ORGANIC ACIDS IN FRUIT JUICES FOR THE PROPHYLACTIC TREATMENT OF RENAL CALCULI

by

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ABSTRACT

ORGANIC ACIDS IN FRUIT JUICES FOR THE PROPHYLACTIC TREATMENT OF RENAL CALCULI

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Calcium composes up to approximately 90% of all kidney stones, with the remaining 10% being made up by oxalate, phosphate, or a mixture of various urinary ions. An increase in the level of ionized calcium can increase the risk of stone formation. A common problem is a deficiency in the excretion of natural inhibitors in the urine of individuals plagued with recurrent stone formation. Organic acids can reduce the risk of stone formation by complexing ionized calcium in urine. This study has demonstrated that citric, malic, and quinic acids reduce the level of ionized calcium *in vitro*. Based on this observation an attempt was made to duplicate this effect *in vivo*. The juices chosen were cranberry juice cocktail and grapefruit juice, since both of these fruit juices are known to contain high concentrations of organic acids. It was concluded from this study

that the ingestion of 10 ounces of these fruit juices has no significant effect in increasing the level of organic acids in urine of nonstoneforming individuals over a 6 hour time period compared to the ingestion of 10 ounces of water.

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CHAPTER I

INTRODUCTION

Calcium is a major constituent of 90% of all renal calculi (Epstein, 1968), occuring as a complex of either calcium oxalate or calcium phosphate. Calcium oxalate is the most prevalent type of calcium stone, constituting two-thirds of all types of kidney stones formed (Dent et al., 1971). Calcium containing stones can be prevented, or at least managed, by certain dietary modifications. Robertson et al. (1978, 1983) reported that the dietary intake of certain risk factors such as calcium, oxalate, glycosaminoglycans, fluids and urinary pH influence the physiochemical environment of urine necessary for the pathogenesis of stones.

Two main theories have been proposed to explain calculi formation: the saturation/crystrallization theory and the matrix theory.

Saturation/crystallization theory

The concentration of ions which saturate urine is important in the initiation of calcium stone formation. In vitro studies

have shown that when the products of free ionized calcium and oxalate (free ion activity product or FIAP) are balanced by one another, an equilibrium solubility product (ESP) is formed. The degree of urinary saturation is determined by the activity product ratio (APR) of FIAP/ESP (Coe and Favus, 1984). Urine is considered oversaturated it the APR is greater than 1 and undersaturated it the APR is less than 1 (Robertson et al., 1968).

Any disturbance in the FIAP affects crystal formation in one of two ways. A decrease in the FIAP produces an undersaturated urine where existing crystals will be solubilized. An increased FIAP increases the saturation of urine and crystal formation will occur. In other words, any increase or decrease in urinary saturation directly affects the FIAP resulting in an increase (crystal formation) or decrease (solubilization of crystals) in the APR.

Urine is normally supersaturated with respect to calcium and oxalate ions but spontaneous crystal formation does not occur, Spontaneous nucleation of crystals will occur, however, if the amount of calcium (or other substances) increases beyond the point at which urine is no longer able to keep it in solution. The area of supersaturation between the activity product and spontaneous crystal formation is the metastable zone of supersaturation. It is within this metastable zone that the precipitation and aggregation of crystals will occur if preexisting nuclei are present in urine. Precipitation of crystals continues as long as urine remains supersaturated. The upper limits of the metastable

zone where the minimum concentration of the APR produces spontaneous crystallization is called the formation product (FP). The APR/FP is called the formation product ratio (FPR), and it is in this zone where the most rapid growth of stones occurs.

Nucleation of calcium crystals depends primarily on the degree of urinary saturation. A study of calcium oxalate crystalluria by Robertson, Peacock, and Nordin (1969) reported that urine obtained from nonstoneformers (people who have never passed a stone) and stoneformers (people who have passed at least one stone) is usually saturated with calcium and oxalate ions, and that there is no significant difference between the two groups (Pak et al., 1969, 1976).

Two types of nucleation can occur depending on the zone of supersaturation. Homogeneous (spontaneous) nucleation appears when the concentration of ionic species increases above the formation product. Although this type of nucleation is uncommon (Holzbach et al., 1974) it is the method by which calcium oxalate monohydrate stones are formed. Heterogeneous nucleation can occur throughout the metastable zone and requires a particle or "seed", such as hydroxyapatite or sodium urate, which serves as a site of attachment for a crystal (Pak et al., 1975). Heterogeneous nucleation is the usual mode of formation for calcium oxalate dihydrate and calcium phosphate stones.

Matrix theory

Another theory for kidney stone formation is the matrix theory. All kidney stones contain a protein-like matrix of approximately 2 to 3% of the total stone weight. Studies concerning the role of matrix in the genesis of stones has produced conflicting results.

Uromucoid is the major macromolecule present in urine and is similar in chemical composition to stone matrix, except uromucoid contains sialic acid whereas matrix does not. Hallson and Rose (1979) added uromucoids in vitro to crystal-free whole urine and found that the added uromucoids invoked calcium oxalate crystal formation in 85% of the urine samples. Hallson and Rose hypothesized that the uromucoids promoted stone formation by acting as nuclei which attracted and "glued" calcium oxalate crystals. They also suggest that atter the uromucoid is precipitated in urine the sialic acid portion is cleaved by the renal enzyme sialidase, thus converting it to stone matrix (Malek and Boyce, 1977).

In 1982 Rose and Sulaiman precipitated a uromucoid, the Tamm-Horsfall (T-H) glycoprotein, from nonstoneforming urine and added it to ultrafiltered crystal-free nonstoneforming urine. Their results showed that the addition of the T-H glycoprotein to the ultrfiltrate significantly increased calcium oxalate crystal formation in 73% ot the samples. Contradictory evidence for the T-H glycoprotein acting as a stone promoter was reported in a study by Kitamura and Pak (1982). Kitamura and Pak precipitated

the T-H glycoprotein from the urine of nonstoneformers and stoneformers and added the glycoprotein to a synthetic urine medium containing calcium oxalate seeds. Their results showed that the addition of the T-H glycoprotein exerted a slight inhibitory effect by increasing the FPR in both nonstoneformers and stoneformers urine. The study also reported that as the concentration of the added T-H glycoprotein increased, the rate of crystal formation decreased. Therefore, T-H glycoprotein has a significantly greater inhibitory effect in the stoneforming group. This evidence presented by Kitamura and Pak refutes the study of Rose and Sulaiman, but the results could be explained by the different methodology used by each research group. Whether the matrix acts as a promoter or inhibitor still requires further investigations, but it unquestionably influences stone formation in some way.

Inhibitors of stone formation

Once crystal nucleation is initiated, growth continues as long as the urine remains supersaturated. However, this level of supersaturation is usually exceeded in healthy individuals without the formation of a kidney stone. The question arises, why do some people form kidney stones while others do not? This can be explained by the concentration of inhibitors in urine.

Urinary inhibitors affect the crystallization, aggregation, and growth of calcium stones by altering the ionic activity of urine. When these urinary inhibitors are present they raise the

FPR, allowing for greater concentrations of calcium and oxalate ions without increasing the risk of stone formation. Dent et al. (1971) and Robertson (Peacock, Marshall et al., 1976) compared the levels of urinary inhibitors in nonstoneformers and stoneformers and found that the inhibitor concentration was lower in stoneformers. Another study (Smith et al., 1980) reported no significant difference between the two groups.

The inhibitory activity can occur by several processes. The inhibitor may chelate the free urinary ionized calcium or oxalate to prevent crystal formation. Another possibility is for the inhibitor to bind to the surface of a crystal, thus disrupting the surface lattice and thereby preventing any further adsorption of other ions onto the crystal. Or the inhibitor can bind calcium or oxalate in the intestinal lumen to form an insoluble complex, thus preventing its absorption and excretion.

A variety of inhibitors have been identified and are known to prevent crystal formation, aggregation or both. One of the first inhibitors identified used rat cartilage as a substrate (Howard et al., 1967; Robertson, Hambleton, and Hodgkinson, 1969). This low molecular anionic inhibitor was isolated (but not completely identified) from urine and found to prevent the in vitro calcification of rat cartilage. Stoneformers are known to be significantly deficient in this urinary inhibitor. Although this inhibitor has been suggested to prevent calcium phosphate crystallization, its role in calcium oxalate inhibition is obscure and further investigation is needed.

Many naturally occurring inhibitors have been proven effective against calcium lithiasis *in vitro*. Citric acid is a low molecular weight organic acid inhibitor of calcium oxalate precipitation and growth (Fleisch, 1978) which forms soluble complexes with urinary calcium. Many stoneformers who are diagnosed as hypocitraturic (urinary citrate $\leq$400 mg/day in men and 200 mg/day in women) (Rudman et al., 1982, Nicar et al., 1983) have normal concentrations of urinary calcium. The deficiency in urinary organic acids has been suggested as an additional risk factor in stone disease (Vogel et al., 1984). Other organic acids, such as hippuric and lactic acids, also increase the *in vitro* solubility of calcium oxalate, while glucuronic and pyruvic acids are ineffective (Elliot and Eusebio, 1965).

Pyrophosphate (-P-O-P-) inhibits calcium oxalate crystallization (Whelshman et al., 1972,) and growth (Meyer and Smith, 1975). However, it is generally believed that the concentration of urinary pyrophosphate is insufficient to create the same inhibitory effect found *in vitro* (Fleisch, 1978, and Ryall et al., 1981). Magnesium ions retard crystal precipitation (Desmars and Tawashi, 1973) by chelating urinary oxalate, thereby increasing the YPR of calcium oxalate. Gershoff and Prien (1967) reported no new stone formation in 83% (30 out of 36) of recurrent stoneformers during a five year treatment plan consisting of dietary supplements of 100 mg MgO and 10 mg vitamin B_6 (pyroxidine) daily.

There is a general consensus that urine from both

stoneformers and nonstoneformers is normally supersaturated with calcium oxalate crystals. It is thus becoming apparent that the more important focus of activity is to determine whether there is a deficiency in aggregation inhibitors that distinguishes the two groups. Glycosaminoglycans, such as heparin and chondroitin sulfate, are known to inhibit the *in vitro* aggregation of calcium oxalate crystals (Robertson, Knowles, Peacock, 1976, Ryall et al., 1981), however, heparin is present in such minute amounts in urine that it has limited applicability *in vivo* (Goldberg and Cotlier, 1972). Ryall and associates (1981) reported that urinary magnesium posesses the ability to inhibit crystal aggregation at a concentration of $5X10^{-3}$ mol/l, which is well within the concentration range of normal urinary magnesium of $1.5\text{-}5.8X10^{-3}$ mol/l (Sutor et al., 1979).

TREATMENT OF CALCIUM OXALATE LITHIASIS

Medical management

People who have passed at least one stone are usually at risk to form one or more additional stones at some time during their lifetime. Medical intervention is usually the first method of treatment upon discovery of stone formation because of its broad specificity of action. Most treatment plans are implemented without taking into consideration the causation of the stone, and may actually increase the risk of forming rather than preventing a stone. Some considerations when treating a stone patient should

be to return the physiochemical nature of urine to normal, to avoid any adverse side effects, and to overcome any physiological deficiencies (i.e., inhibitors or promoters) in the urine.

One type of medical intervention is the oral administration of phosphates. Phosphate is given as a means of reducing urinary pH and to decrease hypercalciuria by chelating calcium within the intestine or urine. Pak et al. (1974) orally administered 10 to 15 mg/day of sodium cellulose phosphate to stoneformers having absorptive hypercalciuria. Cellulose phosphate is a non-absorbable salt which strongly binds divalent cations in the intestine and prevents their absorption from the intestine. This treatment significantly reduced the level of urinary calcium below 200 mg/day in all 16 patients without producing a negative calcium balance. A few of the consequences using this therapy are a reduction of urinary magnesium and an increase in urinary oxalate accompanied by diarrhea.

Orthophosphate is also given orally (1-2 gms) in patients diagnosed with absorptive hypercalciuria in an effort to reduce urinary calcium, but its actions differ from cellulose phosphate. Orthophosphate is a soluble absorbable salt, and when ingested it reduces urinary calcium but to a lesser degree. Because it is absorbable, it increases urinary phosphates, thus increasing the risk of calcium phosphate stones. This risk is offset by the reduction of urinary calcium combined with a two- to three-fold increase of pyrophosphates in the urine (Lewis et al., 1966).

Thiazide **diuretics are often administered to reduce calcium**

excretion in stoneformers suffering from absorptive and renal hypercalciuria, In 1978, Yendt and associates significantly reduced the recurrence of calcium oxalate stones in 90% of stoneformers by the administration of 50 mg of hydrochlorothiazide twice a day. Post-treatment analysis also reported a decrease in urinary oxalate, presumably by increasing fecal calcium, a reduction in urinary citrate, and an increase in magnesium excretion without any significant change in urinary pH.

Although the main effect of diuretics is to reduce urinary saturation and increase urinary output, not all diuretic medication has this hypocalciuric property. Chlorthalidone and metolazone, two thiazide-related drugs, have no significant effect on reducing calcium excretion and may actually increase urinary calcium (Yendt et al., 1978). Many idiopathic stoneformers are diagnosed as normocalciuric yet are still plagued with stones, and the use of these hypocalciuric drugs is ineffective.

Another method of treatment is to increase the amount of urinary inhibitors. An increase in urinary inhibitors has been reported during phosphate or thiazide treatment and is attributed as a secondary effect to the reduction in calcium excretion. Pak and associates (1986) have extensively studied the effects of potassium citrate therapy in stoneformers with absorptive and renal hypercalciuria and idiopathic (normocalciuric) stoneformers diagnosed with hypocitraturia,

Potassium citrate was given to nonstoneformers and stoneformers in oral doses ranging from 30 to 300 mEq/day for more

than 2 years. In all three types of hypercalciuria the stoneformers had a significant sustained increase in urinary pH and restoration of urinary citrate levels to normal with no significant alteration in calcium excretion (Pak et al., 1986). In 86% (21/25) of these patients no new stone formation was observed during the long term treatment. The physiochemical effects of potassium citrate treatment appear to be greater than either phosphate or thiazide therapy. Potassium citrate therapy effectively reduces urinary saturation with respect to calcium oxalate crystals because of the ability of the increased citrate to chelate ionized urinary calcium and increase urinary output (Pak et al., 1985, 1986).

Dietary modifications

Dietary modifications are important in the causation and treatment of urolithiasis by altering or correcting any metabolic abnormalities in the urine. However, dietary restrictions are often imposed on a stone patient without taking into consideration the pathophysiological processes involved in stone formation. The dietary intake of calcium is vital to stone formation. The greatest amount of body calcium is found in bones and teeth. The average Kecommended Daily Allowance (RDA) of calcium for adults is 800 mg/day on a 1400 calorie diet (Nutrition in Health and Disease, 1982). The main sources of calcium are from milk and other dairy products. Approximately 40% of dietary calcium is utilized by the body and the remaining 60% is excreted primarily

in the urine.

Urine analysis provides a good determination of the calcium excretion. Twelve or twenty-four hour urine specimens are collected and analyzed to detect hypercalciuria. Although numerous tests to detect hypercalciuria have been developed, the different methods used and the different times of urine collection have made it difficult to establish a definite level of calcium excretion. Many studies use the upper limits of calcium excretion considered to produce hypercalciuria to be 300 mg/day for men (Smith et al., 1978, and Coe, 1981), 250 mg/day in women (Williams, 1974, and Coe, 1981), or 4 mg/kg body weight per day on a dietary intake of 1000 mg of calcium (Food and Nutrition in Diet Therapy, 1984).

Once a person is determined to be hypercalciuric, the next step is to determine the cause of the hypercalciuria. Three types of hypercalciuria have been identified.

1) Absorptive hypercalciuria, in which there is an increased hyperabsorption of intestinal calcium due to an increase in the renal filtered load of calcium and reduced renal tubular absorption of calcium. This group can be further subdivided into Type I (hypercalciuria on a low calcium diet), Type II (hypercalciuria on a normal calcium diet), and Type III, (hypercalciuria associated with hypophosphatemia),
2) Resorptive hypercalciuria, which involves the

removal of calcium from bone due to hyperparathyroidism, Paget's disease, or tumors, and

3) Renal hypercalciura, a defect in the reabsorption of calcium by renal tubular cells resulting in a leakage of calcium into urine.

The method of treatment differs for each type of hypercalciuria. Dietary restrictions can be used effectively to treat absorptive hypercalciuria but are ineffective against resorptive or renal hypercalciuria (Loening et al., 1980). There are many instances of stoneformers who have an increase in calcium excretion yet do not show any of the causes associated with the ditterent types of hypercalciuria's. These types of people are considered to be "idiopathic" stoneformers because the cause of the hypercalciuria is not known.

Restriction of dietary calcium effectively reduces the level of urinary calcium resulting in lower APR. Extreme caution should be taken when dietary calcium restrictions are imposed so that the calcium balance within the body is not impaired. Calcium restriction lower than 400 mg/day produces a negative calcium balance which depletes bone calcium and may induce osteoporosis (Lindergard et al.,1983). A moderate calcium restriction in the range of 400-600 mg/day is recommended for absorptive hypercalciuria (Pak et al., 1984).

A low oxalate diet should be imposed during dietary calcium restrictions because there is an inverse relationship between the

amount of calcium ingested and the amount of oxalate excreted (Bataille, **1983).** Calcium is predominantly absorbed by the duodenum of the small intestine and oxalate is absorbed in both the ileum and jejunum. A severe reduction in dietary calcium reduces the amount of calcium in the intestinal lumen. Without any free calcium ions to bind to the intestinal oxalate, the oxalate remains in its soluble form and is freely absorbed from the intestine. Once it is absorbed from the intestine it binds to the ionized urinary calcium within the kidney, forming an insoluble complex, and the formation of a stone begins. Although a low concentration of urinary calcium is advantageous in the prevention of stone formation, the amount of urinary oxalate is **10** to **15** times more important in determining the precipitation of calcium oxalate crystals than either urinary calcium or phosphate (Finlayson, **1974).**

An important aspect of calcium lithiasis involves the urinary excretion of oxalate. Calcium oxalate stones are the most common type of kidney stones and a greater emphasis should be placed on their treatment. Oxalic acid is an organic acid that is a metabolic end product excreted primarily in urine. No evidence of oxalate catabolism has been found (Hagler et al., **1973).** In humans it is synthesized endogenously from two precursors, glyoxylic acid and ascorbic acid, or obtained from the ingestion of foods and liquids in the diet. Approximately **85%** of urinary oxalate is produced endogenously, with the metabolism of glyoxylic acid responsible for 40-60% and ascorbic acid accounting for **40%.** Only

2-5% of dietary oxalate is absorbed from the intestine, and *in vitro* studies using the small intestine of rats indicate that this is a passive process occurring most readily in the jejunum (Binder, 1974, and Madorsky et al., 1977). This is considered to be the reason for the increase in oxalate excretion in people who have had ileal bypass surgery (Drach, 1986).

Many diets prescribed to manage calcium oxalate stones stress the avoidance of such oxalate-rich foods as spinach, cocoa, rhubarb, leafy tea, and fruit juices. The daily dietary oxalate intake can vary from as little as 70 mg to 920 mg (Hodgkinson, 1977). For calcium oxalate stoneformers this amount is reduced to 40-50 mg/day (Food and Nutrition in Diet Therapy, 1984) in conjunction with a low calcium diet, for the reasons previously described.

The amount of oxalate ingested is not reflected in the amount of oxalate that appears in urine. Oxalate in foods is often (or will be) found as an insoluble salt which prevents its absorption from the intestine. The bioavailability, or the percent of the total amount of ingested oxalate that is absorbed from the intestine, does not always correlate with the oxalate content of foods and liquids considered to be oxalate-rich. Of the seven oxalate-rich foods tested ,only spinach (1236 mg) was determined to cause hyperoxaluria (Brinkley, 1981). The investigations in oxalate bioavailability are few and the avoidance of foods and liquids having a high oxalate content is still recommended.

Even though the bioavailability of ingested oxalate is low it

can have a dramatic effect on stone formation as previously mentioned. Dietary restriction of oxalate is encouraged to decrease the concentration of oxalate in urine to prevent hyperoxaluria (urinay oxalate greater than 44 mg/day). Hyperoxaluria can occur either by an increase in the endogenous production of oxalate or by hyperabsorption of intestinal oxalate.

An increase in the endogenous production of oxalate is associated with a aysfunction in glyoxylate metabolism and may occur in several ways. First, an increase in a dietary precursor, such as glycine or serine, leads to an increased production of oxalate. Secondly, there are the primary hyperoxaluria types I and II. Both are congenital illnesses caused by a genetic defect of an enzyme important in the synthesis of glyoxylate. Pyridoxine (vitamin B_6) administration is effective in treating type I patients by significantly reducing urinary oxalate because of its ability to transaminate glyoxalate to glycine instead of to oxalate (Balcke et al., 1983).

Increased absorption of oxalate (or enteric hyperoxaluria) is caused by the malabsorption of intestinal fat (Chadwick et al., 1973, Earnest et al., 1974, Smith et al., 1978). Unabsorbed fats bind calcium in the intestinal lumen, thus allowing for a greater amount of oxalate to be absorbed from the intestine and excreted.

One universally recommended treatment is increasing the ingestion of fluids. A high fluid intake dilutes urine, lowers the urinary concentration of stone-forming ions, and decreases the saturation level of calcium salts. A recommended minimum urine

output of 2 liters/day is achieved by drinking approximately 3 liters of fluid per day (Pak et al., 1984), or 8 ounces (240 ml) of fluid hourly while awake (Hosking et al., 1983). At least 50% of the fluid intake should be water and the other half can be fluids of the patient's choosing, as long as the patient does not choose fluids containing high contents of calcium or oxalate. Fluid intake should be balanced throughout the day to maintain a consistently high urine flow. Hosking and associates (1983) treated 108 idiopathic calcium stoneformers with a fluid intake of 8 ounces/hour while awake, and reported that 58% of the patients had no new stone formation for up to 5 years. Hosking concluded that drug therapy in patients with idiopathic calcium lithiasis may not be justified if fluid therapy is proven to be effective.

Theoretically, an increase in urine volume would not only dilute the saturation of calcium salts but would also decrease the concentration of urinary inhibitors. In 1980 Pak and associates studied the *in vivo* effects of urinary dilution on calcium phosphate and oxalate supersaturation in patients with absorptive hypercalciuria. Results from this study reported that a urine volume ranging from 1 to 2.3 liters/day (on a fluid intake of 1.8 to 3.3 liters/day) significantly increased the formation product ratio of calcium oxalate, thus indicating that an increase in fluid intake can inhibit nucleation of calcium oxalate stones. Conversely, the dilution effect did not significantly alter urinary excretion of stone-forming inhibitors, particularily magnesium, citrate, and pyrophosphate. This indicates that the increase in

fluids was accompanied by an increase in inhibitor absorption.

The amount of dietary proteins ingested is also crucial to calcium stone formation. Fellstrom and associates (1984) fed high and low protein diets to recurrent stoneformers and reported an increase in urinary calcium accompanied by a decrease in urinary citrate on the high protein diet. This evidence suggests that the effect of a diet low in calcium and oxalate may be negated if the diet still contains a high level of protein.

Although numerous treatments have been proposed for the treatment of kidney stone disease, it appears that one of the most effective treatments is to increase the drinking of fluids and to take some form of medication to increase the level of urinary inhibitors and decrease the urinary concentrations of calcium and oxalate. The purpose of this study is to investigate the role of fruit juices in the treatment of calcium lithiasis. These juices contain high levels of organic acids which have the potential to reduce the level of ionized calcium in the urine, and the ingestion of fruit juices would also contribute to increased fluid intake, which is also very desirable in stoneforming patients.

CHAPTER II

MATERIALS AND METHODS

Measurement of calcium

All glassware was washed with a 5% solution of ethylenediamine tetraacetic acid (Na_2EDTA) and rinsed thoroughly with deionized water to remove any extraneous calcium that might interfere with the analysis of calcium in the test samples. Stock solutions were prepared from reagent grade chemicals purchased from the Sigma Chemical Co.; distilled, deionized water was used in their preparation.

A Corning calcium-specific electrode (Cat. no. 476041) was used to measure the ionized calcium against a Corning glass reference electrode (Cat. no. 476002). All samples were measured in a water bath at 37^{o} C with constant stirring. The calibration of the electrode was a modification of the method used by Jacobson et al. (1979). A synthetic urine was prepared containing intermediate concentrations of the nitrate salts of the following ions found in urine: Na^{+1} (0.16 M), K^{+1} (3.35×10^{-5} M), Mg^{+2} (3.45×10^{-3} M), Zn^{+2} (5.5×10^{-6} M), Fe^{+2} (3.0×10^{-5} M) Cu^{+2} (4.0×10^{-6} M), and Pb^{+2} (5.0×10^{-7} M). These concentrations represent an approximate average for these ions found in normal

urine. Fifty ml of synthetic urine was added to 50 ml of 2 M KCl. A calibration curve was then generated by the addition of five aliquots of differing amounts of calcium concentrations. Because the mobilities of K^{+1} and Cl^{-1} in solution are nearly equal, KC1 is added to the synthetic urine to act as a supporting electrolyte. This ensures that the potential difference at the liquid junction of the ion-specific electrode is at a minimum so as not to interfere with the calcium concentration reading. The final calcium concentrations were 2.5 x 10-4 M, 5.0 x 10^{-4} M, 2.5 x 10^{-3} M, 5.0 x 10^{-3} M, and 2.44 x 10^{-2} M. A standard curve was generated by plotting the electrode reading in millivolts on the ordinate and the calcium concentration on the abscissa. A representative example of a standard curve for ionized calcium is presented in Figure 1. Ion-selective electrodes give a logarithmic response to the activities of ions rather than to their concentrations. Therefore the calibration was considered complete when a straight line was plotted on three cycle semilog paper. The electrode was calibrated at the beginning of each day prior to sample analysis. A stabilization period of two minutes was used before the final reading of the electrode was taken for the sample being analyzed

Effect of organic acids in vitro

The calcium concentration was adjusted to 1x10^{-3} M in the synthetic urine (Garti et al., 1980) and a test solution was prepared containing equal parts of synthetic urine and 2 M KCl.

One ml of 1mg/ml solution of malic, citric, and quinic acid was then added to 100 ml of the test solution. One ml of deionized water served as a control. The pH of the test solutions was adjusted by the addition of either NaOH or H_2SO_4. The decrease in the level of ionized calcium in the test solution was then measured over a period of 10 minutes (Sarig et al., 1982). Each organic acid was tested at a pH of 4, 5, and 6.

Analysis of urine samples

Urine samples were collected at 2 hour intervals up to and including 6 hours thereafter. There was no specific time of day in which the sample was obtained from each test subject. The pH was measured in each fresh sample using a glass electrode. Total calcium was measured by a Sigma diagnostic kit (No. 586). The basis of this test is the reaction of total calcium in the sample with o-cresolphthalein to produce an intense purple color which is proportional to the calcium concentration. The samples were quantified spectrophotometrically at 575 nm using a Bausch and Lomb Spectronic 20. Oxalate, citric, malic, and quinic acids were analyzed using high-performance liquid chromatography (HPLC). All subjects were on an unrestricted diet, except for cautioning them not to ingest an extraordinary amount of liquid. All urine samples were collected, the pH was measured, and each sample was immediately stored at -20^{o} C if further analysis was not performed.

High performance liquid chromatography

Chromatography was performed at room temperature with a Bio-Rad Aminex HPX-87H cation-exchange column (300 X 7.8 mm) with a Bio-Rad guard column (55 mm) attached. Ten microliters of prepared organic acid standards (1 mg/dl) and each urine sample were injected into the column and run for 30 minutes at a flow rate of 0.4 ml/min delivered by a Perkin-Elmer ISS-100 autoanalyzer solvent delivery system. Eluting compounds were detected at 210 nm with a Perkin-Elmer UV spectrophotometric detector. The mobile phase used to separate the samples into their components was 0.1 M H_2SO_4 (Coppola et al., 1986) (5.6 ml doubly distilled Velcor H_2SO_4 in 1 liter deionized water). One mg/ml standards of citric acid, malic acid, oxalic acid, quinic acid were injected and their retention times were used as reference points on the chromatogram for the appearance of the organic acids in the urine samples. Urine samples were syringe filtered through a 0.45 micron Millipore ultrafilter. Cranberry juice and grapefruit juice were centrifuged twice at 10,000 RPM for 10 min. each time to remove any pigment or pulp from the juice and ultrafiltered. The presence of urinary organic acids was determined by comparison of their retention times with those of the standards. The amount of urinary organic acids was calculated by dividing the area under the curve for each urinary organic acid and dividing by the area under the curve of the corresponding standard and multiplied by the concentration of the standard (mg/dl).

Test subjects

Eight subjects were used, 4 women and 4 men. None of the participants had any previous history ~~of~~ kidney stones or urinary tract infection. A zero time urine sample was collected and on three different days, the subjects were then asked to drink 10 ounces of water, cranberry juice cocktail, and grapefruit juice. Urine samples were then collected at 2, 4, and 6 hours post-ingestion. Total calcium, ionized calcium, and pH were measured immediately following the collection of each sample. Oxalate, citric acid, malic acid, and quinic acid were measured in each sample as previously described above.

Statistical analysis

Twenty-five runs of each ~~in~~ *vitro* organic acid addition and the controls were performed. The one way ANOVA (analysis of variance) was used to verify the significance between test samples and controls in both the ~~in~~ *vitro* and ~~in~~ *vivo* studies at the $p \leq 0.05$ level.

CHAPTER III

RESULTS

Effect of pH on the calcium complexing ability of three organic acids

One ml of a 1 mg/ml solution of either citric, malic, or quinic acid was added to 100 ml of synthetic urine containing 1.0 mM Ca^{+2}. One ml of deionized water served as a control. Aliquots of each solution were then brought to a pH of 4, 5, and 6, and the amount of free calcium in each solution was determined after 10 minutes with a calcium ion-selective electrode.

It can be observed from the Table 1 that the addition of 1 ml of water to the synthetic urine increased the level of ionized calcium at all three pH's. It can also be observed that as the pH increases, there is also an increase in the level of ionized calcium. The addition of 1 ml of citric acid to the synthetic urine results in a decrease of ionized calcium; the greatest decrease occurried at pH 4 with a lesser decrease at pH 5 and 6. Malic acid also decreased the concentration of ionized calcium in the solution with the greatest decrease occurring at the lowest pH. However, in contrast to citric acid, the smallest decrease

occurred at pH 5. For quinic acid, the greatest decrease in calcium concentration occurred at pH 5, with a smaller decrease at pH 4 and only a slight decrease at pH 6.

All three organic acids, at each of the three pH's, significantly reduced the amount of ionized calcium in the synthetic urine when compared to the control. At pH 4 the decrease in ionized calcium after the addition of citric and malic acids was significantly different from quinic acid but not from each other, At pH 5 the decrease in the level of ionized calcium caused by the organic acids was significantly different from the control but not from each other. At pH 6 the decrease in ionized calcium caused by citric and malic acids was significantly different from quinic acid but not from each other.

HPLC analysis of organic acids, cranberry juice cocktail, and grapefruit juice

Ten microliters of 1 mg/ml standards of citric, malic, quinic, and oxalic acids were analyzed by high performance liquid chromatography. The resulting chromatograms are presented in Figures 2 thru 5, Single peaks were detected for citric acid at 14.78 min., malic acid at 16.69 min., and oxalic acid at 15.68 min. Two peaks were detected for quinic acid at 15.81 and 17.03 min. (Figure 2).

Cranberry juice cocktail was also analyzed by HPLC. The resulting chromatogram is presented in Figure 3. The following organic acids were identified in the chromatographs: citric acid at 14.72 min., and malic acid at 16.64 min. Based on the

chromatographic results the concentrations of citric and malic acids in cranberry juice cocktail were quantified to be 2.4 and 2.6 mg/ml respectively. Chromatographic results for grapefruit juice (Figure 4) demonstrated a concentration of 9.75 mg/ml of citric acid and 5.75 mg/ml of quinic acid. A typical chromatogram of a urine sample is presented in Figure 5. Citric acid was identified at 14.80 min. and malic acid at 16.68 min. Due to poor column separation, quinic acid and oxalic acid could not be quantified in either of the juices or in the urine samples.

Effect of ingestion of ten ounces water, cranberry juice cocktail, and grapefruit juice on the level of ionized calcium in urine.

A total of 8 subjects participated in the in vivo study. Urine samples were collected prior to and 2, 4, and 6 hours after each subject had ingested 10 ounces of tap water, grapefruit juice, or cranberry juice cocktail. Changes in pH, total and ionic calcium, and citric and malic acids are presented in Tables 2, 3, and 4, respectively.

It can be observed from Table 2 that there is no significant difference in the urinary pH at time zero, having a mean pH of 5.34. In the control group a steady increase in urinary pH was observed, ranging from 5.35 at time zero to 6.23 at 6 hours. This was a significant increase in urinary pH at 6 hours compared to zero hour. In contrast, when these same subjects drank either grapefruit juice or cranberry juice cocktail, a significant increase in urinary pH occurred up to 4 hours followed by a decrease in pH.

The concentration of ionic calcium after drinking water was quite variable, showing a decrease after 2 hours, increasing after 4 hours, followed by another decrease at 6 hours. However, none of these increases or decreases was significant. Total calcium followed this same trend. The ratio of ionic to total calcium remained relatively stable, ranging from 31.7 to 36.6% (Table 3), and showing significant increase at 2 hours.

The change in total and ionic calcium after drinking grapefruit juice can also be observed from Table 3. The overall trend was similar to that observed after drinking water, also showing no significant changes in calcium concentration. A decrease in both total and ionized calcium was observed at two hours, followed by an increase in both values at 4 hours and very little change occurring at 6 hours. The ratio of total calcium was again relatively stable at 0, 2, 4 hours, ranging from 31.2 at 2 hours to 34.7% at four hours but decreased to 33.8% at 6 hours.

The data after drinking cranberry juice cocktail shows the greatest variability (Table 3). In this group there was a relatively large increase in both ionized and total calcium after 2 hours, followed by a decrease to control values at 4 hours and further decreasing at 6 hours. The ratio of ionic to total calcium ranged from a low of 34.9% at 6 hours to a high of 40.8% at 4 hours. As can be observed from the data, the values for these ratios were generally higher than those observed for either the control or the grapefruit juice groups. None of these changes was significant.

The values for citric and malic acids were also quite variable, apparently showing no particular trends during the course of the time study (Tables 4 and 5, respectively). The amount of citric acid increased after drinking water and remained relatively constant at 4 and 6 hours. Malic acid decreased at 2 hours followed by an increase at 4 and 6 hours. Ingestion of grapefruit juice resulted in a slight increase in citric acid at 2 hours, decreasing at 4 hours, and increasing again at 6 hours. Malic acid followed this same pattern. Drinking cranberry juice cocktail resulted in an decrease in citric acid after 2 hours followed by a slight increase at 4 hours and a large increase at 6 hours. Malic acid showed a decrease at 2 and 4 hours, followed by an increase at 6 hours.

CHAPTER IV

DISCUSSION

Calcium is a major constituent of up to 90% of all renal calculi. The concentration of ionized calcium in the urine has been determined to be a major risk factor in inducing precipitation and growth of calcium-containing kidney stones (Robertson et al., 1978). An additional risk factor that appears to influence stone formation is the occurrence of naturally found inhibitors in urine. Studies have demonstrated that urine from normal individuals has an increased ability to inhibit the crystallization of calcium salts, whereas urine from urolithiasis patients shows a reduced capacity to prevent crystallization (Fleisch, 1978). The identity of these inhibitors has not been firmly established, but a study by Vogel et al. (1984) suggests that a diminished excretion of organic acids may contribute to the overall inhibitory potential of urine because of their ability to inactivate ionized calcium and other divalent cations by the formation of complexes or ion pairs. Their study reported that urolithiais patients have lower levels of urinary organic acids when compared to urine from normal persons, which may contribute to the causation of stones in these patients. These findings

support an earlier study by Elliot and Eusebio (1965) that determined that urinary organic acids increase the solubility of calcium oxalate stones.

Citric acid appears to possess the greatest inhibitory activity of all the organic acids found in urine, accounting for 10 to 11% of the total organic acids excreted (Canary et al., 1961). It has been shown to contribute up to 50% of the total inhibitory activity against the precipitation of calcium phosphate crystals in normal urine (Bisaz et al., 1978). From 19 to 63% of urolithiasis patients have been reported to be hypocitraturic (Rudman et al., 1980).

Thus it has become apparent that it would be desirable to increase the level of organic acids in urine, especially in stone-forming individuals. This study was undertaken to first demonstrate the potential of organic acids to complex calcium ions in vitro, and secondly to determine if the in vitro effect could be observed in vivo.

The first aspect of this problem to be examined was the complexing ability of various organic acids in vitro. Previous investigations have demonstrated that organic acids have the ability to complex calcium ions in various solutions (Elliot and Eusebio, 1965, Vogel et al., 1984). These studies were extended in this investigation by examining the complexing ability of citric, malic, and quinic acids in a simulated urine environment. In addition, it has been demonstrated that pH can affect the complexing ability of organic acids, and since the physiological

pH of urine can vary from 4.5 to 8.0 (Modern Urine Chemistry, 1982), the complexing ability of various organic acids was examined at three different pH values.

This study demonstrates that citric, malic, and quinic acids significantly reduce the concentration of ionized calcium in a synthetic urine at pH 4, 5, and 6. Citric and malic acids have the greatest ability to chelate ionized calcium, with quinic acid having less than one-half of the ability of these two acids. In addition, it was found that both citric and malic acid lost approximately 30% of their ability as the pH increased from 4 to 6, thus having their greatest ability at lower pH's. This observation is in contrast to an investigation by Elliot and Eusebio (1965), who reported that citric acid had a greater ability to complex calcium ions at pH 7 than at pH 5. This discrepancy may be accounted for by the fact that Elliot and Eusebio performed their investigation with organic acids in simple salt solutions rather than in a synthetic urine. Urine consists of a complex ionic environment. The presence or absence of ions that are present in urine can interfere with the complexing power of the organic acid if these ions are not accounted for in solution. Based on these in vitro results, it would appear that all three organic acids possess the ability to reduce the level of ionized calcium in urine and decrease the potential for crystallization. It would follow that it would be beneficial to increase the level of organic acids in the urine, especially in the urine of stoneformers, in order to reduce the risk of stone

formation.

There have been several investigations suggesting the use of organic acids, in particular citric acid, for the treatment of urolithiasis. A recent investigation by Pak et al. (1984) reported that the oral ingestion of 60 mEq/day (3840 mg) of potassium citrate increased the level ot urinary citrate by approximately 30% while decreasing the level of urinary calcium by 40%. Cranberry juice cocktail contains approximately 720 mg of citric acid and a total of 2460 mg of organic acids per 10 ounces of juice. Therefore 10 ounces of cranberry juice 2 times a day should provide more than enough organic acids in the diet. Grapefruit juice contains 2925 mg of citric acid and 1725 mg of quinic acid, for a total of 4650 mg of organic acids per 10 ounces of juice, and should also be able to increase the level ot organic acids in the urine. From this evidence it was concluded that either cranberry juice cocktail or grapefruit juice should provide sufficient dietary organic acids which would result in a measurable increase in the excretion of organic acids.

Based on the work by Pak and others, and on the in vitro observations of this study, an investigation was undertaken to determine it organic acids could be supplied in the diet from cranberry juice cocktail and grapefruit juice. Both fruit juices are known to contain appreciable amounts of organic acids, particularily citric acid.

Results from this in vivo study were quite variable. Urinary pH increased steadily during the time study for all three groups,

peaking at 6.23 atter 6 hours for the group drinking water, and 6.31 and 6.63 for grapefruit and cranberry juice, respectively, at 4 hours and then declining at 6 hours. This increase in pH can not be explained and is unexpected, since the organic acids should decrease urinary pH. Excretion of ionic and total calcium also showed wide variances. In the control group ionic calcium decreased by 19.4% atter 2 hours and then increased by 20.2% after 4 hours, followed by a decrease of 17% after 6 hours. Total calcium showed a similar trend. For the subjects drinking grapefruit juice, there was a 43.4% decrease in ionic calcium after 2 hours, increasing by 28% at 4 hours, followed by a 4% decrease at 6 hours. Again total calcium followed a similar trend. The study in which cranberry juice cocktail was ingested showed the same variability as the other two groups with the minor exception that an increase of approximately 27.1% in ionic calcium occurred after 2 hours. Several factors probably contributed to the variability of these results. First, there was no attempt to control the diet of the individuals participating in the study. Because the in vivo study was initiated in the morning, it was assumed that most individuals consumed at least one meal during the 6-hour time period which would contribute to the variability ot dietary calcium intake. Also, the majority ot studies investigating urolithiasis perform urine analysis using 24 hour samples (Light et al., 1973, Menon and Mahle, 1983) rather than collecting samples at 2-hour intervals. In defense of the protocol used in the in vivo study, the short sampling period is

justified based on a previous observation by Sobota (1984), who reported that antiadherence effects associated with cranberry juice cocktail could be observed in urine within a 2- to 4- hour period. Because the antiadherance effects are due at least in part to the excretion of organic acids, it was hypothesized that the potential effects of the organic acids on ionized calcium would also be observed within 2 to 6 hours after drinking the juice. It is also more practical to measure samples in intervals rather than 24-hour samples because sudden increases in urinary saturation of calcium complexing salts increase the risk of crystal formation. Analysis of a 24 hour urine sample provides an overview of the physiochemical environment of urine but does not take into consideration the effect of treatment over short periods of time.

An additional factor which may have contributed to the variation in urinary calcium is the fact that the urine samples were not corrected for dilution effects. In this regard it might be expected that the samples taken at later time periods would exhibit the greatest variation due to a dilution effect. This did not prove to be the case. Normally a correction for dilution is made by observing the creatinine clearance (Menon and Mahle, 1983). In this study, as in previous studies that have analyzed total and ionized urinary calcium, a different method was used (Light et al., 1973). A ratio of ionic to total calcium was used as an indicator that would take into account any dilution effect, since this study is concerned with the relative concentration of

the ionic species of calcium. In this regard, the ratio of the control sample was very stable, ranging within three percentage points. This indicates that while there may be variability in terms of total calcium over short periods of time, the relative concentration of ionic species is stable, having an average value of **34.1%**. The ratio of ionic to total calcium is also quite stable after drinking grapefruit juice, ranging from **31.2** to **34.7%** with an average value of **33.1%**. This value is quite close to the ratio observed in the control group. In contrast, the ratio observed after drinking cranberry juice cocktail showed the greatest variability, ranging from **34.9** to 40.8%, with an average value of **38.2%** for the 6-hour time period. From the in vivo results it appears that neither cranberry juice nor grapefruit juice reduces the level of calcium ions (Table **3C)** in the urine of normal people, at least in short term. This is in agreement with Light et al. (1973), who demonstrated that nonstoneforming subjects had no significant change in the level of ionized calcium after drinking cranberry juice cocktail.

In conclusion, the following observations were made: 1) Organic acids have the ability, in vitro, to complex ionized calcium, 2) Fruit juices such as cranberry juice cocktail and grapefruit juice contain sufficient amounts of organic acids to theoretically increase the level of organic acids excreted in urine. However, the results indicate that these acids are not excreted in any significant amounts. And 3) Drinking 10 ounces of fruit juice did not increase the level of organic acids in the

urine of nonstoneforming patients nor did it significantly alter urinary ionized calcium in these subjects. However, based on the reports of Pak et al. **(1984)** and Light et al. (1973), and the results presented here, it might be expected that drinking fruit juices should increase the level of organic acids excreted in the urine of stoneforming patients and thus provide some protection against the formation of calcium-containing stones.

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TABLE 1

comparison of the effect of organic acids on calcium ion concentration at pH 4, 5, and 6, in vitro

	Ionized Calcium Concentration		
	pH 4	pH 5	pH 6
CONTROL	1.05 ± 0.10 mM	11.06 ± 0.10 mM	1.10 ± 0.19 mM
CITRIC ACID	-23.2 ± 0.05% *	-20.0 ± 0.04%*	-16.2 ± 0.20%*
MALIC ACID	-25.4 ± 0.13% *	-18.7 ± 0.04%*	-19.6 ± 0.15%*
QUINIC ACID	-10.7 ± 0.10% *	-14.9 ± 0.06%*	-2.92 ± 0.30%*

n = 25

Values expressed in mean + sem

* significantly different from control ($p \leq 0.05$)

TABLE 2

Urinary pH after drinking water, cranberry juice cocktail (CJC), and grapefruit juice (GJ)

	0 Hour	2 Hour	4 Hour	6 Hour
Water	5.35 ± 0.20	5.90 ± 0.24	6.13 ± 0.38	6.23 ± 0.35*
CJC	5.36 ± 0.27	5.88 ± 0.19	6.63 ± 0.30*	5.59 ± 0.37
GJ	5.31 ± 0.14	5.91 ± 0.19*	6.31 ± 0.34*	6.07 ± 0.35

n = 8

Values expressed in mean + sem

* significantly different from 0 Hour ($p \leq 0.05$)

TABLE 3

Urinary calcium after drinking water, cranberry juice cocktail (CJC), and grapefruit juice (GJ)

A) Ionic Calcium[1,2]

	0 Hour	2 Hour	4 Hour	6 hour
Water	9.63 ± 1.23	7.76 ± 3.05	9.33 ± 2.43	7.74 ± 1.95
CJC	9.68 ± 1.92	12.3 ± 3.49	9.47 ± 1.58	7.03 ± 1.51
GJ	12.3 ± 3.46	6.95 ± 1.45	8.89 ± 1.32	8.54 ± 1.26

B) Total Calcium[1,2]

	0 Hour	2 Hour	4 Hour	6 Hour
Water	29.4 ± 2.65	21.2 ± 9.73	26.1 ± 6.65	24.4 ± 5.58
CJC	25.4 ± 3.91	31.6 ± 8.65	23.2 ± 4.30	20.1 ± 4.15
GJ	31.5 ± 6.68	22.3 ± 5.34	25.6 ± 4.83	25.2 ± 4.58

C) % Ionized/Total Calcium[2]

	0 Hour	2 Hour	4 Hour	6 Hour
Water	32.7 ± 0.46%	36.6 ± 0.31%*	35.7 ± 0.37%	31.7 ± 0.35%
CJC	38.1 ± 0.49%	38.9 ± 0.40%	40.8 ± 0.37%	34.9 ± 0.36%
GJ	32.8 ± 0.52%	31.2 ± 0.27%	34.7 ± 0.28%	33.8 ± 0.28%

n = 8

1) Values expressed in mg/dl

2) Values expressed in mean ± sem

* Significantly different from 0 Hour

TABLE 4

Urinary citric acid after drinking water, cranberry juice cocktail (CJC), and grapefruit juice (GJ)

	0 **Hour**	2 **Hour**	4 **Hour**	6 **Hour**
Water	**35.6** ± 17.4	54.9 ± 22.6	49.3 ± 20.4	48.1 ± 14.9
CJC	59.1 ± 29.5	44.0 ± 16.0	47.2 ± 18.9	86.8 ± 35.9
GJ	65.7 ± 24.4	68.8 ± 26.2	41.9 ± 13.9	76.8 ± 23.4

n = 8

Values expressed in mg/dl

Values expressed in mean ± sem

TABLE 5

Urinary malic acid after drinking water, cranberry juice cocktail (CJC), and grapefruit juice (GJ)

	0 Hour	2 Hour	4 Hour	6 Hour
Water	16.0 ± 6.48	15.3 ± 7.37	21.2 ± 8.45	26.2 ± 7.32
CJC	23.9 ± 12.1	12.8 ± 6.21	8.78 ± 4.56	19.3 ± 9.72
GJ	20.0 ± 7.98	14.1 ± 6.41	10.8 ± 4.51	16.2 ± 7.98

n = 8

Values expressed in mg/dl

Values expressed in mean ± sem

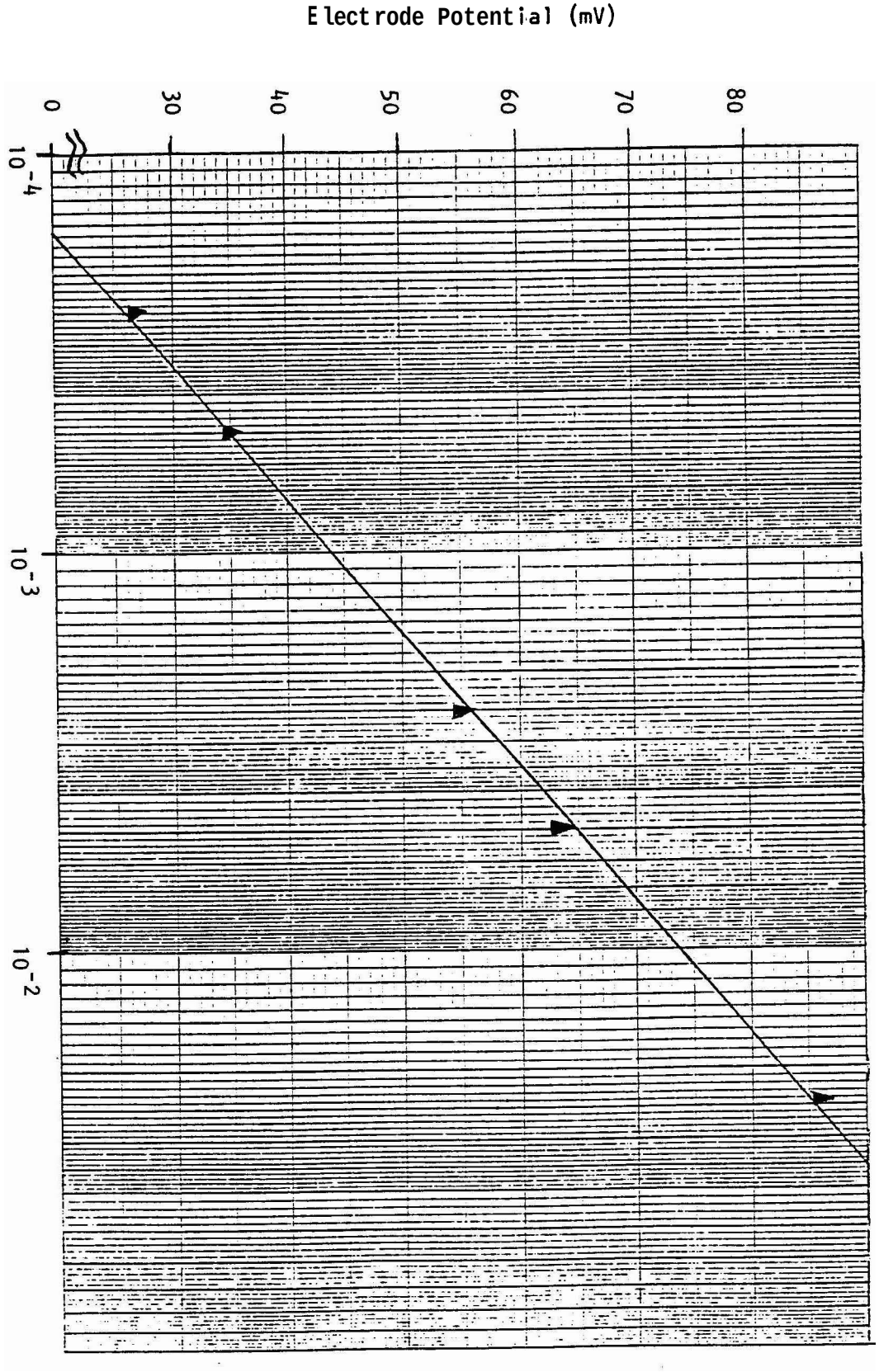


FIGURE 1

Calibration curve for ionized calcium in synthetic urine

FIGURE 2

Organic acid chromatograms

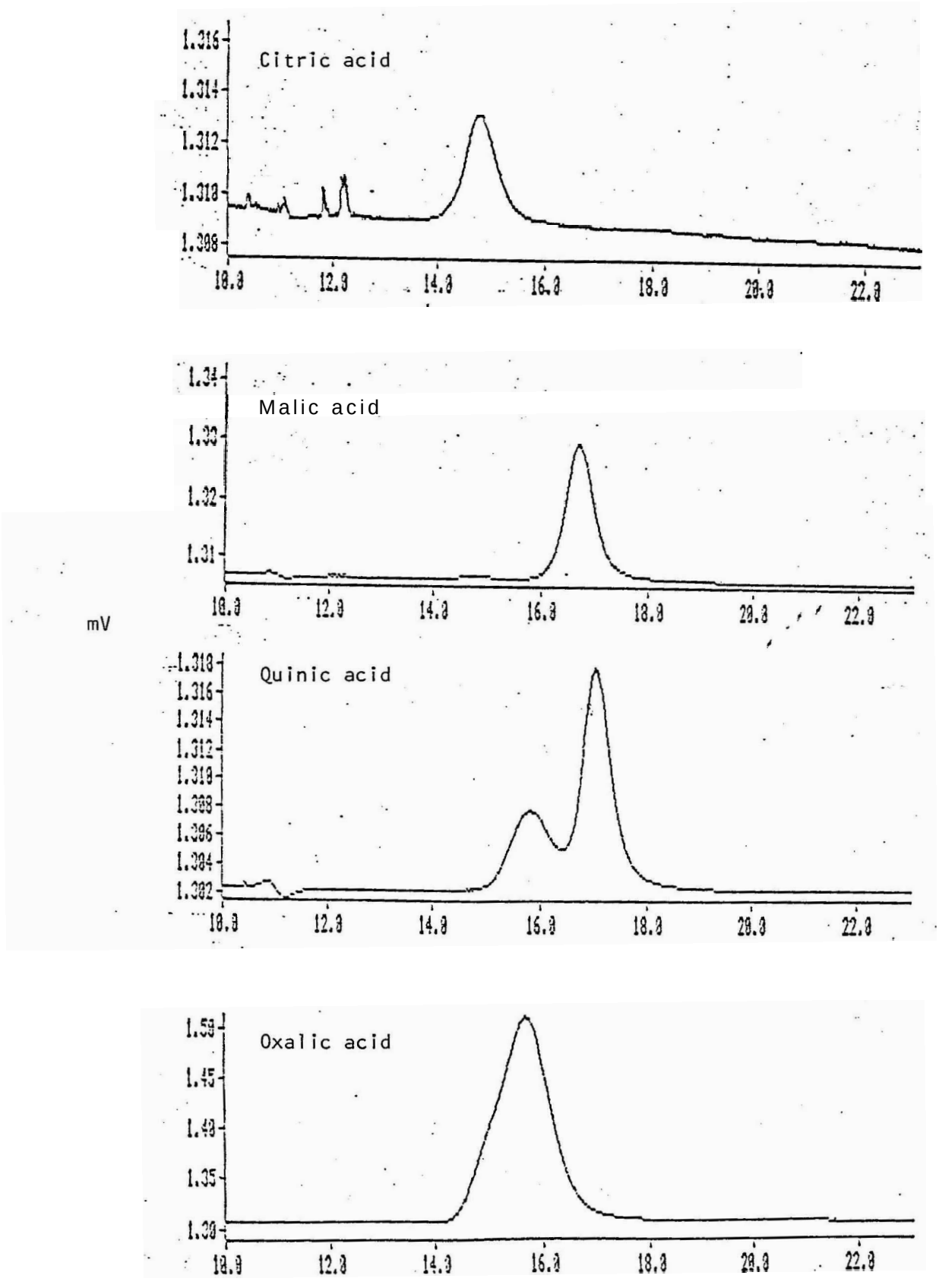


FIGURE 3

Chromatogram of cranberry juice cocktail

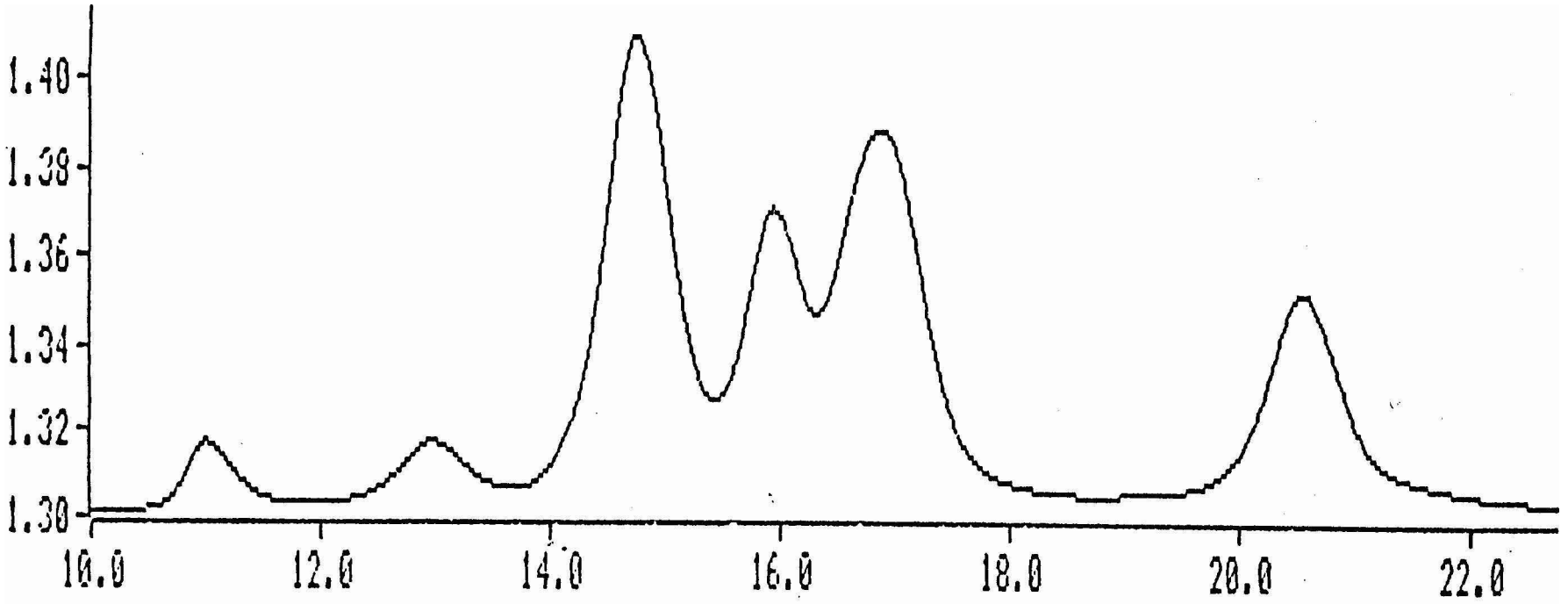


FIGURE 4

Chromatogram of grapefruit juice

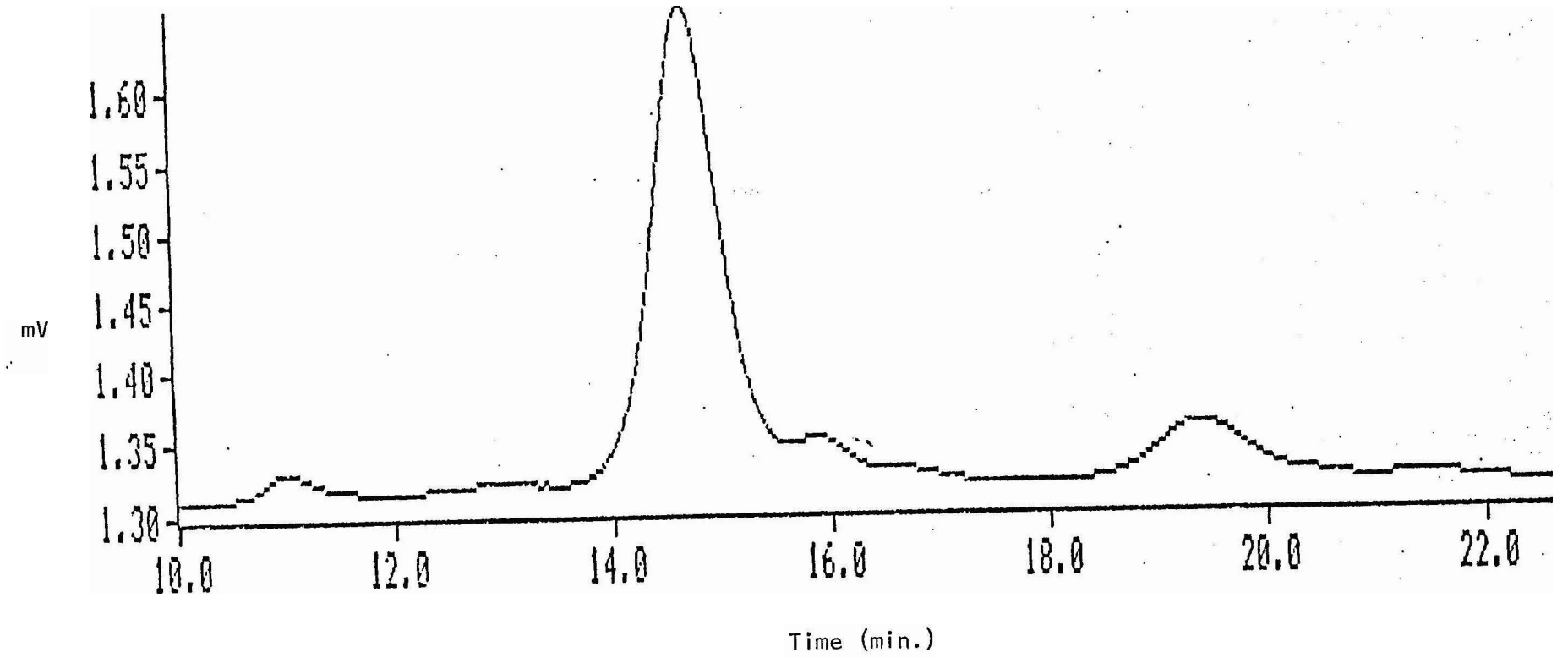
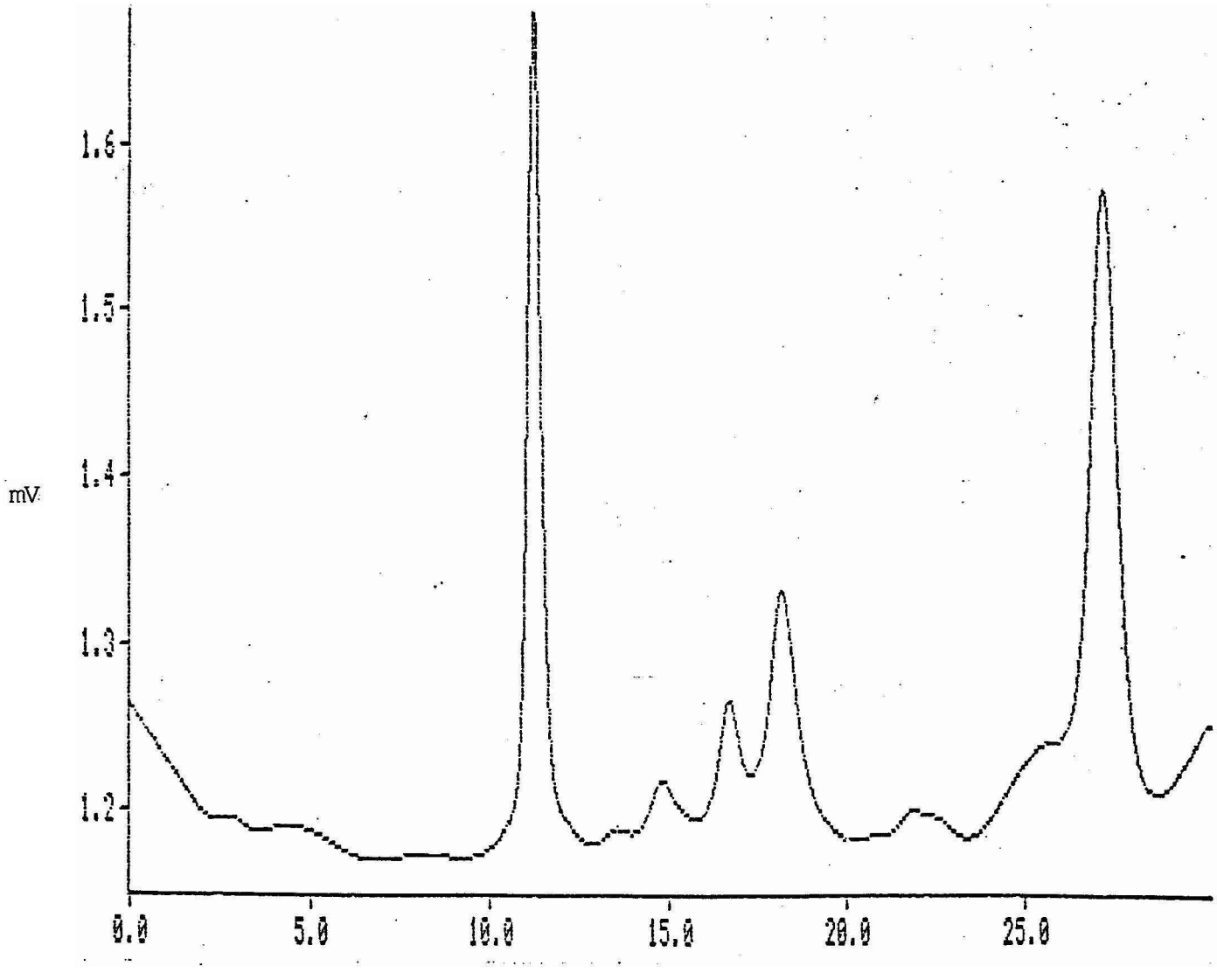


FIGURE 5

Chromatogram of a typical urine sample



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