracellular acidosis produced by this drug rather than to a direct effect of this agent on citrate reabsorption or metabolism or to a response of citrate reabsorption to the alkaline tubular fluid.

Another condition in which citrate excretion is probably altered by intracellular acid-base changes is potassium depletion. There is much evidence suggesting that potassium depletion causes an intracellular metabolic acidosis in the renal cortex. In keeping with this evidence, potassium depletioncauses a fall in citrate excretion and a decrease in citrate concentration in the renal cortex (29).

2.ORGANIC ACIDS

The influence of citric acid cycle compounds other than citrate on renal citrate handling, like the effect of metabolic alkalosis, was first noted many years ago (30) and subsequently was the subject of many investigations. Infusion of malate, succinate or fumarate causes an increased excretion of citrate (31,32). The general effects on citrate metabolism of these compounds resemble in many, but not all, respects those produced by metabolic alkalosis. Malate infusion, for example, results in decreased citrate reabsorption, decreased peritubular and total renal citrate uptake, and increased cortical citrate concentration. Indeed, malate and succinate infusion may have a more profound effect than metabolic alkalosis and can cause

citrate excretion to approximate or to exceed the filtered load (32). In one study, citrate clearence during malate infusion exceeded the filtered load by 25%, indicating citrate secretion by the nephron (22).

Studies with labeled citrate precursors have also demonstrated that during infusion of these precursors some of the citrate in the urine is derived from intrarenal synthesis (33). Runeberg and Lotspeich (34) infused [^{14}C]succinate into one renalartery of a dog and compared [^{14}C]citrate excretion by the two kidneys. The specific activity of citrate in the urine from the kidney receiving the infusion of leveled precursor was up to 6 times grater than in the urine from the other kidney, thus demonstrating intrarenal synthesis and secretion of citrate. However, the total contribution of citrate derived from labled succinate was small, representing less than 15% of the excreted citrate, so that most of the citrate in the urine still was derived from filtered citrate.

These studies indicate that when metabolic precursors of citrate are infused, citrate synthesis by the kidney from these compounds increases. A small amount of the synthesized citrate escapes into the urine, but the predominant effect of the increased intracellular citrate levels is to inhibit tubular and peritubular uptake of citrate. When enough precursor is infused, citrate reabsorption may be completely blocked and synthesized citrate may be added to the tubular fluid, so that citrate

excretion exceeds the filtered load.

3.METABOLIC INHIBITORS

Administration of inhibitors of the citric acid cycle may also result in increased citrate excretion, presumably by blocking intrarenal citrate metabolism, increasing intracellular citrate levels, and blocking citrate reabsorption. Malonate, which inhibits succinic dehydrogenase, shows this type of effect (32). Maleate also increases citrate clearance (35). Resent evidence suggests that this compound forms a relatively inert CoA derivative in renal tissue, thus reducing the availability of CoA for normal metabolic reactions (36). Whether or not the effect of maleate on citrate excretion is due to this mechanism has not yet been established.

As might be anticipated, fluoroacitate and fluorocitrate, potent inhibitors of citrate metabolism, greatly alter renal citrate handling. However, a striking and unexplained difference in the effects of these two compounds has been reported. In a study by Gordon (37), administration of fluoroacetate resulted in a 30-fold increase in the level of citrate in the kidney; despite this change, plus a three fold increase infiltered load of citrate, citrate excretion did not change . In contrast, fluorocitrate caused only a sixfold increase in tissue citrate but a fourfold increase in citrate excretion. The failure of fluoroacetate to increase citrate excretion, the

TABLE 1. Conditions affecting citrate excretion

Group	↑ Citrate Excretion	↓ Citrate Excretion
Acid–base changes	alkalosis	acidosis K depletion acetazolamide volume expansion starvation
Organic acids	malate succinate fumarate	
Metabolic inhibitors	fluorocitrate fluoroacetate malonate maleate	
Miscellaneous	calcitonin lithium vitamin D calcium magnesium	heat ethacrynic acid menstual period calcium

ability of fluorocitrate to do so , and the increase in tissue citrate levels in the rat caused by both agents have been confirmed in other studies (38,39).

4. MISCELLANEOUS

Table 1 summerizes the different conditions and agents known to influence citrate excretion. Besides the effects of acid-base changes and of the organic acids and of metabolic inhibitors alresdy discussed, a variety of other substances has been shown to increase or decrease citrate excretion. In general, little is known about the effects of these substances on overall renal citrate handling or about possible mechanisms underlying their action. With vitamin D, however, there is evidence for a metabolic action responsible for its effect on citrate excretion. Administration of vitamin D to normal or rachitogenic rats increases citrate excretion and renal tissue levels of citrate (40). Pursuing this lead, it was found that vitamin D treatment of intact animals inhibited citrate oxidation by homogenates and mitochondria prepared from these animals (41). It was also found that addition of vitamin D directly to the mediuminhibited citrate oxidation by kidney mitochondria (42). Thus the effect of vitamin D on citrate excretion appears to arise from inhibition by this agent of mitochondrial citrate metabolism in the kidney.

Detection and quantification of citric acid cycle intermediates is of fundamental biological interest. Important metabolites of carbohydrate, amino acid, and lipid metabolism are involved, so that separation and measurement is important in many metabolic studies and in the assessment of food quality. The structural similarities among the citric cycle intermediates and their lack of distinctive spectral properties have, however , conspired to make quantification and separation most difficult.

The purpose of the present study was to establish a specific and stable method for the determination of carboxylic acids by combining the above esterification with high-performance liquid chromatography, to obtain additional data on the urinary excretion of citrate and other TCA cycle intermediates in patients with renal tubular acidosis and renal stone. For purposes of comparison we have studied normal subjects, from Bangkok and from the northeast region, and patients with acidification defect and with renal stone.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

REAGENTS

Name	Supplier
Acetone	BDH Chemicals Ltd. (England)
Citric acid (Trisodium salt dihydrate)	SIGMA (USA)
Dicyclohexano-18-crown-6	SIGMA (USA)
Fumaric acid (Disodium salt)	SIGMA (USA)
Helium gas	Thai industrial gases limited
DL-Isocitric acid lactone	SIGMA (USA)
-Ketoglutaric acid	SIGMA (USA)
L-Malic acid	SIGMA (USA)
Methanol (HPLC)	MAY&BAKER (England)
Methylmalonic acid	SIGMA (USA)
Oxalacetic acid	SIGMA (USA)
Phenacyl bromide (2-Bromoacetophenone)	SIGMA (USA)
Potassium hydroxide	MERCK (Germany)
Potassium dihydrogen phosphate	Bakers Analyzed (USA)
Sodium hydrogen phosphate	MERCK (Germany)
Succinic acid	SIGMA (USA)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

INSTRUMENT

Name	Manufacturer
1. Model CM 4000 multiple solvent delivery system	LDC/Milton Roy, FL(USA)
2. Model A 1000 auto Injector	LDC/Milton Roy, FL(USA)
3. Spectro Monitor 3000 variable wavelength detector	LDC/Milton Roy, FL(USA)
4. CI-10B integrator	LDC/Milton Roy, FL(USA)

COLUMNS

Name	Manufacturer
1. Spherisorb S5 ODS 2 (P/N 415141505, 250x4.6 mm, particle size 10 um)	LDC/Milton Roy, FL(USA)
2. Uptight precolumn filter	Upchurch scientific,INC.(USA)

CHROMATOGRAPHIC CONDITIONS

Mobile phase : Freshly triple distilled water: Methanol (35:65)

Flow rate : 1.0 ml per minute

Injection volume : 5 ul

Detector : UV set at 254 nm

Integrator

Attenuation : 6

Chart speed : 5 mm per minute

METHODS

Normal subjects

Normal 24 hours urine samples were obtained from two groups of healthy subjects. First, 30 healthy medical students, 15 males, and 15 female ages ranged from 19 to 20 years. All were proved to be healthy on the basis of clinical assessment and biochemical screening. Another group, 15 healthy subjects from Khonkaen, 6 males and 9 females.

Patients

Two groups of villagers were stuied, the first group had acidification defect as assessed from NH_4Cl loading test. Patients with urinary tract infection, and systemic diseases known to cause dRTA, as well as cases with serum creatinine over 2.0 mg/dl were excluded. Patients in this group comprised of 15 cases, 7 males and 8 females. Another group had renal stone, comprised of 15 cases, 8 males and 7 females.

ESTERIFICATION PROCEDURE

Preparation of buffer solution

A 0.08 M phosphate buffer, pH 6.8, was prepared by dissolving 5.44 g KH_2PO_4 and 14.3 g $Na_2HPO_4.12H_2O$ in 1 L of deionized water. To this solution, methylmalonic acid, used as internal standard, was added to give a concentration of 1 g/l.

Preparation of esterification agent

The esterification agent was prepared by dissolving in acetone both phenacyl bromide and dicyclohexano-18-crown-6 to give concentrations of 30 and 1.5 g/l, respectively.

Preparation of working standards

Citrate standard solution (1mmol) :

Dissolve 29.4 mg trisodium citrate.$2H_2O$ in 1 ml distilled water, dilute an aliquot 1:100 with distilled water.

Fumarate standard solution (1mmol) :

Dissolve 16.2 mg disodium fumarate in 1 ml distilled water (0.1 M stock solution, store frozen).

Just before use dilute 1:100 with distilled water.

Isocitrate standard solution (1mmol) :

Dissolve 26 mg isocitrate in 1 ml distilled water.

Dilute an aliquot 1:100 with distilled water.

-Ketoglutarate standard solution (1mmol) :

Dissolve 14.6 mg -ketoglutaric acid in 1 ml distilled water (freeze this 0.1 M stock solution; the free acid is more stable at -20 C than its salts). Before use dilute an aliquot 1:100 with distilled water.

L-Malate standard solution (1mmol) :

Dissolve 13.4 mg L-malic acid in 1 ml distilled water (0.1 M stock solution, store frozen). Just before use dilute 1:100 with distilled water.

Oxalacetate standard solution (1mmol) :

Dissolve 13.2 mg oxalacetic acid in 1 ml of distilled water (store stock solution at -80 C); just before use dilute 1:100 with distilled water.

Succinate standard solution (10mmol) :

Dissolve 11.8 mg succinic acid in water, adjust pH to 7 with 1 N KOH, and make up to 10 ml with water.

PROCEDURE

In a 18 ml pyrex test tube with a screwcap, 100 ul of a neutralized urine sample or of the working standard were mixed with 400 ul of the phosphate buffer solution, 750 ul of phenacyl bromide solution and 750 ul of dicyclohexano-18-crown-6 solution. Place the test tube in a boiling water-bath for 40 min, then cool. The cooled solution is ready for the chromatographic analysis. No other pre-treatment or clean-up was necessary. The solutions, after derivatization, were stable for several weeks.

STANDARD CURVE

A series of standard solutions of citrate, fumarate, isocitrate, α-ketoglutarate, L-malate, oxalacetate and succinate was prepared to give final concentrations of 0.02, 0.04, 0.06, 0.08, and 0.10 mmol for the first standard curve and 0.25, 0.50, 1.00, and 2.00 mmol for another. They were analyzed as described under assay procedure. The chromatograms of these analytes were calculated as the peak area ratio of each analyte to methylmalonic acid and plotted against each analyte concentration for preparation of standard curves as shown in figure .

RECOVERY STUDY

The analytical recovery of the acids of interest was evaluated by adding known amounts of them to ten different urine samples and reassaying (TABLE 4).

PRECISION DETERMINATION

To evaluate the precision of the method, a urine specimen was analyzed ten times. The results of within-run precision are reported in TABLE 5. The between-run precision of this method was evaluated from the standard mixture of intermediates which was used for calibration by internal standard method in each analysis. The results of between-run precision are reported in TABLE .

STATISTICAL ANALYSIS

7

Data on the intermediates levels were expressed as mean value ± standard deviation. Differences between means were assessed by the one-way analysis of variance (ANOVA) and the linear regression analysis.

CHAPTER III

RESULTS

Krebs cycle, tricarboxylic acid (TCA) cycle or citric acid cycle intermediates are carboxylic acid. The establishment of high-performance liquid chromatography (HPLC) conditions was accomplished by examining the retention characteristics of tricarboxylic acids on reversed-phase columns. Optimal resolution of all components from each others and from phenacyl ester derivatizes of urine was accomplished using a 25-cm column of spherisorb ODS utilizing a mobile phase of methanol and water (65:35 v/v). Figure 2 shows a typical chromatogram obtained from a standard mixture of the phynacyl ester derivative and internal standard, methylmalonic acid. No peaks of alpha ketoglutarate and oxalacetate were detected in this chromatogram, and it was noticed that these intermediates had a keto group in their structure. Figure 3 shows the chromatogram of a urine sample, derivatized according to the above procedure. No interfering peaks from urine esterification components were detected in control sample. Comparative retention times for tricarboxylic acids are presented in Table 2. It was noticed that with standard HPLC elution conditions, dicarboxylic acids elute first and tricarboxylic acids elute last. Maximum sensitivity ranges from 1000 umol of isocitrate to 1 umol of fumarate at a signal-to-noise ratio of 2:1 (Table 3).

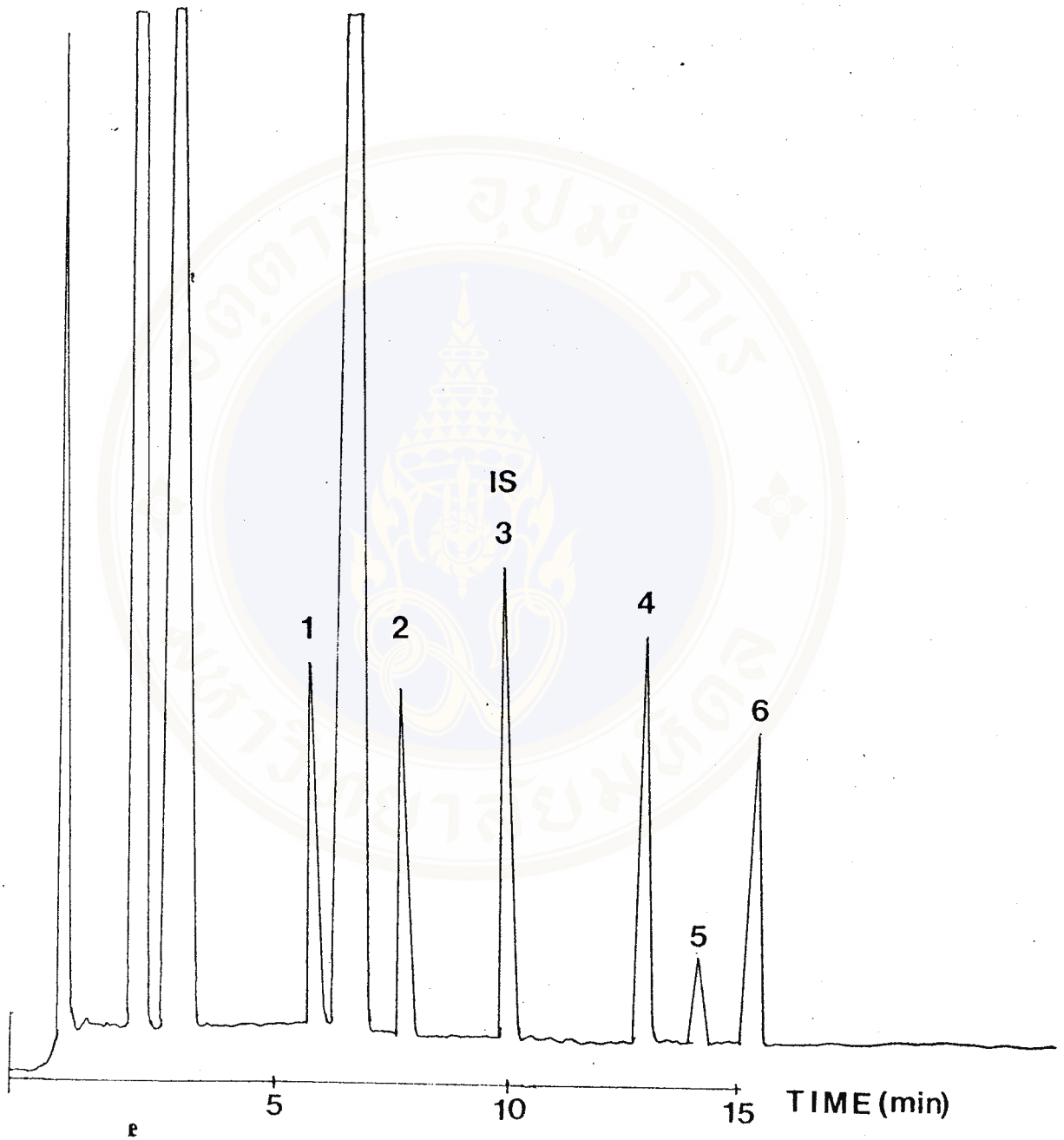


FIG. 2 HPLC separation of standard carboxylic acids (1 mM) according to the described procedure. Peaks:1=malate; 2=succinate; 3=methylmalonic acid (Internal Standard,IS) 4=fumarate; 5=isocitrate; 6=citrate.

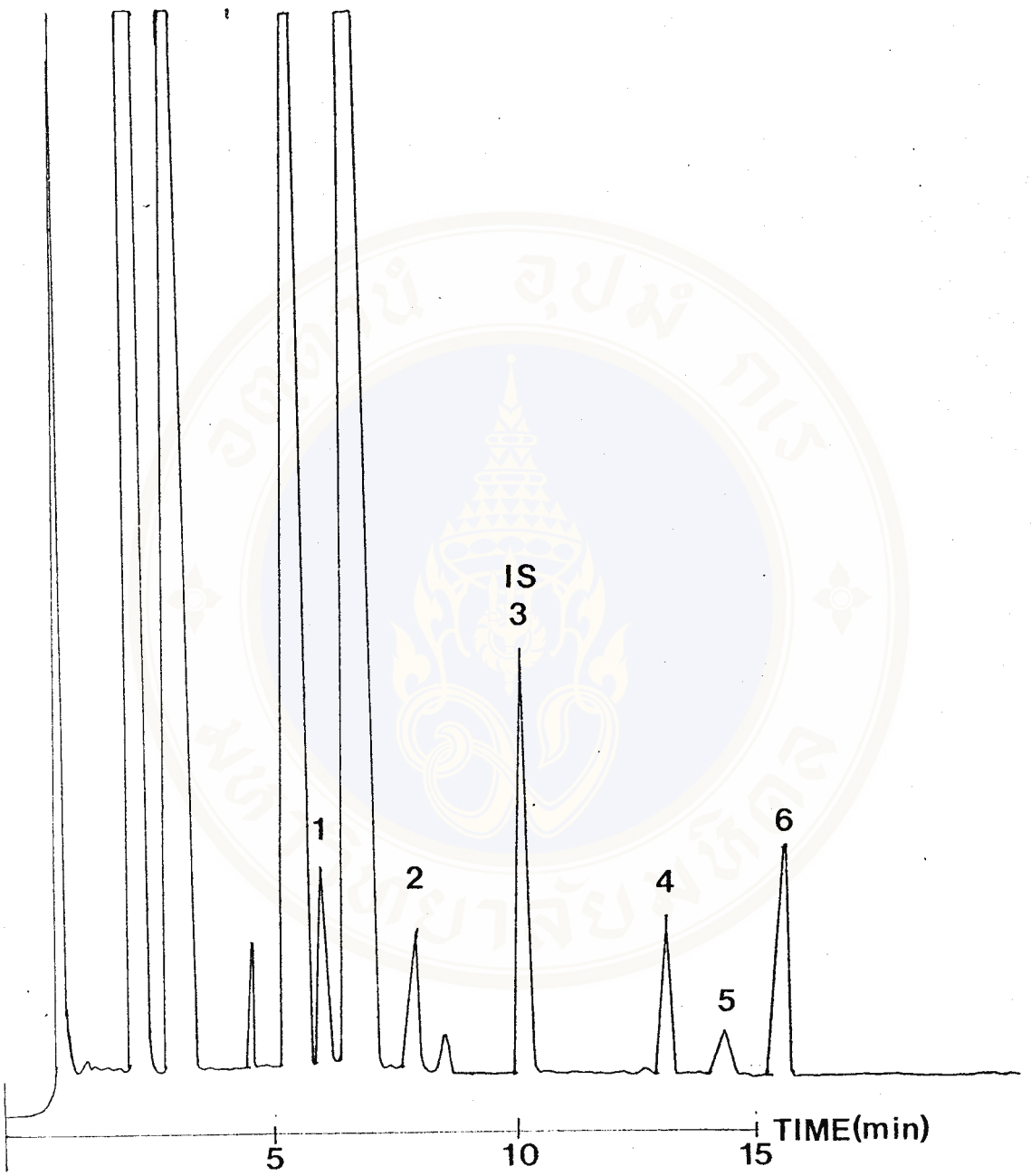


FIG.3 Chromatogram of a urine sample. Peaks:1=malate; 2=succinate; 3=methylmalonic acid (Internal Standard,IS) 4=fumarate; 5=isocitrate; 6=citrate.

TABLE 2 RETENTION TIMES OF THE INVESTIGATED ACIDS DERIVATIZED ACCORDING TO THE DESCRIBED PROCEDURE

Analyte	t_R (min)
Malate	5.28
Succinate	7.79
Methylmalonate	9.38
Fumarate	11.47
Isocitrate	13.63
Citrate	15.59

TABLE 3 DETECTION LIMITS OF CITRIC ACID CYCLE INTERMEDIATES

Signal-to-noise ratio is 2.

Analyte	Detection limit (umol)
Malate	20
Succinate	40
Fumarate	1
Isocitrate	1000
Citrate	60

Linear calibration curves of the peak area ratio for TCA cycle intermediates/internal standard versus the amount of each intermediates, were constructed. In order to cover a large (0.02-2 mmol) concentration range , without sacrificing accuracy or precision, it was advisable to use two standard curves for all of intermediates, except for isocitrate which had only one because of it high sensitivity. The lower-end standard curve was designed for 0.02-0.1 mmol concentration range and the higher end standard curve for 0.25-2 mmol. First standard curve consisted of five points and second standard one (0.25-2 mmol) consisted of four points (Figure 4-8). Each standard curve covered the anticipated assay range.

The analytical recovery of the acids of interest was evaluated by adding known amounts of them to ten different urine samples and reassaying. The good agreement between the results for the amounts added and the values found (see Table 4) indicates a very low matrix effect of urine on the measurements performed.

To evaluate the precision of the method, a urine specimen was analyzed ten times. The results of within-run precision are reported in Table 5. The between-run precision for stantard mixture of intermediates are reported in Table 6 .

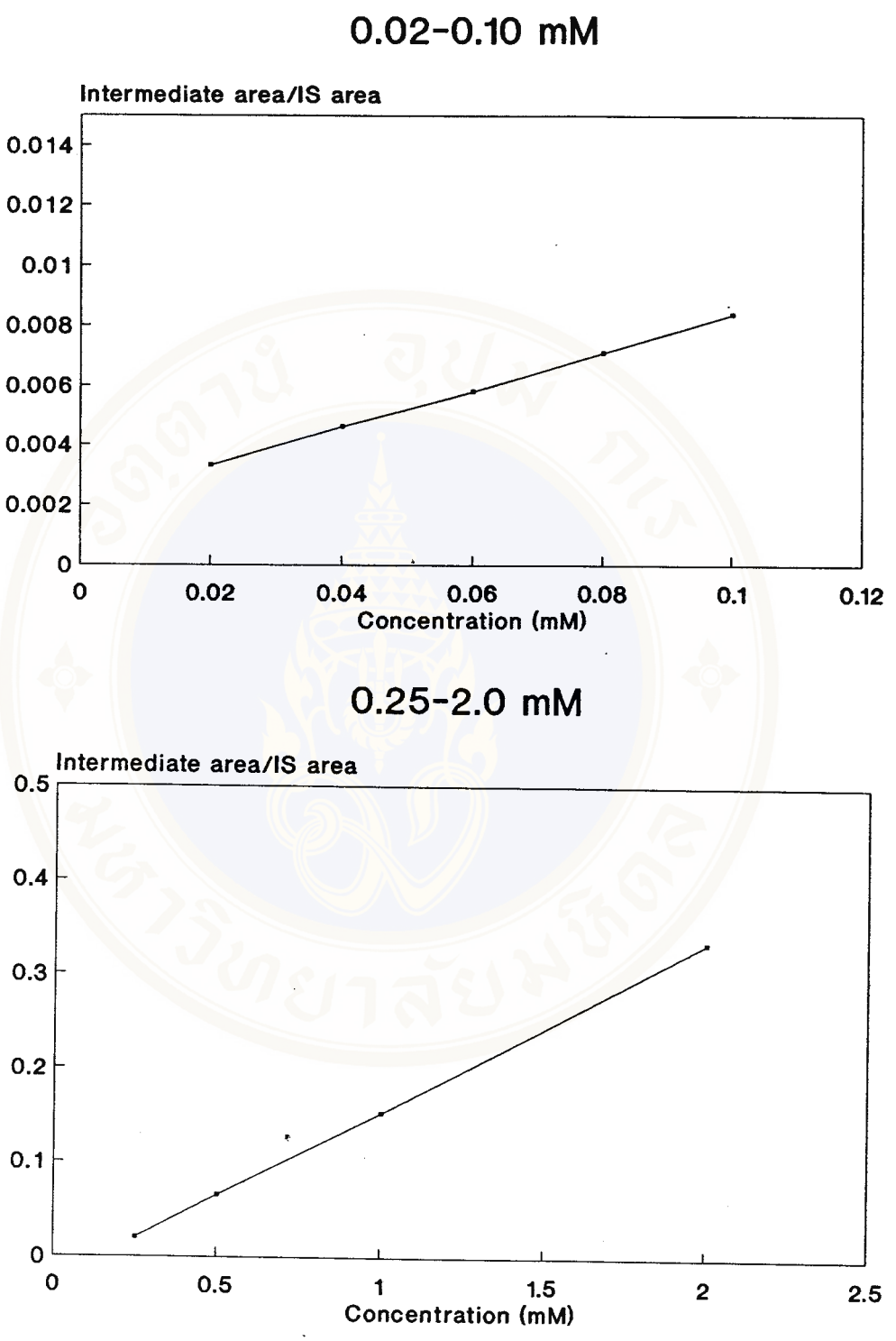
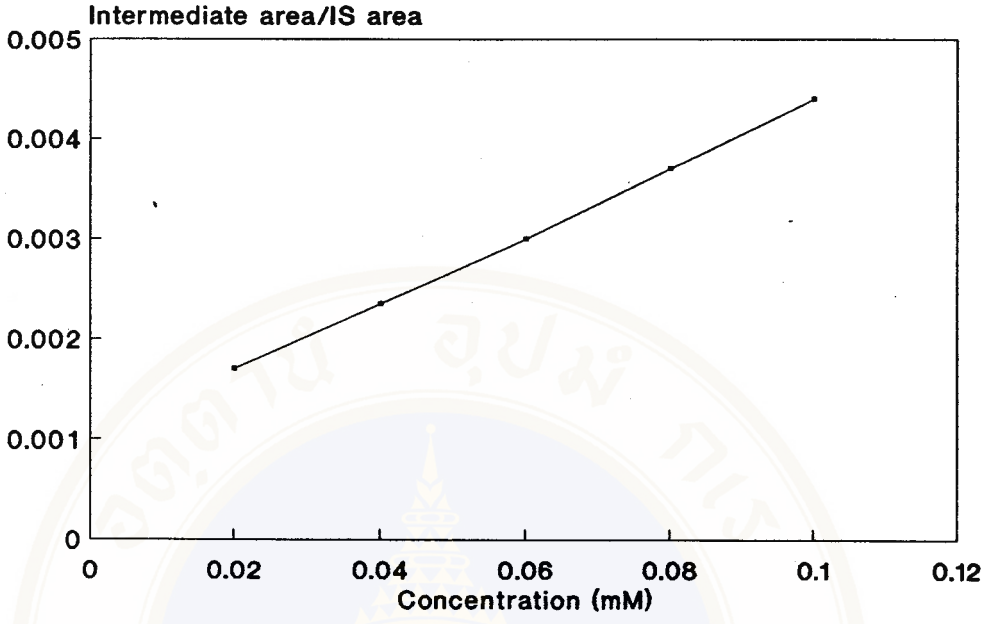


FIG.4 Standard curve of malate

0.02-0.10 mM



0.25-2.0 mM

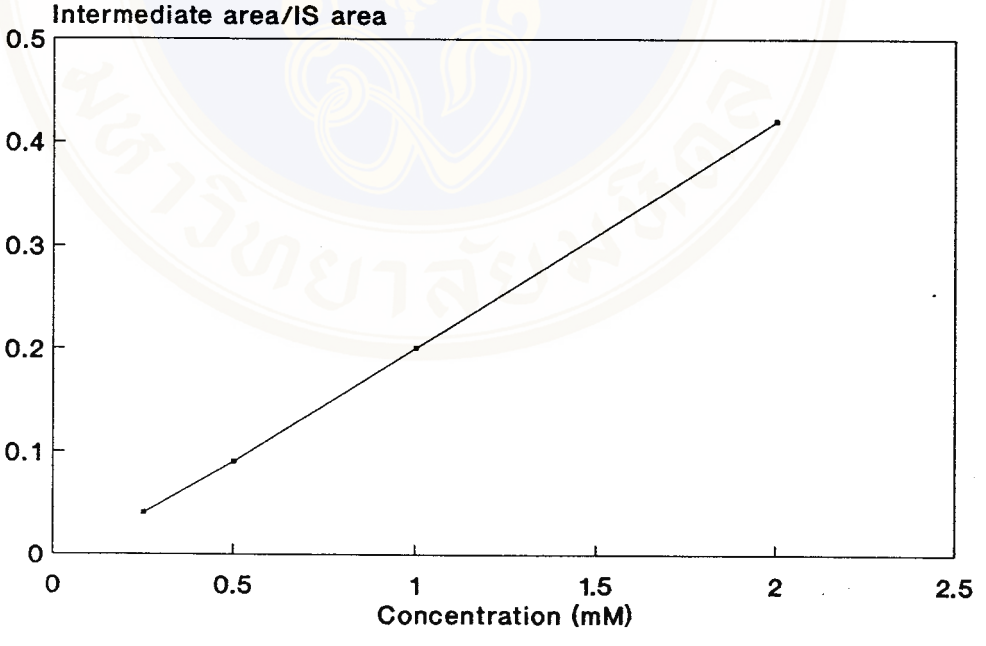


FIG.5 Standard curve of succinate

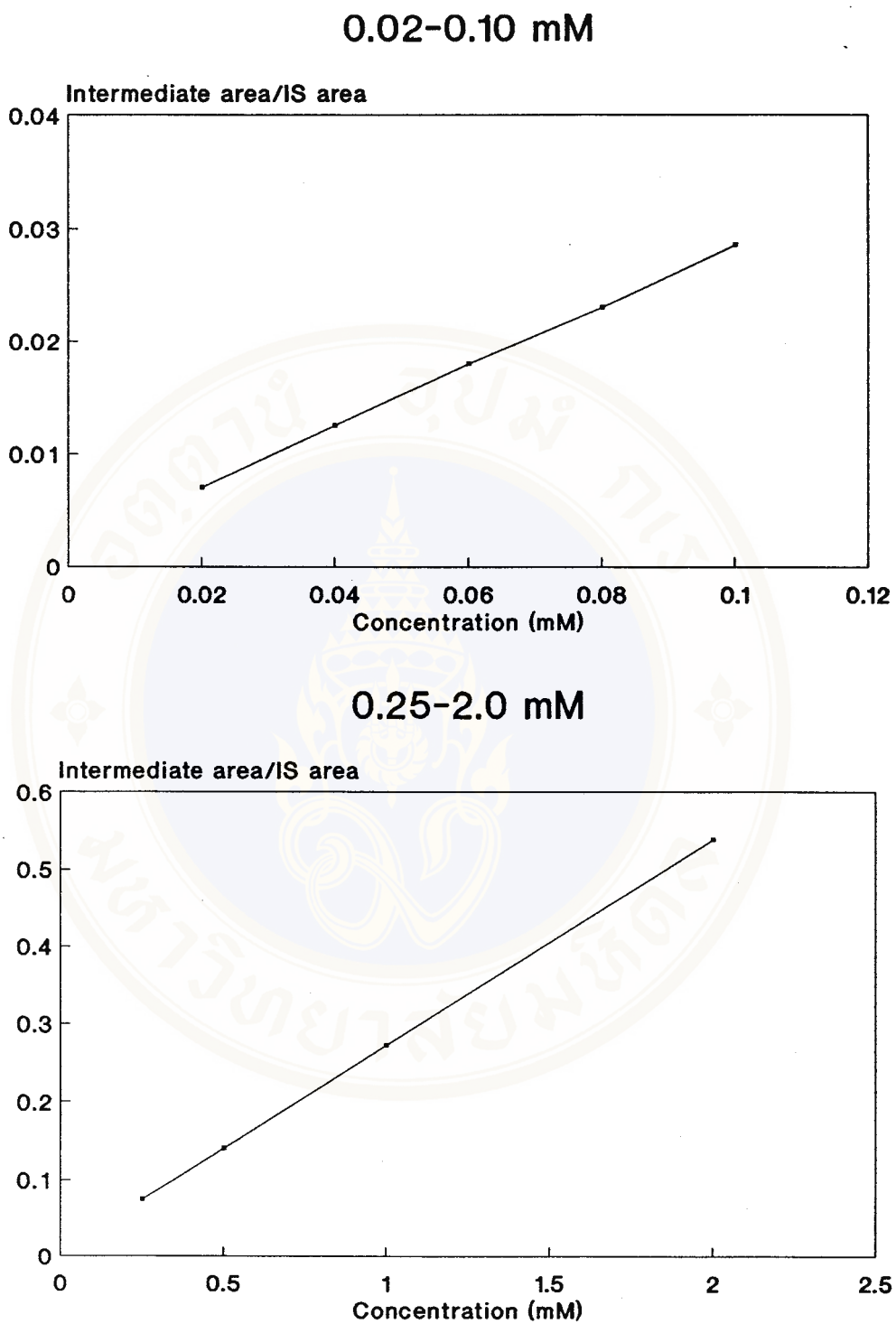


FIG.6 Standard curve of fumarate

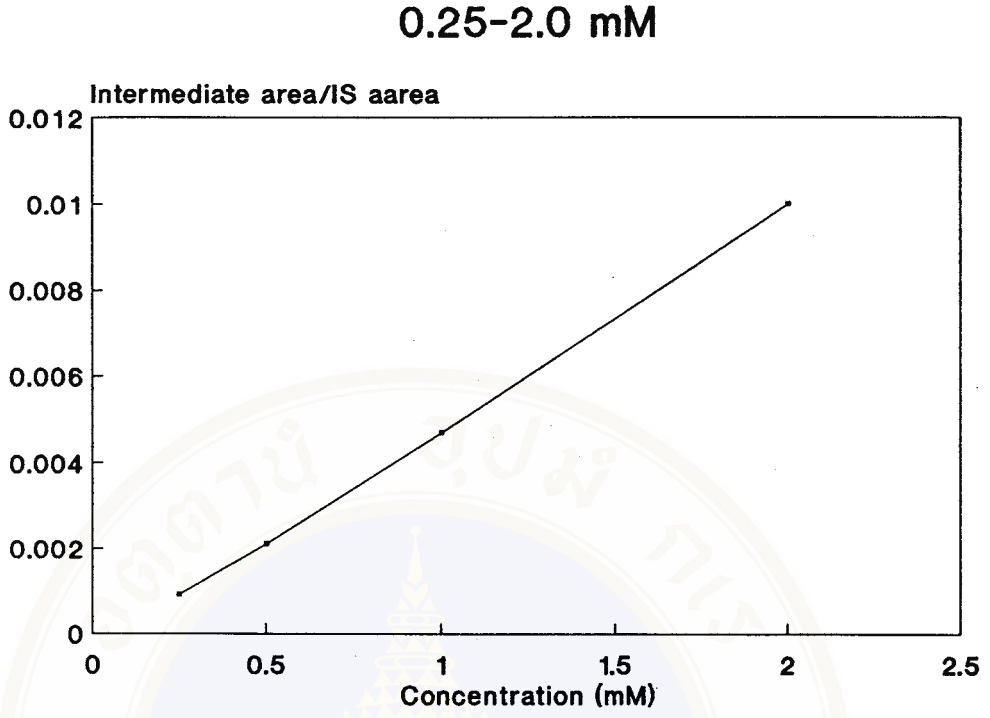


FIG.7 Standard curve of isocitrate

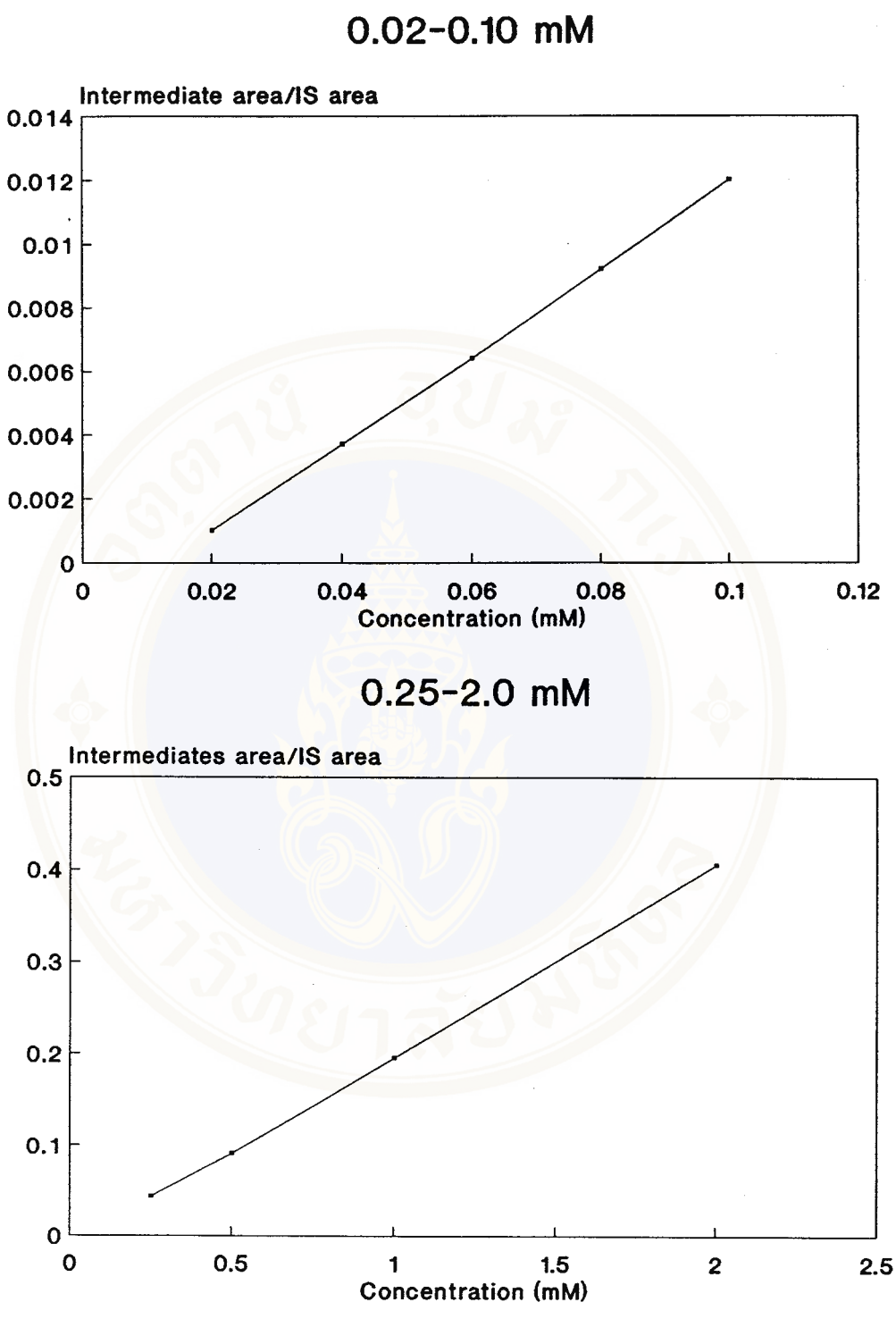


FIG.8 Standard curve of citrate

TABLE 4 MEAN RECOVERY OF INTERMEDIATES FROM URINE

Analyte	Concentration added (mmol)	Found±S.D. (n=10)	Recovery(%) and range
Malate	1.00	0.91±0.047	91.87(85.60-99.50)
Succinate	1.00	0.95±0.055	95.50(87.00-100.62)
Fumarate	1.00	0.98±0.041	98.56(92.20-100.62)
Isocitrate	1.00	0.93±0.058	93.27(82.40-99.50)
Citrate	1.00	1.02±0.087	100.26(91.30-115.50)

TABLE 5 WITHIN-RUN PRECISION FOR INTERMEDIATES IN A URINE SAMPLE

Analyte	Intermediate area/ IS area (Mean ± S.D.) n = 10	C.V.%
Malate	0.002 ±0.000	2.4
Succinate	0.046 ±0.001	2.6
Fumarate	0.034 ±0.000	2.7
Isocitrate	0.011 ±0.000	3.3
Citrate	0.255 ±0.003	1.3

TABLE 6 BETWEEN-RUN PRECISION FOR STANDARD MIXTURE OF INTERMEDIATES

Analyte	Intermediate Area/IS Area (MEAN±S.D.) (n=10)	C.V.%
Malate	0.106 ±0.006	6.26
Succinate	0.118 ±0.008	7.26
Fumarate	0.192 ±0.011	5.79
Isocitrate	0.006 ±0.000	5.00
Citrate	0.117 ±0.007	6.16

The extent of background interference from impurities in the reagents and by-products of the reaction was evaluated by heating the reaction mixture in the absence of acids. The corresponding chromatographic profile showed a series of small peaks appearing in the vicinity of those for malic.

Figure 9 illustrate the relationship between two methods in citrate analysis, enzymatic method (44) and high-performance liquid chromatographic method, there was a highly significant correlation ($p<0.001$, $r=0.755$).

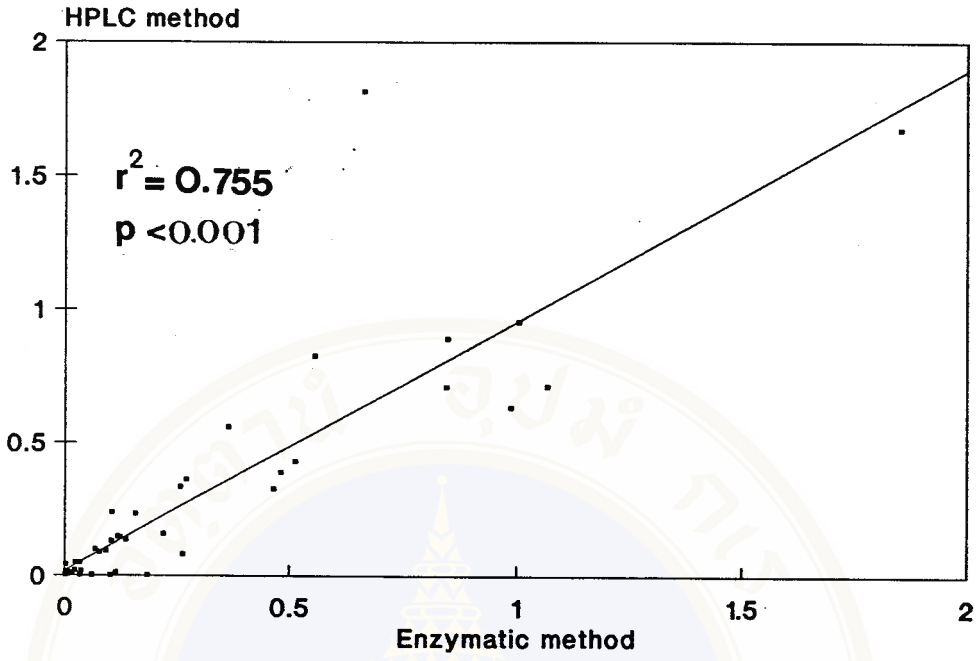


FIG.9 Correlation between enzymatic (44) and HPLC method in citrate analysis

The urinary citric acid cycle intermediates excretion in four study groups; normal in Bangkok, normal in Northeast region, acidification defect, and renal stone was compared as shown in figure 10-14. Urinary malate excretion of normal from Bangkok, 0.21±0.29 mM, shows sligthly higher but not significantly different than in any other group; 0.17± 0.08, 0.11±0.09, and 0.09±0.07 mM for normal from northeast region, acidification defect, and renal stone, respectively, as show in Figure 10. Urinary succinate excretion shows the same pattern of excretion like malate, that normal from Bangkok, 0.49±0.21 mM, was significantly higher ($p<0.001$) than in any other group; 0.27±0.08, 0.24±0.06, and 0.25±0.13 mM for normal from northeast region, acidification defect, and renal stone, respectively, as shown in Figure 11. Urinary fumarate excretion shows no significantly different at the 0.05 level between four study groups (Figure 12); 0.09±0.07, 0.08±0.04, 0.13±0.10, and 0.13±0.16 mM for normal from Bangkok and northeast, acidification defect, and renal stone, respectively. Urinary isocitrate excretion between two groups, 0.63±0.40 mM for normal from Bangkok and 0.57±0.31 mM for acidification defect at the 0.05 level (Figure 13). Finally, Urinary citrate excretion between normal from Bangkok, 0.48±0.35 mM, and normal from northeast, 0.51±0.48 mM show no significantly different, but both two normal groups show significantly higher ($p<0.002$) than both two patient groups; 0.10±0.19 and 0.20±0.32 mM for acidification defect and renal stone, and no significantly difference between two patient groups.

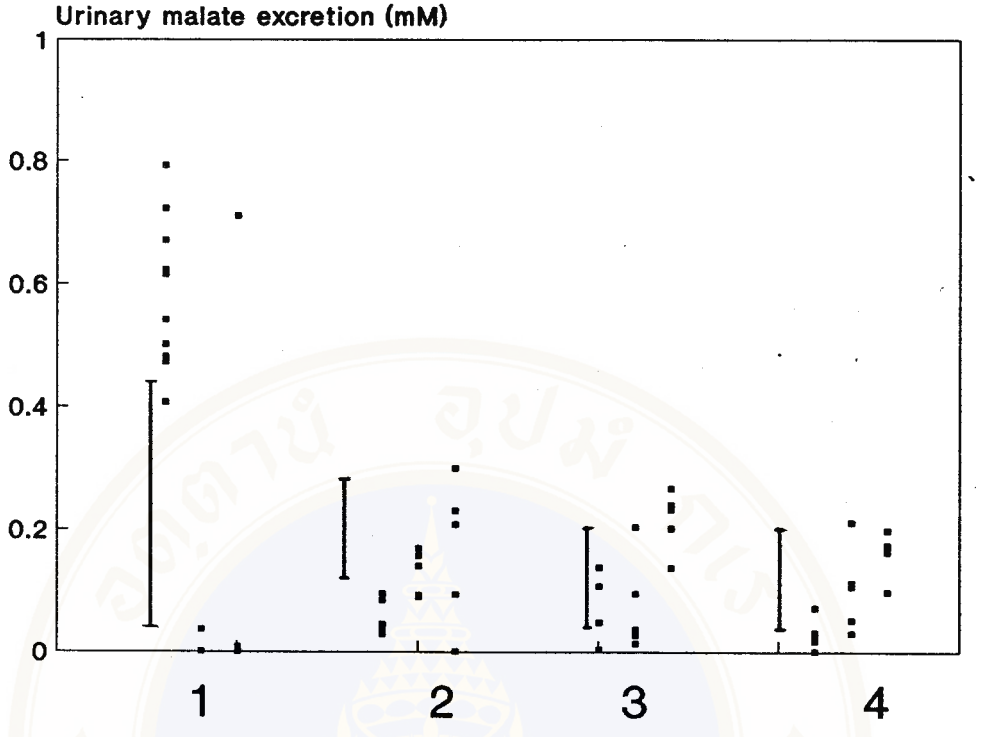


FIG. 10 Urinary malate excretion of normal from Bangkok, normal from northeast of Thailand, acidification defect and renal stone

1 = Normal from Bangkok (n=30)
2 = Normal from northeast of Thailand (n=15)
3 = Acidification defect (n=15)
4 = Renal stone (n=15)

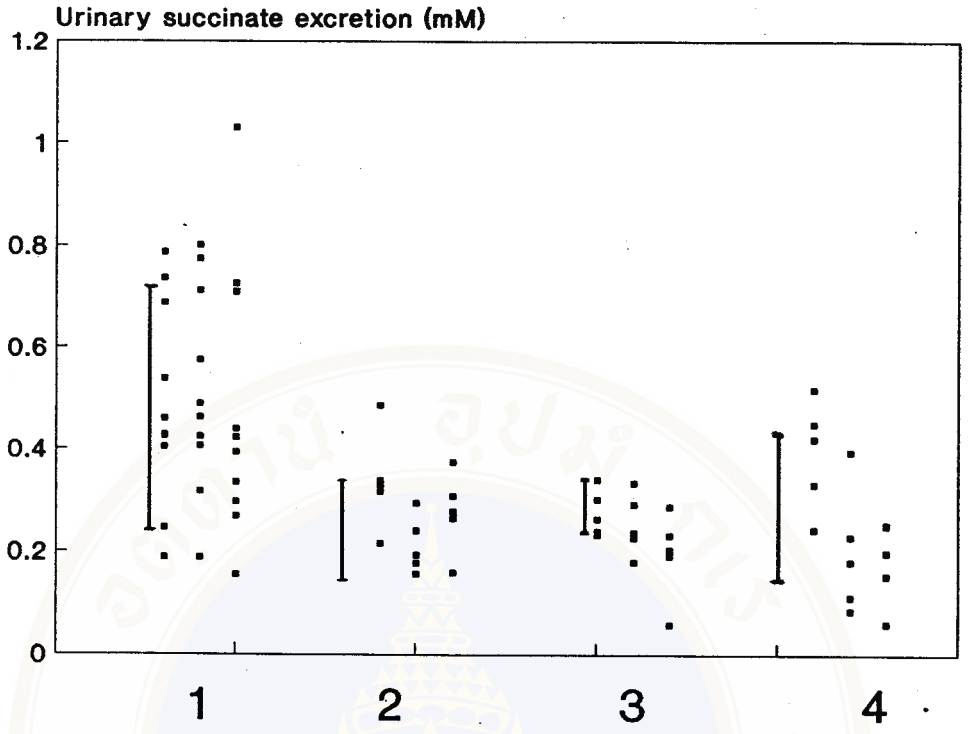


FIG. 11 Urinary succinate excretion of normal from Bangkok, normal from northeast of Thailand, acidification defect and renal stone

1 = Normal from Bangkok (n=30)
2 = Normal from northeast of Thailand (n=15)
3 = Acidification defect (n=15)
4 = Renal stone (n=15)

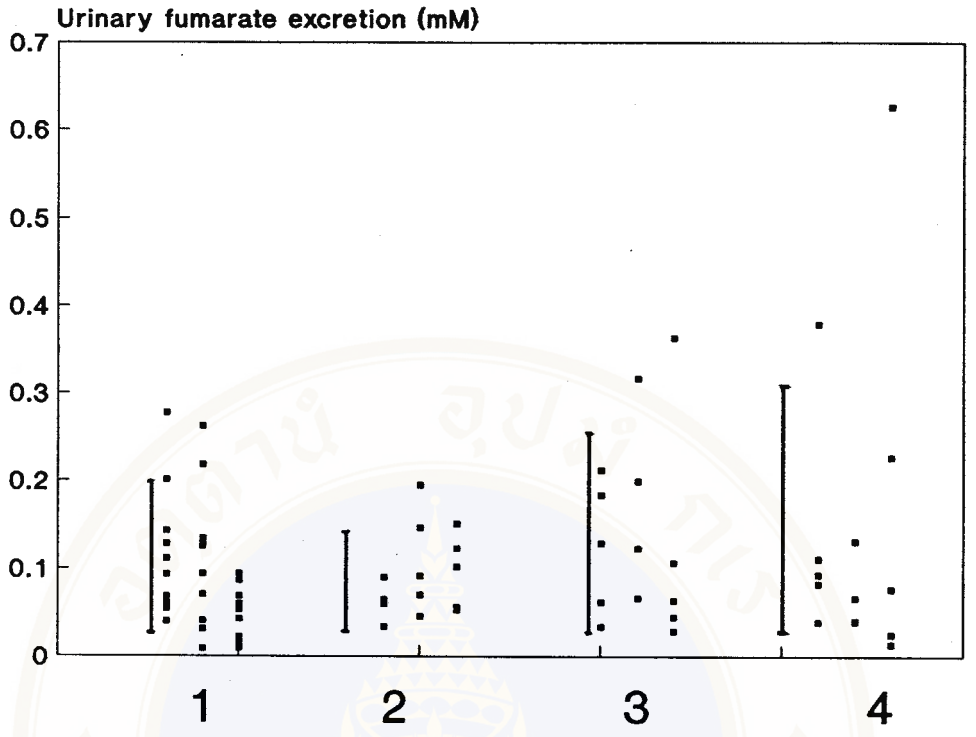


FIG. 12 Urinary fumarate excretion of normal from Bangkok, normal from northeast of Thailand, acidification defect and renal stone

1 = Normal from Bangkok (n=30)
2 = Normal from northeast of Thailand (n=15)
3 = Acidification defect (n=15)
4 = Renal stone (n=15)

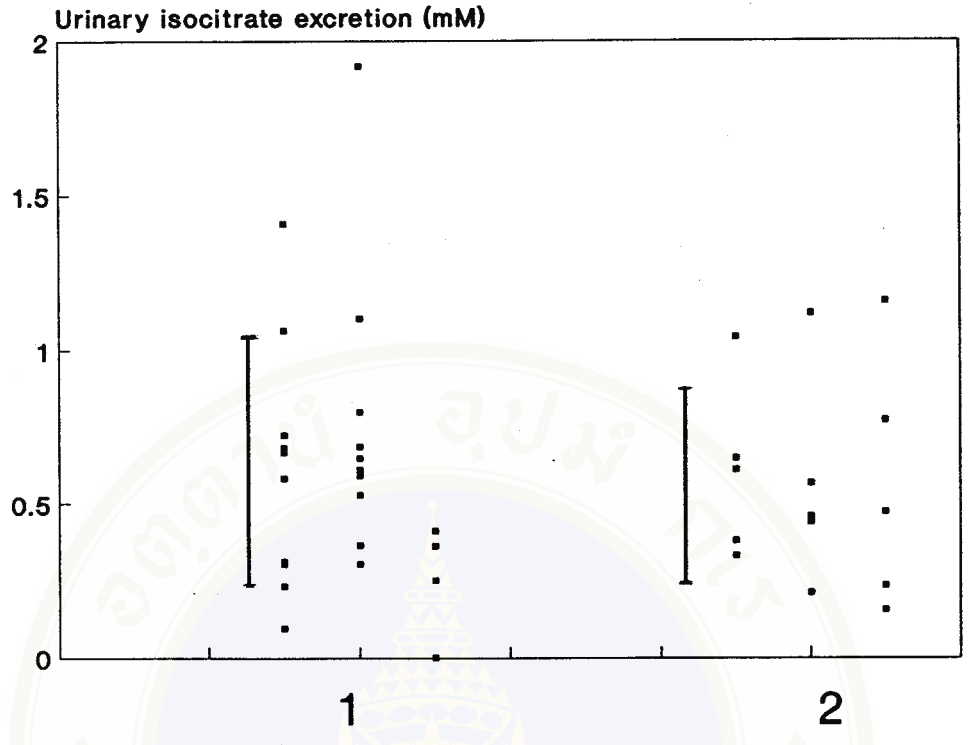


FIG. 13 Urinary isocitrate excretion of normal from Bangkok, and acidification defect

1 = Normal from Bangkok (n=30)

2 = Acidification defect (n=15)

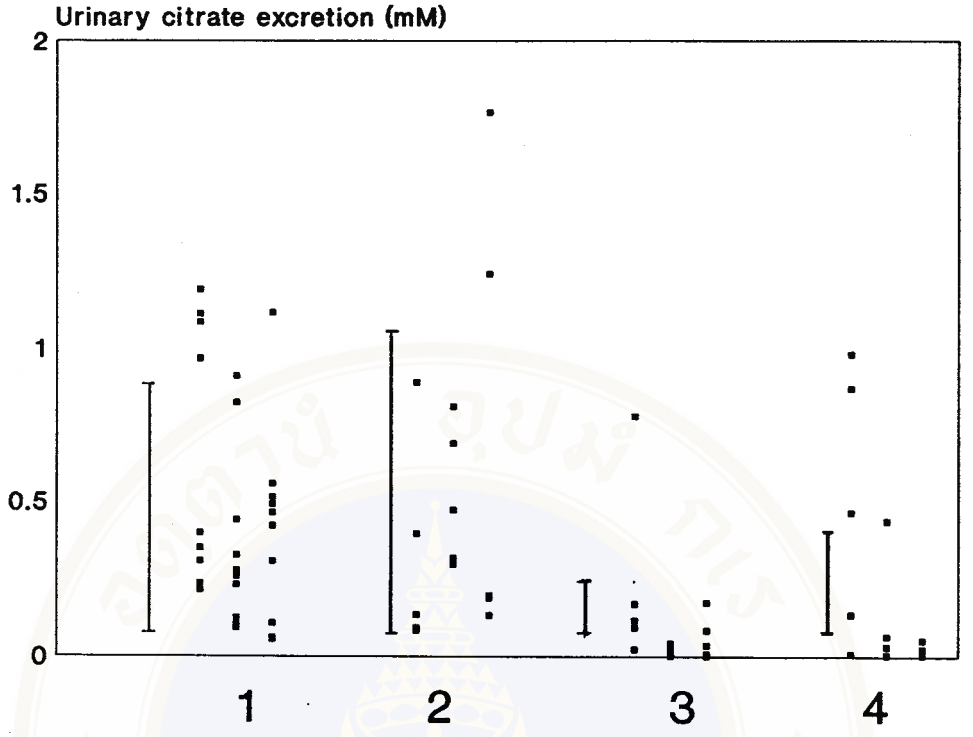


FIG. 14 Urinary citrate excretion of normal from Bangkok, normal from northeast of Thailand, acidification defect and renal stone

1 = Normal from Bangkok (n=30)
2 = Normal from northeast of Thailand (n=15)
3 = Acidification defect (n=15)
4 = Renal stone (n=15)

Table 7 summarizes the excretion values (means±SD) of all intermediates in four study groups.

Table 7 Urinary TCA cycle intermediates excretion (mean+SD),mM

	1	2	3	4	p values
Citrate	0.48+0.35	0.51+0.48	0.10+0.19	0.20+0.32	1>3,4 (p<0.002) 2>3,4 (p<0.002)
Succinate	0.49+0.21	0.27+0.08	0.24+0.06	0.25+0.13	1>2,3,4 (p<0.001)
Malate	0.21+0.29	0.17+0.08	0.11+0.09	0.09+0.07	ns
Fumarate	0.09+0.07	0.08+0.04	0.13+0.10	0.13+0.16	ns
Isocitrate	0.63+0.40	-	0.57+0.31	-	ns

ns = No two groups are significantly different at the 0.05 level

1 = Normal in Bangkok (n=30)

2 = Normal in Northeast region of Thailand (n=15)

3 = Acidification defect (n=15)

4 = Renal stone (n=15)

CHAPTER IV

DISCUSSION

A high performance liquid chromatographic method for measuring low concentration of urinary citric acid cycle intermediates are described. The method, which improved from Caccamo F et al's method (6), employs a one-step esterification procedure with phenacyl bromide as esterification reagent. The analysis, which is simple and rapid, is performed at 254 nm, avoiding interferences that occur at lower wavelengths (43), the eluent is a simple water-methanol mixture, which make the column long life. This proposed method gives high enough recovery to determine the citric acid cycle intermediates in urine with high accuracy and precision. Various attempts have been made to determine simultaneously these acids, however, none of these methods meets the requirements of simplicity, rapidity and, mostly, reliability. Moreover, the results of urinary citrate excretion obtained by this high performance liquid chromatographic method were compared with those obtained by the previously reported enzymatic method (44), for 45 urine specimens, as shown in figure 9. Linear regression analysis of the two methods were well correlated ($r=0.755$, $p<0.001$).

As noted in previous case reports, the excretion of citrate was found to be diminished in renal tubular acidification defects, including renal tubular acidosis (RTA). The levels of urinary citrate excretion expressed as mean values ± standard deviation obtained from these studies were 0.48± 0.35 mM for normal in Bangkok, 0.51±0.48 mM for normal from northeast, 0.10±0.19 mM and 0.20±0.32 mM for acidification defect and renal stone, respectively. The urinary citrate excretion in normal groups were significantly higher than patient groups ($p<0.002$), as shown in figure 8. The results of normal groups in Bangkok were slightly lower than the previous report by Ong-Aj-Yooth et al (14) with the normal value of urinary citrate > 0.3 mM. In this study, we founded that there were 14 out of 15 cases of acidification defect and 11 out of 15 cases had a urinary citrate excretion less than 0.3 mM. The reduced excretion of citrate in renal tubular acidosis seems to be fairly specific for this syndrome, for patients with renal stone which was not associated with tubular acidosis had normal rates of citrate excretion. Thus a reduced excretion of citrate may prove a useful means of distinguishing the renal stone of renal tubular acidosis from that due to other conditions.

The present study also supported the using of urinary citrate excretion as a good screening test for detecting dRTA cases than the other citric acid cycle intermediates; succinate, malate, fumarate, and isocitrate, which needs further detail study.

In conclusion, the proposed method allows the identification, separation and determination of carboxylic acids as constituents of urine. The analysis which is simple and rapid gives high enough recovery to determine the citric acid cycle intermediates in urine with high accuracy and precision.

REFERENCES

1. Schwarzenbach R. High-performance liquid chromatography of carboxylic acids. J Chromatogr 1982; 251: 339.

2. Richards M. Separation of mono- and dicarboxylic acids by liquid chromatography. J Chromatogr 1975; 115:259.

3. Horvath CS, Melander W, and Molnar I. Liquid chromatography of ionogenic substances with nonpolar stationary phases. Anal Chem 1977; 49: 142.

4. Persson BA, and Lagerstrom PO. Ion-pair partition chromatography in the analysis of drugs and biogenic substances in plasma and urine. J Chromatogr 1976; 122: 305.

5. Mentasti E, Gennaro MC, Sarzanini C, Baiocchi C, and Savigliano M. Derivatization, identification and separation of carboxylic acids in wines and beverages by high-performance liquid chromatography. J Chromatogr 1985; 322: 177-189.

6. Caccamo F, Carfagnini G, Corcia AD, and Samperi R. Improved high-performance liquid chromatographic assay determining organic acids in wines. J chromatogr 1986; 362: 47-53.

7. Borch RF. Separation of long chain fatty acids as phynacyl esters by high performance liquid chromatography. Anal Chem 1975; 47: 2437.

8. Miller JM, Brindle ID, Cater SR, and So KH. Potassium fluoride assisted derivatization of carboxylic acids to phynacyl esters for determination by high perfor-

mance liquid chromatography. Anal Chem 1980; 52: 2430.

9. Durst HD, Milano M, Kikta EJ, Connelly SA, and Grushka E. Phynacyl esters of fatty acids via crown ether catalysis for enhanced ultraviolet detection in liquid chromatography. Anal Chem 1975; 47: 1797.

10. Buckalew VM Jr, McCurdy DK, and Ludwig GD. Incomplete renal tubular acidosis. Am J Med 1968; 45: 32.

11. Nilwarangkur S. Renal tubular acidosis. The Bulletin of the Nephrology Society of Thailand 1987; 6: 6-17.

12. Milne MD, Scribneer BA, and Crawford MA. Non-ionic diffusion and the excretion of weak acids and bases, Am J Med 1958; 24: 709.

13. Norman ME, Feldman NI, Colm RM, Roth KS, and McCurdy DK. Urinary citrate excretion in the diagnosis of distal renal tubular acidosis. J Pediatr 1978; 92: 394-400.

14. Ong-Aj-Yooth S, Vasuvattakul S, Nimmannit S, Nilwarangkur S, Ong-Aj-Yooth L, Malasit P. Urinary citrate excretion in normal Thai adults. Abs. 1st National Symposium in Urinary Stone and Renal Tubular Acidosis. Khon Kaen, Thailand.

15. Edwards KDG, Mody NJ, and Crawford MA. Citrate utilization by the renal tubules in primates. J Med Sci 1962; 27: 45-50.

16. Dedmon RE, and Wrong O. The excretion of organic anion in renal tubular acidosis with particular to citrate, Clin Sci 1962; 22: 19.

17. Greenspan EM. Hyperchloremic acidosis and nephrocalcinosis: the syndrome of pure "lower nephron" insufficiency. Arch Int Med 1949; 83: 271.

18. Owen EE, and Verner JV. Renal tubular disease with muscle paralysis and hypokalaemia. Am J Med 1960; 28: 8.

19. Yaffe SJ, Craig JM, Fellers FX. Studies on renal enzymes in a patient with renal tubular acidosis. Am J Med 1960; 29: 168.

20. Cohen RD, and Prout RES. The origin of urinary citrate. Clin Sci 1964; 26: 237-245.

21. Nieth H, and Schollmeyer P. Substrate-utilization of the human kidney. Nature London 1966; 209: 1244-1245.

22. Baruch SB, Burich RL, Eun CE, and King VF. Renal metabolism of citrate. Med Clin North Am 1975; 59: 569-582.

23. Evans BM, MacIntyre I, Macpherson CR, and Milne MD. Alkalosis in sodium and potassium depletion (with especial reference to organic acid excretion). Clin Sci 1957; 16: 53-65.

24. Clarke E, Evans BM, MacIntyre I, and Milne MD. Acidosis in experimental electrolyte depletion. Clin Sci 1955; 14: 421-440.

25. Adler S, Anderson B, and Zemotel L. Metabolic acid-base effect on tissue citrate content and metabolism in the rat. Am J Physiol 1971; 220: 986-992.

26. Baruch S, Burich RL, and King VF. Effects of alkalosis on renal citrate metabolism in dogs infused with citrate. Am J Physiol 1973; 225: 385-388.

27. Crawford MA, Milne MD, and Scibner BH. The effects of changes in acid-base balance on urinary citrate in the rat. J Physiol London 1959; 149: 413-423.

28. Simpson DP. Effect of acetazolamide on citrate excretion in the dog. Am J Physiol 1964; 206: 883-886.

29. Fourman P, and Robinson JR. Diminished urinary excretion of citrate during deficiencies of potassium in man. Lancet 1953; 2: 656-657.

30. Orten JM, and Smith AH. A study of certain metabolites and related compounds as precursors of endogenous citric acid. J Biol Chem 1937; 117: 555-567.

31. Crawford MA. The effects of fluoroacetate, malonate and acid-base balance on the renal disposal of citrate. Biochem J 1963; 88: 115-120.

32. Grollman AP, Harrison HC, and Harrison HE. The renal excretion of citrate. J Clin Invest 1961; 40: 1290-1296.

33. Kook YJ, and Lotspeich WD. Citrate excretion during intrarenal arterial precursor infusion in the alkalotic dog. Am J Physiol 1968; 215: 282-288.

34. Runberg L, and Lotspeich WD. Krebs cycle acid excretion with isotopic split renal function techniques. Am J Physiol 1966; 211: 467-475.

35. Krebs HA, Salvin E, and Johnson WA. The formation of citric and alpha ketoglutaric acids in mammalian body. Biochem J 1938; 32: 113-117.

36. Pacanis A, and Rogulski J. Studies on chemical and enzymatic synthesis of maleyl-CoA. J Biol Chem 1981; 256: 13030-13034.

37. Gordon EE. The metabolism of citrate-C^{14} in normal and fluoroinhibitor poisoned rats. J Clin Invest 1961; 40: 1719-1726.

38. Harrison HE, and Harrison HC. Cortisol and citrate metabolism. Am J Physiol 1959; 196: 943-945.

39. Linderbaum A, Write MR, and Schubert J. Citrate formation in vivo induced by non-lethal doses of fluoroacetate. J Biol Chem 1951; 190: 585-593.

40. Steenbock H, and Bellin MA. Vitamin D and tissue citrate. J Biol Chem 1953; 205: 985-991.

41. DeLuca HF, Gran FC, and Steenbock H. Vitamin D and citrate oxidation. J Biol Chem 1957; 224: 201-208.

42. DeLuca HF, and Steenbock H. An in vitro effect of vitamin D on citrate oxidation by kidney mitochondria. J Biol Chem 1957; 228:469-474.

43. Turkelson VT and Richards M. High-performance liquid chromatography of fatty acids. Anal chem 1978; 50: 1420.

44. Nielsen TT. A method for enzymatic determination and citrate in serum and urine. Scand J Clin Lab Invest 1976; 36: 513-9.