THE DETERMINATION OF 5-OXOPROLINE BY

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

by

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ABSTRACT

THE DETERMINATION OF 5-OXOPROLINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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This work deals with the development of a simple procedure for the quantification of 5-oxoproline in human urine. It is an attempt to make it applicable to routine use in the clinical laboratory. This is an extension of the study done by Robert Edwards M.S Thesis (Youngstown State University, 1978) and involves the adaptation of an existing "qualitative HPLC method for the general screening of organic acid disorders".

An HPLC with UV detector and a Bio-Rad Aminex HPX-87H column were used. This column containing the sulfonated polystyrene-divinyl benzene co-polymer was very stable with prolonged good column condition and provided a very good separation system.

The assay procedure was very simple. 20 µL of filtered urine was injected onto the column using 8 mM sulfuric acid as the mobile phase at a flow rate of 1.0 mL/min. The peak of the unknown was identified using

the retention time and peak height ratios at two different wave lengths in the UV. To quantify 5-oxoproline, an external standard was used. A calibration curve was made with different concentrations of 5-oxoproline by plotting the amount of 5-oxoproline injected in nanomoles against peak height in mm (absorbance). The peak height of the unknown was then read from the chromatogram obtained and the amount was interpolated from the calibration curve. The calibration curve was a typical Beer's Law plot with a linearity range of 0 to 20 nanomoles. The limit of detection was approximately 2.0 nanomoles.

This assay involves about 15 minutes, but the column needs to be cleaned for the next assay, Since urine contains numerous compounds, an average of 45 minutes is required for cleaning after each assay,

Clinical studies were made on five different diseased urines. Two homocystinuric urines were found to contain substantial amounts of 5-oxoproline. Test samples from two patients with creatinine clearance problems were examined, but only one patient excreted 5-oxoproline; the other chromatogram showed no trace of 5-oxoproline.

This study has shown that the method is simple, fairly rapid, sensitive and can be utilized in any small clinical laboratory which has an HPLC with UV detection.

For further research the assay should be performed on a statistical basis, in order to develop a baseline for the normal values in different populations, e.g., male vs. female, adult vs. child, etc.

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ABBREVIATION AND DEFINITIONS

Dead Volumes	any volume between the injector and detector where separation does not occur.
Elution chromatography	the removal of the components of interest from the column with an eluent.
Flow rate	milliliters of mobile phase which are pumped during a given time frame.
K' values	# of moles of solute in the stationary phase divided by the # of moles of solute in the mobile phase - retention of the component compared to the 'solvent front (or dead volume)
Mobile phase	solvents which are pumped through the system coursing over the column and eluting components.
Pattern of elution	Order of appearance of the peaks in the chromatogram
Regulatory enzymes	regulate the metabolic pathways in which they are involved.
Relative Standard Deviation (S.D.)	the measure of precision (also known as coefficient of variation)
TMS	Trimethyl silyl
AUFS	Absorbance unit full scale

CHAPTER I

INTRODUCTION

STRUCTURE OF 5-OXOPROLINE

The cyclization of glutamic acid forms an anhydride, which is commonly known as 5-oxoproline. The synonyms of 5-oxoproline are pyroglutamic acid, 5-oxo-2-pyrrolidine carboxylic acid, 2-pyrrolidone-5-carboxylic acid, glutiminic acid, α- amino glutaric acid lactam and glutamic acid lactam.

The correct structure of 5-oxoproline was reported in 1890's by Menozzi and Appiani (1,2). Pattabhi and Venkatesan (3) determined the detailed crystal structure of 5-oxoproline by the method of symbolic addition, from 934 observed three-dimensional photographic data and reported that the "crystals are monoclinic with a = 8.14, b = 8.86, c = 9.32A (all $\pm$ 0.02A) and B = 116.5 (2), Z = 4, space group P2./c. The structure (shown in fig. 1) was refined by full matrix least-squares method to R 0.091." The structure is stablized by N.....H and O — H.....O type hydrogen bonds, The dimensions of the amide group which is significantly non-planar are comparable to those found in the peptides.

The five-membered ring with the cis-type amide bond causes the non-planarity of the amide group.

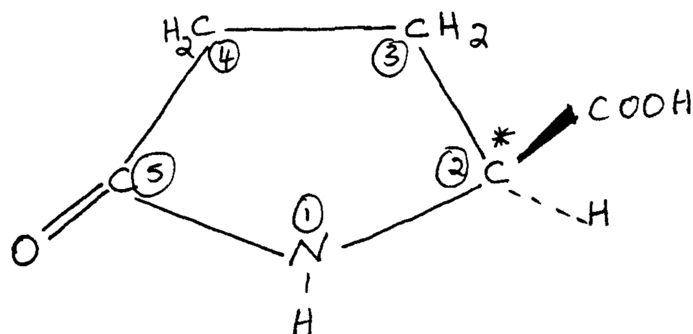


Fig. 1. Structure of **5-Oxo-L-Proline**

The carbon atom at position 5 has a double bond with an oxygen atom which makes the compound play an important role in ultra-violet spectroscopy.

CHEMICAL BACKGROUND

5-Oxoproline exists in the form of D and L enantiomers (2), which is due to the chiral carbon atom-at position 2, having a carboxylic acid substituent and the H-atom. As usually designated, the wedge representation in fig. 1 signifies that the carboxyl group lies above the plane of the paper, while the dotted line signifies that the hydrogen atom lies below the plane of the paper. On reversing the substituents on the carbon atom at position 2, the D form would result.

5-Oxoproline is obtained by heating glutamic acid at 180-190°C (4). Wilson and Canon (5) demonstrated that in an aqueous solution, 5-oxoproline and glutamic acid

are present in equilibrium. The equilibrium position is dependent upon pH and temperature. Some of the typical equilibrium values at a pH of 0.13, 3.96, 11.81 and 13.38 at 25° C are 8.0, 97.8, 62.7 and 1.9% of 5-oxoproline respectively.

5-Oxoproline is a derivative of the amino acid **proline;** as can easily be noted by numbering the ring atoms in the molecule.

The molecular weight of this compond is 129.11. The specific rotation of **5-oxo-L-proline (2g/100 mL** water) at **20° C** and using the D line of **sodium,** $[\alpha]_D^{20°}$, is **-11.9°** C (6). It is soluble in alcohol, acetone and water. The melting point of the DL form is about 182° C.

A number of derivatives of 5-oxoproline have been encountered. The most commonly found derivative of 5-oxoproline is theanine (glutamic acid ethyl amide), a major component in tea extracts (7). It is formed by the following reaction.

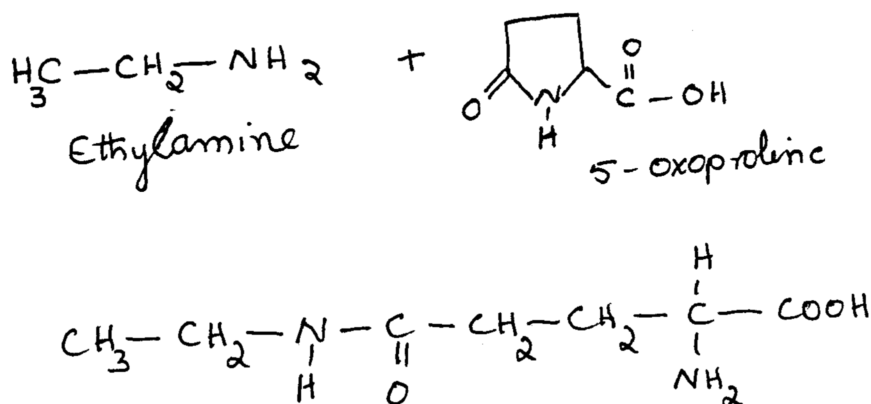


Fig. 2. Theanine

(glutamic acid ethyl amide)

Kanso *et al* (8), determined the structure of another derivative, N-hydroxymethyl-pyrrolidone carboxylic acid, which is produced by the reaction of pyroglutamic acid with formaldehyde.

Recently one more derivative of 5-oxoproline has been determined by a group of Japanese workers (9). The derivative is a new antibiotic designated as FR-900148 which is produced by a strain of Streptomyces xanthocidicus. The structure has been established as 1-N-valyl-3-chloro-2,5-dihydro-5-oxo-1H-pyrrole-2-carboxylic acid, on the basis of spectroscopic and chemical evidence.

Pyroglutamic acid has been reported by various workers as being tasteless (10,11), bitter (12) and a flavour enhancer (13) that could be formed from glutamate during processing or reconstitution of dried food products (5). It is not detected by most glutamate analyses (14).

PHYSIOLOGICAL BACKGROUND

"5-Oxoprolinuria" or "pyroglutamic aciduria" may be due to an inborn error of glutathione metabolism. Glutathione is an ubiquitous tripeptide (L-γ-glutamyl-L-cysteinylglycine) found in relatively higher concentrations in many mammalian tissues, in millimolar concentrations intracellularly and in relatively lower concentrations in blood plasma and urine. Glutathione also occurs in several polypeptides including fibrinogen,

immunoglobulins, and collagen as the N-terminal residue. It serves as a storage form of cysteine.

The γ-glutamyl linkage and the sulfhydryl group of the glutathione molecule play an important role in glutathione metabolism (or the γ-glutamyl cycle). Glutathione has important functions in the central nervous system. Patients with defects of the γ-glutamyl cycle are mentally retarded and exhibit other brain defects. The functions, metabolism and the five different categories of diseases related to glutathione metabolism are described below (15).

Functions of Glutathione

(i) Glutathione is a participant in transhydrogenation reactions that function in the formation and maintenance of the sulfhydryl groups of other molecules (e.g., coenzyme A and various enzymes and other proteins).

(ii) It provides a reducing capacity for some reactions, e.g., the formation of deoxyribonucleotides by ribonucleotide reductase.

(iii) Glutathione functions in the detoxification of hydrogen peroxide, other peroxides and free radicals.

(iv) Glutathione also functions in the detoxification of a variety of foreign compounds which interact with glutathione, and which are ultimately excreted in the urine or feces in the form of mercapturic acids. Similar

derivatives of glutathione are formed in endogenous metabolism, **e.g.,** in the metabolism of steroids, prostaglandins, leukotrienes, and melanins.

(v) The **γ-glutamyl** moiety of glutathione plays an important role in the transport of amino acids, **peptides** and amines.

THE γ-GLUTAMYL CYCLE

The **γ-glutamyl** cycle (16) shown in fig. 3 involves the biosynthesis and the degradation of glutathione. The cycle runs through six enzyme-catalyzed reactions which are described below:

(i) **γ-Glutamyl** transpeptidase: this membrane - bound enzyme catalyzes the reaction between an extracellular amino acid and glutathione to form cysteinylglycine and the **γ-glutmyl** amino acid in the cytoplasm.

(ii) Peptidase: this enzyme catalyzes the dissociation of cysteinylglycine in the cytoplasm to form glycine and cysteine (reaction 6).

(iii) **γ-Glutamyl** cyclotransferase: the **"release"** of the amino acid to the cellular pool and simultaneous cyclization of the **γ-glutamyl** moiety to 5-oxoproline is catalyzed by this enzyme. (reaction 2)

(iv) 5-Oxoprolinase: this enzyme catalyzes the hydrolysis of 5-oxoproline to glutamic acid; the energy

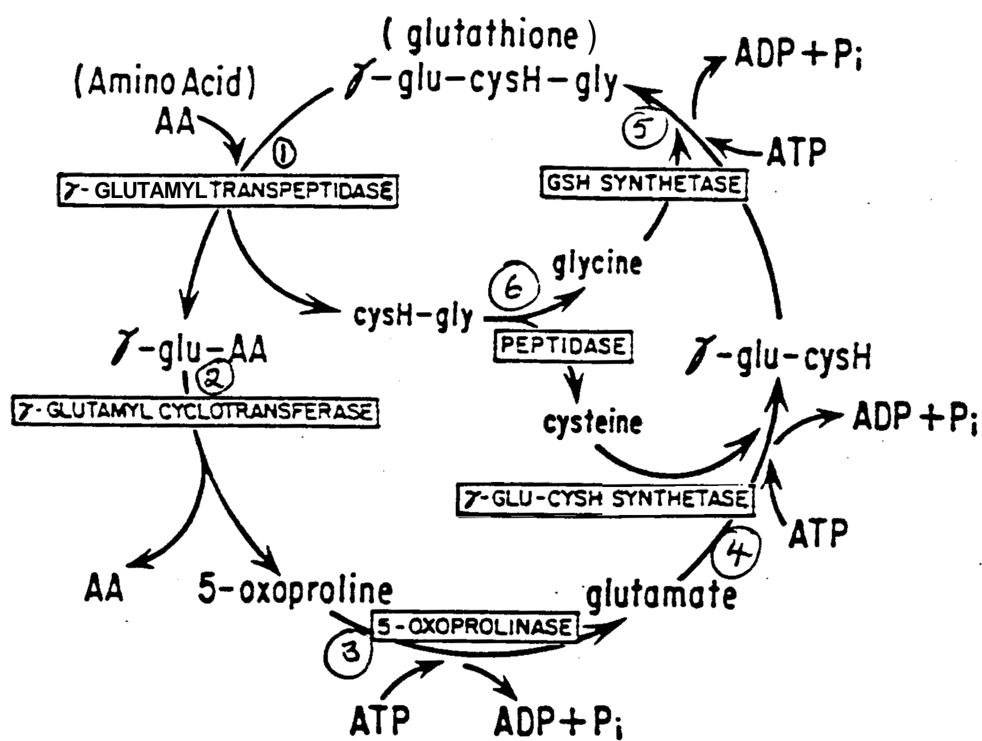


Fig.3 The γ-glutamyl cycle (16)

for **this reaction is** supplied by the cleavage of ATP to ADP and inorganic phosphate (Pi). (reaction 3)

(v) **γ-Glutamylcysteine** synthetase: this enzyme catalyzes the formation of **γ-glutamylcysteine** from glutamic acid and cysteine. ATP is cleaved to form ADP and inorganic phosphate (Pi). (reaction 4)

(vi) Glutathione synthetase: the cycle is completed by the action of this enzyme on glycine and γ-glutamylcysteine to form glutathione and involves the expenditure of an ATP. (reaction 5)

Apart from these pathways described in this γ-glutamyl cycle, **Eldjarn,** **et al** (17) have speculated that there are three alternate pathways of 5-oxoproline degradation.

(i) The acetylation of 5-oxoproline to yield **N-acetyl-5-**oxoproline and its subsequent hydrolysis to **N-acetyl-**glutamate.

(ii) The conversion of 5-oxoproline to **5-hydroxyproline** followed by dehydration to **Δ^1-pyrroline-5-carboxylate.**

(iii) 2-hydroxylation of 5-oxoproline forms **2-hydroxy-5-**oxoproline which undergoes further reaction to yield α-ketoglutaramate.

γ-Glutamyl transpeptidase is the only **membrane-**bound enzyme of the cycle. It is found abundantly in the

epithelial cells of nephron, choroid plexus, jejunum ciliary body and other tissues which are extensively involved in transport (18). Transpeptidase is found on the surface of cell membranes, but glutathione is found intracellularly. This evidence led to the postulate that there must be an intracellular glutathione transporting mechanism. The *in vivo* studies made by injecting animals with inhibitors of transpeptidase produced extensive glutathionuria (19). This study also showed that although this enzyme can catalyze hydrolysis of glutathione and other **γ-glutamyl** compounds *in vitro*, transpeptidation is a major function of it *in vivo*. The most active amino acid acceptors are cystine (20) glutathione, glycine, methionine and other neutral amino acids, but not **proline.** Tissues that have low transpeptidase activity, such as liver and muscle, export glutathione to the blood plasma.

Normally the plasma glutathione levels are in the micromolar range (human 1 to 3 μM) (21) but on inhibition of transpeptidase, the plasma glutathione levels increase. On the contrary when buthionine sulfoximine, an inhibitor of glutathione synthesis, is administered, there is a prompt decrease in the tissue glutathione levels and thereby in the plasma glutathione levels. Anephric animals treated with transpeptidase inhibitors show that about two-thirds of the plasma glutathione is used by the kidney and the remainder by extrarenal

transpeptidase. About 80 percent of arterial plasma glutathione is removed during passage through the kidney. Extracellularly glutathione is oxidized to glutathione disulfide by a non-enzymatic process (22).

It is postulated that the transport mechanism for γ-glutamyl amino acids is separate from those that transport free amino acids, but similar to the transport of some other dipeptides. Direct evidence for this phenomenon has been obtained. The γ-glutamyl cyclotransferase is most active towards the L-γ-glutamyl derivatives of the L-isomers of several amino acids, e.g., glutamine, methionine, alanine, cysteine, cystine and serine. This enzyme is also highly active towards a wide variety of di-γ-glutamyl amino acids, which may be formed by the action of transpeptidase (23).

5-Oxoprolinase is the least active enzyme *in vitro* among the enzymes of the cycle (24). The other enzymes of the cycle, like the glutathione synthetase, γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase have lower activities *in vivo* than the activities found *in vitro* (25). The reaction catalyzed by 5-oxoprolinase is the slowest step in the γ-glutamyl cycle and is considered to be the rate-limiting step. Even a complete block of 5-oxoprolinase activity would not stop the cycle because glutamate can be produced by other pathways from glutamine and α-ketoglutarate. The effect of a competitive inhibitor of 5-oxoprolinase in

rats produced an increase in the tissue concentration and urinary excretion of 5-oxoproline. Specific enzyme inhibitors were applied and blocks of the γ-glutamyl cycle were achieved (16, 26, 27). Administration of amino acids increases transpeptidation, which ultimately produces 5-oxoproline by the action of γ-glutamyl cyclotransferase. Administration of larger amounts of amino acids produces a decrease in the renal glutathione levels (28), but administration of γ-glutamyl transpeptidase inhibitors does not decrease the renal glutathione levels.

Glutathione inhibits γ-glutamylcysteine synthetase in a non-allosteric manner. Glutathione is a competitive inhibitor with respect to glutamate. The other less effective inhibitors are γ-glutamyl-α-aminobutyrate and γ-glutamyl-α-aminobutyrylglycine. The synthesis of glutathione is inhibited by buthionine sulfoximine. γ-Glutamylcysteine synthetase is inhibited *in vivo* by both buthionine sulfoximine and methionine sulfoximine. Glutamine synthesis is inhibited by α-ethylmethionine sulfoximine.

Thus, due to all these different inhibitors and deficiencies there is a pronounced change in the γ-glutamyl cycle. This change in the cycle has been very closely studied with reference to 5-oxoprolinuria and the modified γ-glutamyl cycle in 5-oxoprolinuria has been postulated by Wellner *et al* (29), which is shown in fig.4.

Diseases related to glutathione metabolism (15)

1. 5-Oxoprolinuria: this is due to the deficiency of glutathione synthetase, except in erythrocytes, which leads to the deficiency of glutathione. Glutathione normally regulates its own biosynthesis by inhibiting γ-glutamylcysteine synthetase - the enzyme that catalyzes the first step in the biosynthesis of glutathione. Due to this marked reduction of glutathione levels, there is increased formation of γ-glutamylcysteine, which is converted to 5-oxoproline by the action of γ-glutamyl cyclotransferase (fig. 5). The overproduction of 5-oxoproline exceeds the capacity of 5-oxoprolinase to convert this substrate to glutamate, and some of the 5-oxoproline is therefore excreted in the urine. The metabolic defect leads to a modified γ-glutamyl cycle in which there is a futile synthesis of γ-glutamylcysteine followed by its convertion to 5-oxoproline and cysteine.

Thus 5-oxoprolinuria, an inborn error of glutathione metabolism, is characterized by massive urinary excretion of 5-oxoproline, elevated levels of 5-oxoproline in blood and cerebrospinal fluid, severe metabolic acidosis, tendency towards hemolysis and defective central nervous system function.

2. Erythrocyte glutathione synthetase deficiency: this is associated with the synthesis of an unstable

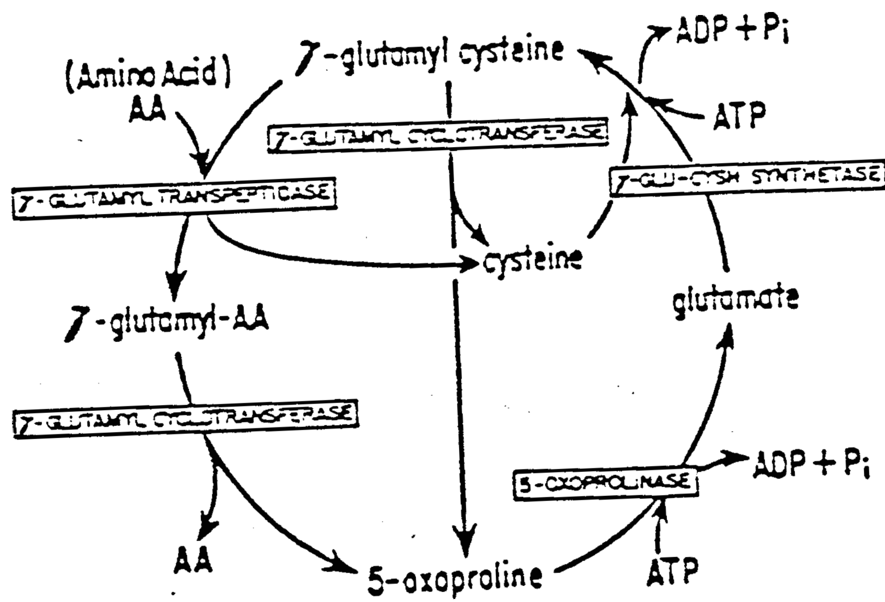


Fig.4 Modified γ-glutamyl cycle (29)

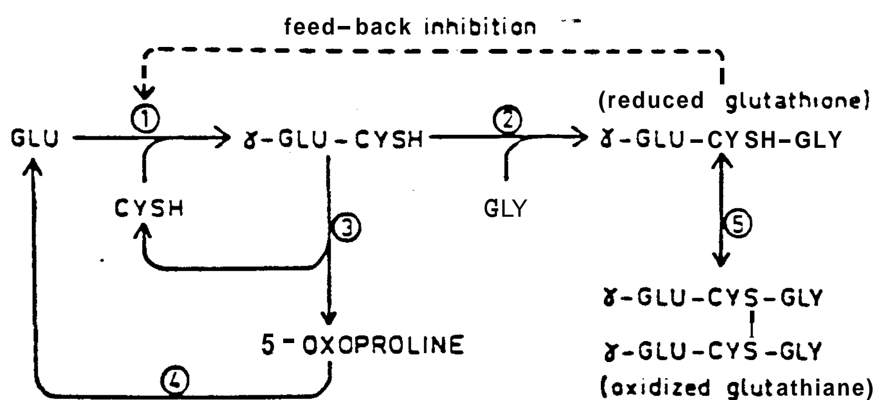


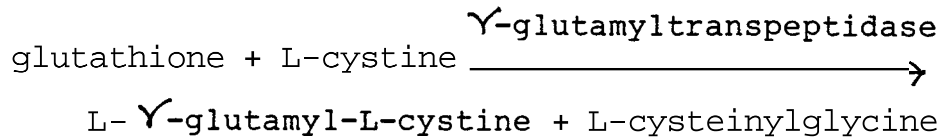
FIG. 5 Mechanism of 5-oxoproline overproduction in patients with glutathione synthetase deficiency. Enzymatic steps involved in the biosynthesis of glutathione (1) γ-glutamylcysteine synthetase; (2) glutathione synthetase; (3) γ-glutamylcyclo tnnsferase: (4) 5-oxoprolinase; (5) glutathione reductase. (16)

glutathione synthetase molecule. The turnover of this defective but active enzyme, is so rapid that in most tissues it compensates well for the defect, but this is not the case in the erythrocyte, where protein synthesis does not take place. Thus glutathione synthetase deficiency, which is apparently restricted to the erythrocyte, is associated with reduced erythrocyte glutathione levels and well compensated for hemolytic disease. 5-Oxoprolinuria does not occur.

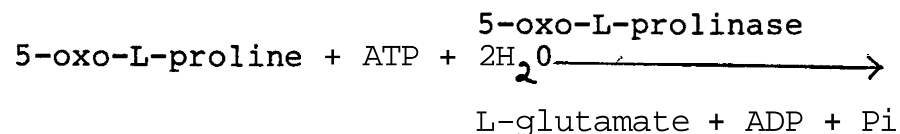
3. <u>γ-glutamylcysteine synthetase deficiency</u>: this disease is associated with generalized glutathione deficiency and marked deficiency in the synthesis of γ-glutamyl compounds. These patients exhibit hemolytic anemia, spinocerebellar degeneration, peripheral neuropathy, myopathy and aminoaciduria.

4. <u>γ-glutamyl transpeptidase deficiency</u>: this disease is associated with patients exhibiting central nervous system involvement, glutathionemia and urinary excretion of substantial amounts of glutathione, γ-glutamylcysteine and cysteine moieties. The urinary excretion of these sulfur containing compounds suggests, that the physiologic function of γ-glutamyl transpeptidase is associated with their metabolism and or their transport. The transport of γ-glutamyl amino acids is found to be inhibited by high concentrations of glutathione.

The γ-glutamyl transpeptidase activity is not completely absent in the patients. Even in the experimental animals treated with potent γ-glutamyl transpeptidase inhibitors, the activity of the enzyme is not completely abolished.



5. <u>5-Oxoprolinase</u> <u>deficiency</u>: These patients are associated with excretion of moderate amounts of 5-oxoproline in their urine, but have higher than normal blood plasma levels of 5-oxoproline. Although 5-oxoprolinase deficiency would decrease conversion of 5-oxoproline to glutamate, there is no deficiency of glutamate, and also there is no complete lack of 5-oxoprolinase in the diseased state.



STATEMENT OF THE PROBLEM

Primary disorders of organic acid metabolism, although rare, are being studied in many large clinical laboratories. One of the main reasons for the lack of widespread testing is due to the unavailabilty of a simple, inexpensive technique for their detection. Hitherto, these disorders could only be diagnosed by the use of a complex analytical procedure involving gas-chromatography and/or the coupling of gas-chromatography and mass spectrometry (GC-MS). These techniques are labor-intensive and require highly skilled operators. The sample preparation also involves a series of steps for their extractions and derivatizations.

The purpose of this research is to employ the qualitative procedure of Bennett and Bradey (30) - "simpler liquid - chromatographic screening of organic acid disorders" by the use of High-Performance Liquid - Chromatography (HPLC), to develop a technique for the quantification of 5-oxoproline in human urine. It is an attempt to make it applicable to routine use in the clinical laboratory. This technique would not only be helpful in the diagnosis, of 5-oxoprolinuria but may also prove that this disorder is more prevalent than is currently appreciated.

This research work is aimed at developing a quantitative technique which is simple, fairly rapid, sensitive and can be utilized in any small clinical

laboratory which has an HPLC with ultra-violet (UV) detection.

HPLC is often preferred because derivatization of the compounds are not usually required for the analysis.

CHAPTER II

REVIEW OF LITERATURE

CURRENT METHOD OF 5-OXOPROLINE DETECTION

The different qualitative and quantitative methods employed until 1977-78 for the detection of 5-oxoproline have been well described by Robert Edwards (6). These and some of the recently developed methods will be briefly discussed here in four different categories,

1. Colorimetric methods: colorimetric studies were made by several workers. Ramkrishna and Krishnaswamy (31) separated 5-oxoproline from urine extracts by paper chromatograpy. Then by the application of hydroxylamine hydrochloride, the 5-oxoproline was converted to γ-glutamylhydroxamate, which could be detected by the application of ferric chloride. The ferric chloride-hydroxamic acid complex was measured **colorimetrically** at 535 nm after elution from the chromatogram.

For the quantitative estimation of 5-oxoproline Lin, *et al* (32) developed a technique which was similar to the one employed by Rydon and Smith (33). In this technique 5-oxoproline is brominated using bromine in aqueous acetic acid; then the unreacted bromine is extracted into chloroform and the N-bromo-5-oxoproline

is mixed with a solution of potassium iodide. Hydroiodic acid (HI) formed in the solution from the potassium iodide is oxidized to iodine (I_2) and N-bromo-5-oxoproline reduced to 5-oxoproline. The yellow solution resulting from the iodine formed is measured spectrophotometrically at 400 nm. By this method a linear standard curve in the range of only 1 to 8 mM could be produced. Thus this method was not sensitive enough for determinations in biological fluids.

Robert Edwards (6) employed the triiodide assay procedure. This method involved a series of steps (shown in fig. 6) leading to the formation of a characteristic yellow-colored triiodide ion, which was measured spectrophotometrically at 390 - 393 nm. Micromolar quantities of 5-oxoproline could be detected by this method but in practice it involved too many interfering substances and an average time of 65 minutes for each assay.

2. <u>Chromatographic methods</u>: gas chromatography was employed by Wilk and Orlowski (34) for the determination of 5-oxoproline in serum and cerebrospinal fluid. 5-Oxoproline was determined as an esterified derivative after reaction with pentafluoropropanol in pentafluoropropionic anhydride.

Rydon and Smith (33) in 1952 employed paper chromatographic methods for detection of peptides and

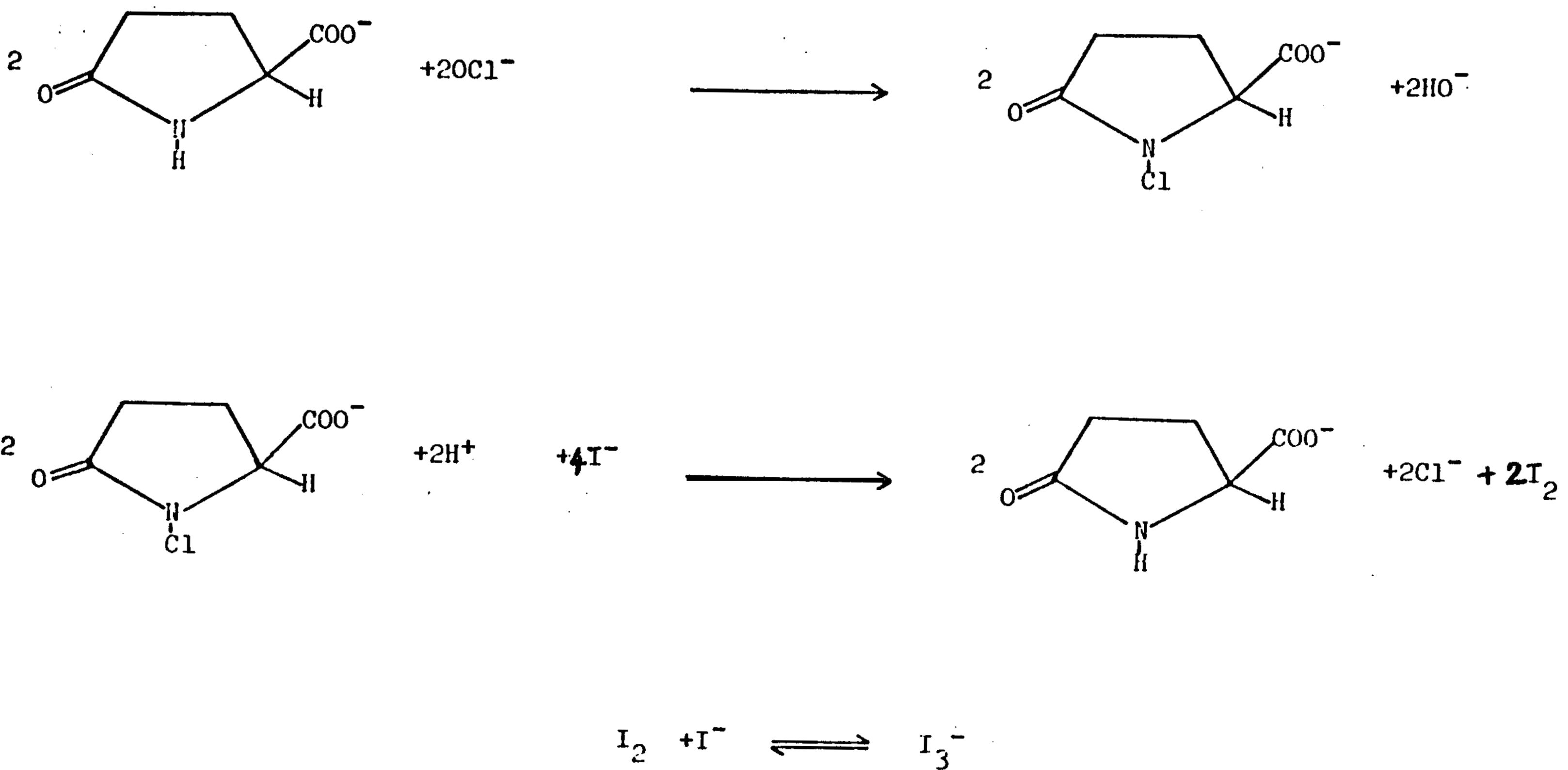


Fig. 6 Reactions in the Triiodide Assay Procedure

amino acids by the ultimate formation of a starch iodine complex. In this technique, **peptides** and amino acids are N-chlorinated with chlorine gas and sprayed with a solution of 1% starch - 1% potassium iodide after sufficient aeration. By this the N-chloro derivative oxidizes the iodide to iodine and ultimately the starch is complexed to produce a blue-black color.

A similar technique was used by Mazur et al (35), and Schwartz and Pallanch **(36),** but these people used tertiary butyl hypochlorite as a chlorinating agent. Pan and Dutcher **(37)** reported the use of sodium hypochlorite as a chlorinating agent.

A gas-liquid chromatographic method was developed by **Polgar** and Meister (38). A D-glutamate cyclotransferase preparation obtained from rat kidney was employed for the enzymic cyclization of D-glutamic acid, and then 5-oxoproline was converted to the corresponding methyl ester and determined quantitatively.

Heidiger et al (39) also employed the gas chromatographic method for the determination **of** asparagine, glutamine and pyrrolidonecarboxylic acid as TMS derivatives in total enzymic hydrolysates of **peptides** and glycopeptides.

Another application of gas chromatography in the eighties was, "Determination of 2-Pyrrolidone-5-carboxylic and **α-Ketoglutaramic** Acids in Human Cerebrospinal **Fluid"** as TMS derivatives by Cooper et al

(40, 41).

Dousin et al (42) developed the use of capillary column gas chromatography for the identification and determination of non-volatile organic acids. The use of simultaneous dual-column capillary gas chromatography has been introduced for the identification of metabolites diagnostic for organic acidurias as TMS derivatives by Tsai et al (43).

In 1979 Ogura et al (44) employed HPLC for the determination of acetic acid, pyroglutamic acid and alcohol in Soy Sauce. Brechbuhler and Holinger (45) used HPLC for determination of organic acids in body fluids.

In the study of flavours and non-alcoholic beverages Sporns (14) quantified pyroglutamic acid in food **by using HPLC.** In 1983 Dimaline **and Reve** (46) employed reverse-phase high-performance liquid chromatography to monitor enzymatic cleavage of pyrrolidone carboxylic acid from regulatory peptides. For measurement of the urinary aromatic organic acids, vanilmandelic acid and homovanillic acid, in the differential diagnosis of neuroblastoma, pheochromocytoma and related tumors, Binder and Sivorinovsky (47) used HPLC with electrochemical detection. HPLC with a multi-detection system was used in 1984 by Todoriki et al (48) for screening disorders of aromatic acid metabolism. Although pyroglutamic acid was not involved, the importance of their work lies in the detection system

used. They used three single UV detectors set at 260, 280, and 320 nm respectively and confirmed the detection of the particular aromatic acids using retention times and peak height ratios.

Another LC method of interest is by Thanh and Sporns (49) for the determination of flavour enhancers and chloride in food. They have reported the detection limit of 5-oxoproline in food as 20 μg/mL or 0.155 mM by their method using refractive index and UV detection in series.

Buchanan and Thoene (50, 51) utilized two columns in series, an Aminex HPX-87H organic acid column followed by a 15 cm, 5 μm C_{18} analytical column for profiling the urinary organic acids.

Allen et al (52) employed the diode array detector in the investigation of neonatal organic aciduria by isocratic cation-exchange liquid chromatography using Aminex HPX-87H column. This method proved helpful in tentatively identifying the peaks on an abnormal chromatogram while awaiting confirmation by mass spectrometry.

Bennett and Bradey (30), whose qualitative work is being employed here (in this research) for the quantification of 5-oxoproline, described an isocratic cation exchange chromatographic technique with ultraviolet detection for the screening of organic acid disorders in urine. These workers used 5-oxoproline in their system and reported a retention time of 18.6

minutes.

A recent method for determination of organic acids is by Daish and Leonard (53). They used HPLC for the rapid profiling of plasma organic acids. Plasma and serum, unlike urine, need a sample preparation step because of the excess of protein in them. These workers used Bond Elut SAX (quaternary amine) 2.8 mL volume disposible anion exchange column (manufactured by Analytichem International, Harbor City, CA, USA) for the sample preparation. This was then followed by the normal HPLC using Bio-Rad Aminex HPX-87H organic acid column (300 x 7.8 mm) protected by a Bio-Rad Aminex HPX-85H guard cartridge (40 x 4.6 mm) and 5 mM H_2SO as mobile phase.

3. Other methods: in 1973 Kesner et al (54) developed a titrimetric method for the determination of total organic acids in urine by extraction with organic solvents. This involved a very long process. The metabolic carboxylic acids were first extracted with a mixture of equal volumes of t-amyl alcohol-chloroform from an acidified sample of urine, that had been adsorbed to a short column of silica gel. Then the total acid in the extract was measured by titration with tetramethyl-ammonium hydroxide (10mM) to a phenophthalein endpoint.

In 1982 Cachron and Eggermont (55) made an isotachophoretic determination of relative apparent mobility (RAM) against chloride ion as an estimate of the

electrophoretic mobility of various ions, including pyroglutamic acid anion. As seen from their results (table 1) the RAM changes with the change in pH from 4.0 to 6.0 and then is fairly constant from pH 6.0 to 10.0.

TABLE 1

Relative Apparent Mobilities (RAM) of the anions of pyroglutamic acid relative to chloride ions (55).

pH	RAM
4.0	39.5
5.0	66.1
6.0	79.8
6.5	80.8
7.0	81.4
7.5	80.8
8.0	80.1
8.5	80.8
9.0	80.9
9.5	80.8
10.0	80.8

4. Combination Methods : in this category a complex analytical procedure is described, which is a compilation of gas liquid chromatography (GLC), mass spectrometry (MS) and computer analysis. This method was developed by Jellum, Stokke and Eldjarn (17), and used for the multicomponent analysis of biological materials. The gas-liquid chromatography is used for the separation, mass spectrometry for the identification and structure studies and the computer analysis for data handling. By the use of this method about 40 of the known inborn errors can be detected. These workers applied this method for analysis of more than 700 patients which led to the discovery of three new inborn errors of metabolism: methylmalonic aciduria, β-methylcrotonyl - CoA carboxylase deficiency and Pyroglutamicaciduria. Although this method was first started in 1972, even to date it is the most refined method, Since it is cumbersome, time-consuming and also requires state of the art equipment, it is being used only for confirmation purposes.

Thus, the determination of 5-oxoproline covers a wide range of analytical procedures, where the simple ones are not sensitive enough and the complex ones are not suitable enough for day to day work in the clinical laboratories due to their complexity, cost and time consuming features.

State of Diseased Patients

The studies on the enzyme activities involved in the γ-glutamyl cycle made a great contribution to the knowledge of the state of the diseased patients. Some of the most obvious features found in these patients will be summarized here (57, 61, 63).

The first and the most common feature of these patients is the change in the acid-base balance. A study of the placenta obtained at delivery of a patient # 1 with 5-oxoprolinuria revealed that the acid-base balance of the patient was normal for 4 hours after birth, but then she developed metabolic acidosis, and by the time she reached 20 hours of age, the blood pH decreased to 7.3. The urine contained 26 mM 5 oxo-L-proline. She had been maintained on a regular oral dose of sodium bicarbonate. Jaundice had been detected in her between 50 - 70 hours which disappeared by the end of the 1st week of her life. There was also an increase in hemolysis. The placenta of the patient showed normal activities of both γ-glutamylcysteine synthetase and γ-glutamyl cyclotransferase, but the glutathione synthetase activity of the placenta was only about 2 percent of the normal.

Another study of the cultured skin fibroblasts of patient # 2 were made. The study revealed somewhat greater activities for γ-glutamylcysteine synthetase, γ- glutamyl cyclotransferase and 5-oxoprolinase, but the

glutathione synthetase activity was found to be less than 5 percent of the control's.

γ-Glutamylcysteine synthetase and **γ-glutamyl** cyclotransferase from the erythrocytes of patient # 3 were found to be similar to that of the control samples, but there was a marked deficiency of glutathione synthetase.

Thus the enzyme studies indicate a marked deficiency of glutathione synthetase in the different tissues of three patients with 5-oxoprolinuria, which leads to a generalized deficiency of glutathione.

Patients # 2 and # 3 were anemic and had an increased tendency towards hemolysis. The deficiency of glutathione synthetase and thereby glutathione caused marked accumulation of 5-oxoproline which produces the characteristic acidosis in 5-oxoprolinuria. Untreated 5-oxoprolinuria is associated with mental retardation and other central nervous system disturbances.

Palekar et al (56) postulates that accumulation of **5-oxo-L-proline** in the blood and tissue contributes to the toxic **phenomena** observed in patients suffering from anemia.

Accumulation of 5-oxoproline in plasma is found to be associated with patients suffering from end stage renal disease (54).

5-oxoprolinuria has also been observed in homocystinuria (21) in which, accumulation of

homocysteine, appears to involve 5-oxoproline formation because homocysteine is a substrate of γ-glutamylcysteine synthetase (57). The primary metabolic disorders of homocystinuria are the deficiencies of cystathione - β-synthase, N^5, N^{10} - methylene - THF - reductase and N - methyl - THF - methyl - transferase and the secondary metabolic disorders of homocystinuria are Vitamin B_{12} malabsorption or deficiency together with methylmalonic acid and Vitamin B deficiency (58).

Wilkin *et al* (59) in their study of "Hawkinsinuria" - a dominantly inhereted defect of tyrosine metabolism-have reported the excretion of large quantities of 5-oxoproline by their patients.

Thus, in general 5-oxoprolinuria or pyroglutamic aciduria is directly or indirectly related to acidosis, hemolytic anemia, end stage renal disease, hepatic coma (60), homocystinuria, hawkinsinuria, mental retardation, stunted growth and various diseases and disorders of central nervous system including spastic tetraplagia and cerebellar ataxia (61). It is assumed that 5-oxoprolinuria may be associated with arthritis (62).

Normal and Abnormal Levels of 5-Oxoproline in Humans

Since the discovery of this disease about one and one-half decades ago, there have not been many cases reported in the literature. However, Meister (15) in his recent article has reviewed them in detail. A brief description of these will be dealt with here.

The first patient with 5-oxoprolinuria was discovered by Jellum *et al* (63) in 1970. He was a 19-year old mentally-retarded male suffering from spastic tetraparesis and ataxia. He was of normal height and weight but had a diaphragmatic hernia for which he was surgically treated. Postoperatively he developed life-threatening metabolic acidosis. This condition was successfully treated with daily infusions containing potassium and bicarbonate ions. Later he was maintained on oral sodium bicarbonate. The patient excreted 24 to 34.5 g (0.19 to 0.27 mol) of 5-oxoproline per day in his urine. Urea excretion was 35 to 45 percent of the normal value. This urinary 5-oxoproline was identified by thin-layer chromatography, gas-liquid chromatography and mass spectrometry. The serum contained 5-oxoproline and another glutamic acid derivative which was not identified. The concentration of 5-oxoproline in the cerebrospinal fluid was reported to be 30mg/dL (2.3 mM).

The second patient described in the literature by Hagenfelt *et al* (61) in 1974, was a female infant with 5-oxoprolinuria, who developed severe metabolic acidosis on

the third day of her life. This acidosis condition was kept under control by the administration of sodium bicarbonate. Physical examination at 11 and 14 months of age revealed normal weight and height for her age. She looked healthy and showed no signs of neurological or other abnormalities; psychomotor development was normal, This patient excreted 48 to 54 mmoles of 5-oxoproline per day in her urine. This 5-oxoproline was found to be of the L configuration by assays done with L-glutamate dehydrogenase after conversion of 5-oxoproline to glutamate by acid hydrolysis, The blood plasma concentration of 5-oxoproline was 58 mg/dL (4.5 mM). The levels of α-amino acids in plasma and urine were found to be normal. The formation of urinary urea increased as the protein content of the diet was increased, whereas the excretion of pyroglutamate somewhat decreased.

A quick glance at Table 2 containing 5-oxoproline levels found in human urine shows a major discrepancy in the reported values of normal urines. Although the value of normal urine from reference 64 is estimated, it differs from the value of normal urine from reference 56 to such an extent, that it is indicative of the lack of a suitable clinical procedure for its determination.

Table 2

5-OXOPROLINE LEVELS FOUND IN HUMAN URINE

Source of 5-oxoproline	Total Concentration (DL form)
Patient #1 urine (63)	240 mmol/day
Patient #2 urine (61)	50 mmol/day
Normal urine estimated (64)	3.9-39 μmol/day
Patient #3 urine (61)	210 mmol/1.73m body surface
Patient #4 urine (65)	30-35 g/hour
Normal urine (56)	80-430 μmol/day
Patient #5 urine (59)	20 mmol/m mole of creatinine

These normal values of 5-oxoproline are also dependent on various factors. It has been demonstrated by Oberholzer ~~et al~~ (66) that patients being fed on **low-**lactose food **"Nutramigen"** showed increased plasma and urine levels of pyroglutamic acid. Thus, diet plays an important role in urinary level of 5-oxoproline. Thanh and Sporns (49) in their study of flavour enhancers and chloride in food have determined the concentration of pyroglutamic acid in some of the foods, which are shown in table 3.

Table 3

S. No.	Food	Pyroglutamic acid concentration in mg/100g
1	Tomato Soup (canned)	53
2	Beef Consomme (canned)	46
3	Potato Chips	218
4	Japanese restaurant soup (miso-seatangel soup)	59

CHAPTER III

MATERIALS AND APPARATUS

SOLVENTS AND REAGENTS

The main solvent used was 8 mM sulfuric acid. The cleaning and/or regeneration solvent was 40% acetonitrile in 0.5 N sulfuric acid. The cleaning solvents used occasionally for the flow cell were methyl alcohol, acetone, chloroform and cyclohexane. All these were HPLC grade and purchased from Fisher Scientific Co., Pittsburgh, PA.

Water used was either glass-distilled - deionized or double deionized and then passed through a charcoal column to remove organics.

ORGANIC ACIDS

The organic acids used in this experiment were citric acid (J.T. Baker Chemical Co., Phillipsburg, N.J.), L-ascorbic acid, malic acid, quinic acid, L-pyroglutamic acid, pyrrole-2-carboxylic acid and biotin (Sigma Chemical Co., St. Louis, MO) malonic acid, pyruvic acid sodium salt, para-arninobenzoic acid and phenylacetic acid (Eastman Kodak Co., Rochester, NY).

APPARATUS OR EQUIPMENT

The equipment used in this work was as follows:

1. Altex model 110A High Pressure pump (Anspec, Ann Arbor, MI).

2. Bio-Rad Aminex Cation Exchange HPX-87H column (300 x 7.8 mm) protected by a 5 cm guard column also containing HPX-87H resin which is 8% cross-linked and 9 μm in particle diameter (Bio-Rad Laboratories, Richmond, CA).

3. Hitachi model 100-10 UV/Visible spectrophotometric detector, distributed by Beckman Instruments, Fullerton, CA.

4. Linear Instruments strip chart recorder which is fed in with Sargent Welch cat. #1010-1 graph paper and later Sargent Welch model-XKR strip chart recorder with cat. #72101 chart paper. A schematic diagram of the experimental set up is shown in fig. 7.

Centrifugations were done with a Sorvall GLC-1 Centrifuge (Dupont-Sorvall, Wilmington, DE). The filtration and degassing apparatus and the filter were from Millipore Corp. Bedford, MA,

The pH measurements were made on a Sargent-Welch model IP pH meter using a Sargent/Jena combination electrode model S-30070-10 (Sargent-Welch Scientific Co., Skokie, IL). Absorbance measurements were made on a Beckman DU-7 recording spectrophotometer (Beckman Instruments, Fullerton, CA).

EXPERIMENTAL SAMPLES

The urine samples used in this experiment were provided by Dr. Kim Carter of St. Elizabeth Hospital, Youngstown, from two patients with creatinine clearance problems. Ms. Helen Berry (Metabolic Research Laboratory, Children's Hospital Medical Center, Cincinnati, OH) provided urine sample from a **homocystinuric** patient and a 3-hydroxy-3-methyl glutaryl **CoA** lyase deficient patient. Another homosystinuric urine sample was provided by Ruth Korenchan (Special Chemistry Laboratory, Rainbow Babies and Childrens Hospital, Cleveland, OH).

CHAPTER IV

EXPERIMENTAL

PREPARATION OF THE SOLVENT

The solvent used in this experiment was 8 mM sulfuric acid. It was prepared by taking 0.46 mL conc. sulfuric acid (molarity 17.5 M) in a 1000 mL volumetric flask and bringing it up to the mark with either glass distilied, deionized or double deionized water. It was then filtered and degassed simultaneously using the Millipore Filtration and degassing apparatus. The filter used was 0.22 μm average pore size of 47 mm diameter.

PREPARATION OF SOLUTIONS AND STANDARD

The different organic acid solutions made were about 15 mg per 50 mL of 8 mM sulfuric acid. The 9.22 mM 5-oxoproline standard was prepared by taking 0.0595 g solid 5-oxoproline in a 50 mL volumetric flask and bringing it up to the mark with 8 mM sulfuric acid. This stock standard was further diluted 1:10, 1:15, 1:20, 1:40 and 1:80 with 8 mM sulfuric acid and used for preparing the calibration curve.

PREPARATION OF SAMPLES

The test samples used in this procedure were aliquots of urine from normal and diseased patients. These were stored at -20°C and thawed right before the analysis. About 2-5 mL of the thawed urine was centrifuged for 10 minutes at 5000 R.P.M., and the supernatant was filtered through a 0.22 μm millipore filter. A volume of 20 μL of this filtered sample was injected directly onto the column.

HPLC

The HPLC was done at room temperature. The solvent (8 mM sulfuric acid, pH 2.0). Flow rate was set at 1.0 mL/min. The sensitivity of the UV detector, which was set at a wavelength of 210 nm was 0-0.5 AUFS full scale deflection. Recorder chart speed was 1.0 cm/min. A 20 μL full volume injection loop was used and a constant volume (20 μL) of the urine sample was injected and peak height of the unknown was read from the chromatogram obtained. The concentration of the unknown corresponding to the peak height was interpolated from the calibration curve.

CHAPTER V

RESULTS AND DISCUSSION

UV SPECTRUM OF THE SOLVENT

The solvent, which was 8 mM sulfuric acid, was scanned in the ultra-violet from 230 to 190 nm, against a water blank using the Beckman DU-7 ultra-violet/visible spectrophometer. The scanning speed was 600 nm/min and the absorbance full scale was set from 0 to 2.0 AUFS.

The spectrum presented in fig. 8 shows that there is no significant absorbance of 8 mM sulfuric acid in this region. However, the region between 205 and 195 nm can tentatively be marked as the peak absorbance range. At 210 nm which was the wavelength used in this experiment sulfuric acid showed an absorbance of 0.0361, which was then adjusted to zero baseline on the UV detector used in conjunction with the HPLC.

UV SPECTRUM OF 5-OXOPROLINE

A solution of 0.22 mM 5-oxoproline in 8 mM sulfuric acid was scanned in the ultra-violet from 230 to 190 nm against 8 mM sulfuric acid as reference using the Beckman DU-7 ultra-violet spectrophometer. The scanning speed was 600 nm/min and the absorbance was set at a full scale range of 0 to 2.000 AUFS.

The spectrum as shown in fig. 9 shows an absorption maxima at 196 nm which agrees with the literature value (6). This absorbance of 1.85 corresponds to the molar absorptivity of 8.41×10^{3} m^{-1} cm^{-1}. The peak then gradually tapers down to zero at about 250 nm. Thus 5-oxoproline shows the characteristic feature of its internal amide bond which is known to absorb in the low UV region- Although the absorption maxima is at 196 nm, we choose 210 nm in this experiment because the instrument used here i.e., Hitachi model 100-10A UV/visible spectrophotometer covers range of 200 to 380 ± 7 nm.

At 210 nm the absorbance of 0.5091 corresponds to a molar absorptivity of 2.31×10^{3} m^{-1} cm^{-1}.

MASS SPECTRUM OF 5-OXOPROLINE

The chemical ionization mass spectrum of a 10 μg sample of solid 5-oxoproline was determined, by introducing it into the direct probe inlet on the Finnigen MAT model #1020B mass spectrometer.

The reagent gas which was methane was at a pressure of 0.4 torr and the energy of the ionizing electrons was 70 eV. The sample probe was heated from 50 to 350 ^{o}C at 120 ^{o}C/min. The mass spectrum was scanned every 2 seconds from mass 45 to 200 AMU.

The spectrum shown in fig. 10 is an average of 35 such scans and is normalized to mass 130 to 100%. The

other major observable peaks are of mass numbers 84, 158 and 170. As expected the origin of mass 130 peak is due to M + H^+ (129 + 1) where M represents the 5-oxoproline molecule, mass 84 peak is due to M minus HCO, (129-45), mass 158 peak is due to M + C_2H_5 (129 + 29) and mass 170 peak is due to M + C_3H_5 (129 + 41). For further information refer to reference (67).

Both Jellum _et al_ (63) and Tham _et al_ (70) have reported on the mass spectrum of the methyl ester of 5-oxoproline obtained from a gas chromatographic peak in urine as well as an authentic sample. The mass 84 peak which was found by them supports the mass 84 peak found by chemical ionization mass spectrometry.

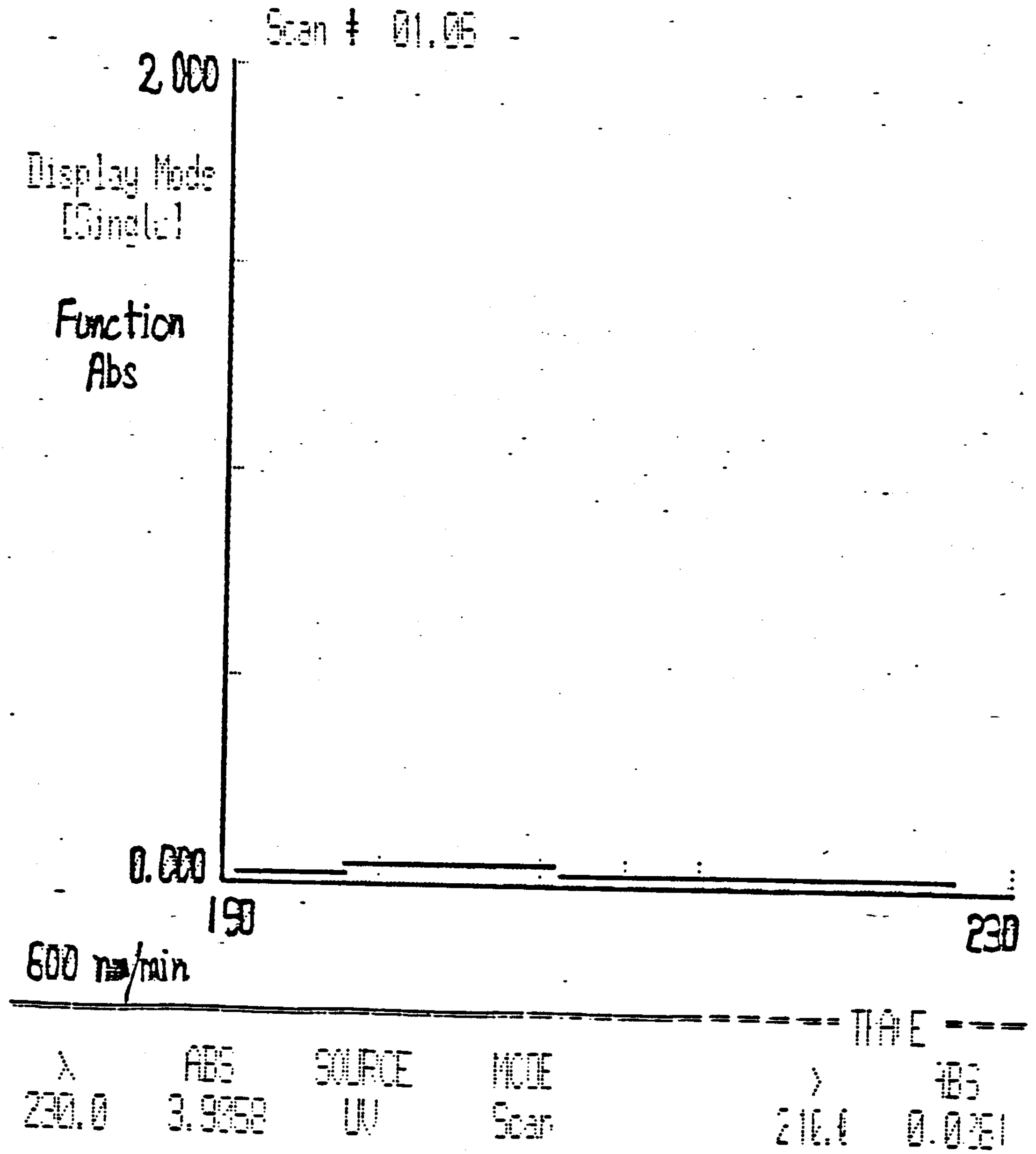


Fig.8 UV Spectrum of 8 mM H_2SO_4

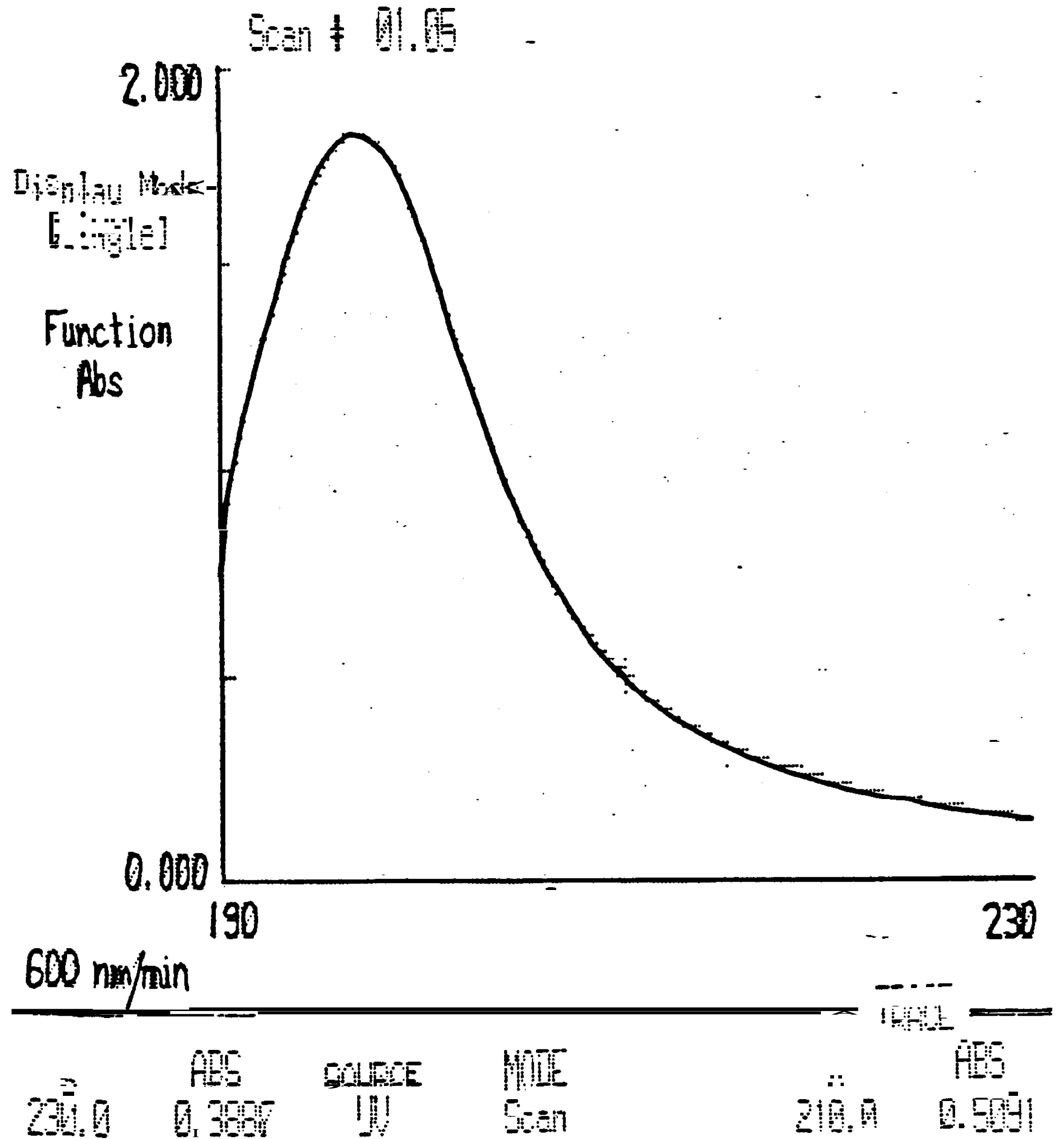


Fig.9 UV Spectrum of a solution of 0.22 mM 5-Oxoproline in 8 mM Sulfuric acid

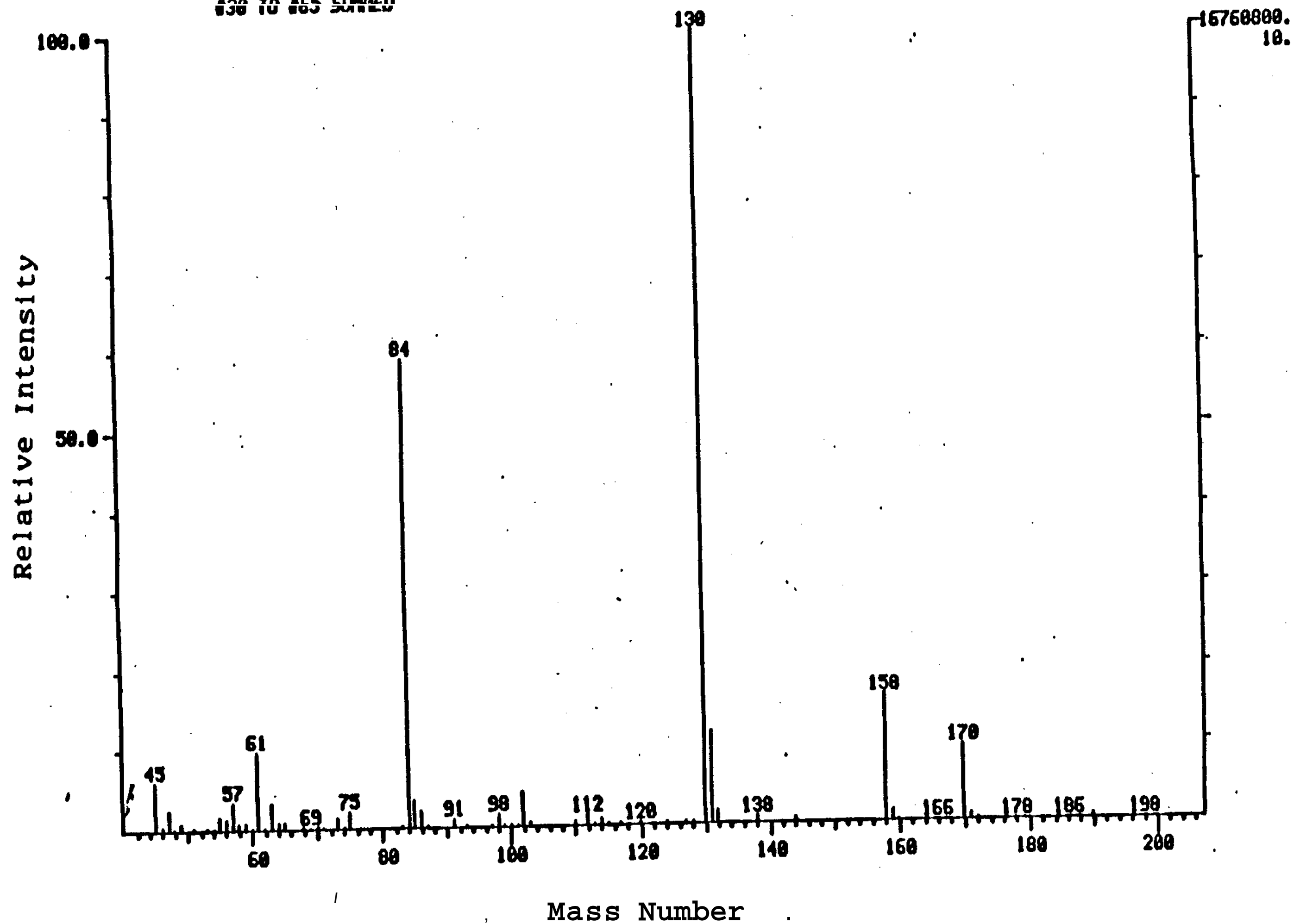


Fig.10 Methane Chemical Ionization Mass Spectrum of 5-Oxoproline

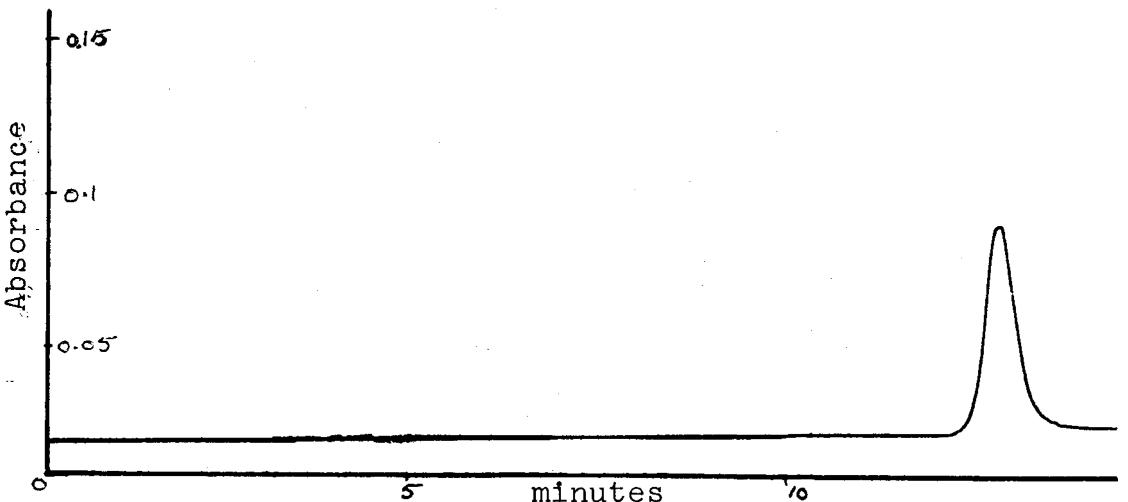


Fig. 11 Chromatogram of 0.92 mM 5-oxoproline (18 4 nmoles) in 8 mM sulfuric acid (monitored at 210 nm)

STANDARD CURVE FOR 5-OXOPROLINE

The standard curve for 5-oxoproline was obtained by plotting the peak height in mm (i.e., absorbance) against the amount of 5-oxoproline injected (i.e., concentration) for different solutions of 5-oxoproline in 8 mM sulfuric acid. These different solutions were made from time to time from the 9.22 mM stock 5-oxoproline, which was stored at 0°C.

The curve as shown in fig. 12 represents a typical Beer's Law plot, The linearity extended from 0 to 20 nmoles per 20μL injected.

Table 4

Data for the calibration curve of 5-oxoproline

Dilution	Amount of 5-oxoproline (n moles)	Peak height (mm)
1:10	18.4	28.0
1:15	12.2	19.0
1:20	9.2	14.0
1:40	4.6	7.0
1:80	2.3	3.0

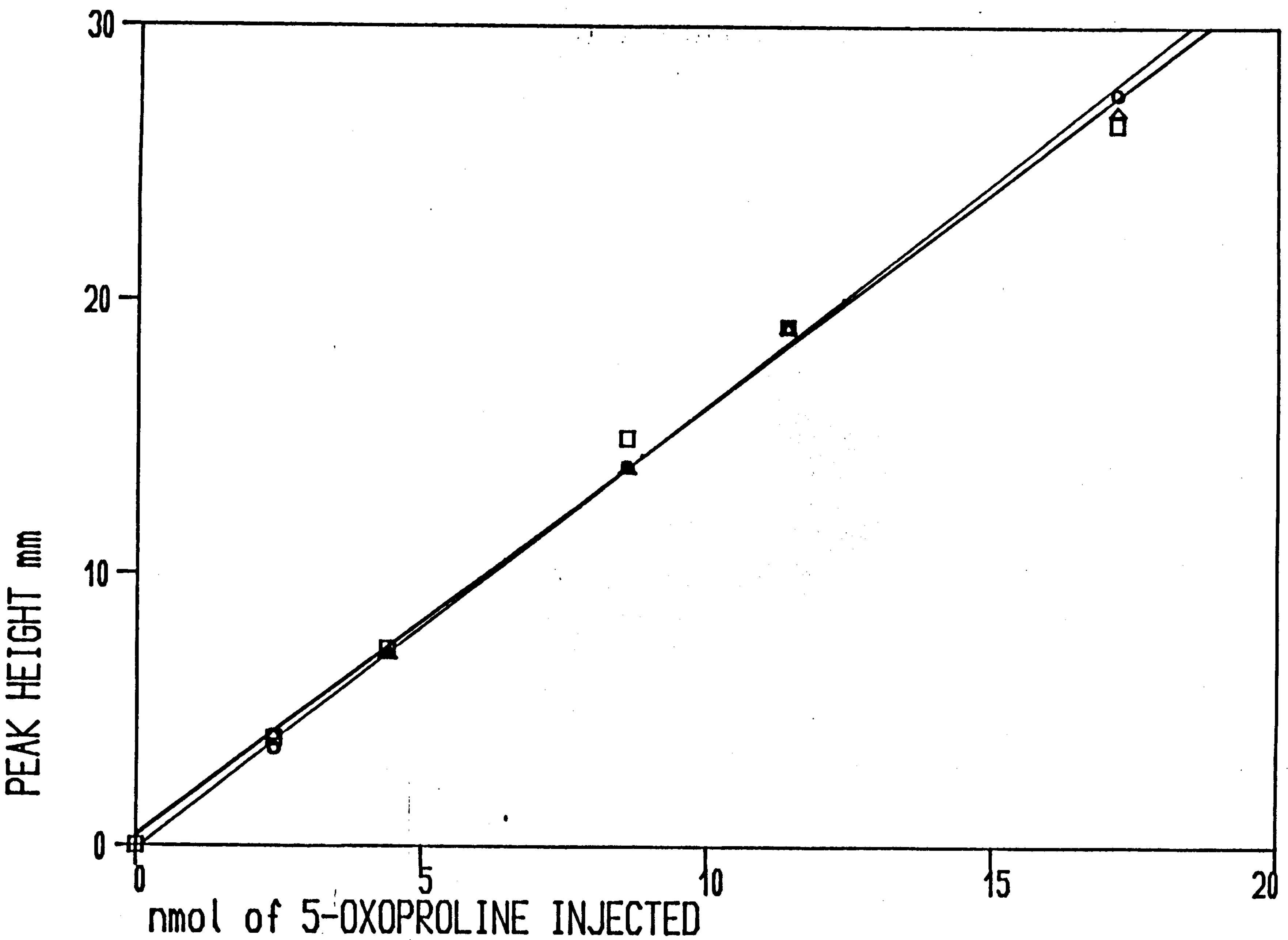


Fig.12 Reproducibility of standard curves of 5-oxoproline

REPRODUCIBILITY OF STANDARD CURVE OF 5-OXOPROLINE

The reproducibility of standard curves of 5-oxoproline is shown **in** fig. 12. As seen in fig. 12, the curves (represented 0 = A, △ = B, • = C and ◇ = D) obtained at four different-occasions are being compared. Curves A and C are an exact copy of the other, but curves B and D show a slight variation. However, the difference in the peak height for any concentration is not more than 2%. The solutions used for obtaining the curves A and C had common concentrations of 5-oxoproline. The solutions used for obtaining curves B and D were made separately and hence had slightly different concentrations.

Thus in general the reproducibility of the curves being 98% and the difference in absorbance of any concentration being not more than 2% proves that the curves are workable, practically identical and reproducible.

RETENTION DATA

Table 5 lists the retention times for the different organic acids used in this study, and monitored at a wavelength of 210 nm. From the data it can be seen that the retention times of most of the organic acids are in the range of 5 to 35 min. The retention times of 3-phenylpropionic acid, iso-vanillic

acid and cacodylic acid are too long. Phenylacetic acid and pyrrole-2-carboxylic acid did not produce any peaks. (Thus, in general this technique is not practical enough for the organic acids with too short or too long retention times). The phenolic non-amino acids have excesively long retention times, and the amino acids are retained on the guard column because of their positive charge at pH 2.0.

These retention times are comparable to the literature values (30, 53) and the pattern of elution is also the same. The lower retention times obtained in this experiment can be attributed to the faster solvent flow rate of 1.0 **mL/min** used here rather than 0.7 **mL/min** used by Buchanan and Thoene (30) and Allen *et al* (53) and also due to the difference in the zero-dead volumes involved in the experimental set up.

Although the K' values of 2.44 (30) and 2.50 for 5-oxoproline in the experiment are close, there is a major discrepancy in the retention time for 3-phenyl propionic acid, which is 68 min in this experiment when compared to 39 minutes (30). Daish and Leonard (50) also found a discrepancy for isovalerate between their HPX-87H column and the one used by Bennett and Brady (30).

Table 5

Retention Data of Organic Acids on Bio-Rad HPX-87H column
At 210 nm; Flow Rate = 1.0 mL/min.

Organic Acid	Retention time (min.)
Citric Acid	4.8
Malic Acid	5.2
Pyruvic Acid	5.6
Ascorbic Acid	5.8
Maleic Acid	6.0
Quinic Acid	6.2
Malonic Acid	6.4
Succinic Acid	7.2
Uric Acid	10.6
Pyroglutamic Acid (5-oxoproline)	12.6
Biotin	35.8
3-Phenylpropionic Acid	68.0
Isovanillic Acid	91.0
Cacodylic Acid	100.0
Phenylacetic Acid	--
Pyrrole-2-Carboxylic Acid	--

PRECISION OF METHOD

To estimate the between batch precision of the column retention repeated periodic injections (over a period of 9 months) of 5-oxoproline were made. The mean retention time (n = 50) was 12.59 (S D =±0.02) min.

CONFIRMATION OF PEAK IDENTIFICATION

The mean retention time of 12.59 (S D =±0.02) min was used for the peak identification, but for confirmation of the peak, the chromatogram was obtained at two different wavelengths (205 and 210 nm) for the pure compound and the unknown and the peak height ratios compared.

The mean (n = 10) peak height ratio of the pure compound was 2.14; S D = 0 and that for the unknown was 2.0; S D = 0. This difference of 0.14 mean peak height ratio may be due to a trace amount of another compound that absorbs more at 210 nm than 5-oxoproline and co-elutes at 12.6 min.

RECOVERY OF 5-OXOPROLINE

To estimate the recovery of 5-oxoproline normal urine was spiked with five different amounts of pure 5-oxoproline. 20 μL of urine which had no 5-oxoproline in it was taken and spiked with 18.4, 12.2, 9.2, 4.6 and 2.3 nmoles of 5-oxoproline each time and chromatographed.

It was found that the recovery was a 100% (n = 3) in all the five cases. The results were as expected because in this experimental procedure, **i.e., HPLC,** there is no sample extraction or derivatization which would otherwise be responsible for any loss in the sample.

Table 6

RECOVERY EXPERIMENT (n = 3)

5-OXOPROLINE ADDED (nmoles)	5-OXOPROLINE FOUND (nmoles)	% RELATIVE RECOVERY
18.4	18.4	100
12.2	12.2	100
9.2	9.2	100
4.6	4.6	100
2.3	2.3	100

CLINICAL STUDIES

Clinical studies were made on the urines from five different **patients.** The chromatograms obtained are shown in fig. 13 to 19.

The chromatograms of the two homocystinuric urines from patient # 9 and patient # 6 were found to contain 5-oxoproline. The 5-oxoproline concentration was 0.825 **mM** in patient # 9 (the peak height of 25 mm from the chromatogram, shows a value of 16.5 **nmol** per 20 μL on the calibration curve. This 16.5 nmole per **20 μL** is equal to 0.825 **mM)** and 0.51 **mM** in patient # 6. Patient # **9's** creatinine level was 0.87 **mg/mL** or 7.7 **mM.** The ratio of mmoles of 5-oxoproline to moles of creatinine is **0.825/7.7** x 10^{-3} or 107. This compares favorably to a value of 120 found by Stokke et **al** (69) in the urine of a 12 year-old boy with homocystinuria who had been receiving pyridoxine treatments for four months. Patient # 9 is also being treated for homocystinuria. Homocystinuria is a human genetic disease and is caused by a defect **in** the enzyme - cystathione **β-synthetase.** The defect or deficiency of this enzyme causes the accumulation of homocysteine, which would otherwise be converted to cystathionine. This excess of **homocysteine,** which causes homocystinuria is associated with 5-oxoprolinuria. The possibility of 5-oxoprolinuria in homocystinuria can be explained by the formation of γ-**glutamyl** homocysteine from excess of homocysteine and

glutathione by the action of γ-glutamyl transpeptidase and then further conversion of γ-glutamyl homocysteine to 5-oxoproline by the action of γ-glutamylcyclotranferase. Thus the production of 5-oxoproline exceeds the capacity of the enzyme 5-oxoprolinase to form L-glutamate, which results in 5-oxoprolinuria (21).

The deamination of homocysteine forms α-keto -γ thiobutyrate. Hence, there is a possibility of finding this in the homocysteinuric urines. The other organic acids found in patient # 6 were citric acid and ascorbic acid and in patient # 9, in addition to citric acid and ascorbic acid, malic acid and succinic acid were also found. (1) The work done by Buchanon and Thoene (51) on the urines of patients with generalized aminoaciduria, organic aciduria due to renal tubular defect and methylmalonic aciduria, however, does not show the presence of any 5-oxoproline.

The urine from patient (10), with 3-hydroxy-3-methylglutaryl CoA lyase deficiency showed no trace of 5-oxoproline. The chromatogram is shown in fig. 19. Hydroxy methylglutaryl-CoA lyase is responsible for the conversion of 3-hydroxy-3-methylglutaryl-CoA to acetoacetate and acetyl CoA. 3-Hydroxy-3-methyglutaryl CoA is an important intermediate in cholestrol biosynthesis.

The urines of patients # 7 and # 8 from the St. Elizabeth's Hospital were chromatographed and are shown

in fig. 15 and 16. They showed the peaks of the normal constituents of urine - citric acid, ascorbic acid, succinic acid, uric acid and malic acid. Both the patients had creatinine clearance problems,but only patient # 7 had 0.33 mM 5-oxoproline; there was no 5-oxoproline found in the chromatogram of patient # 8. These patients #7 and #8 had some abnormal peaks also which were not identified in this experiment.

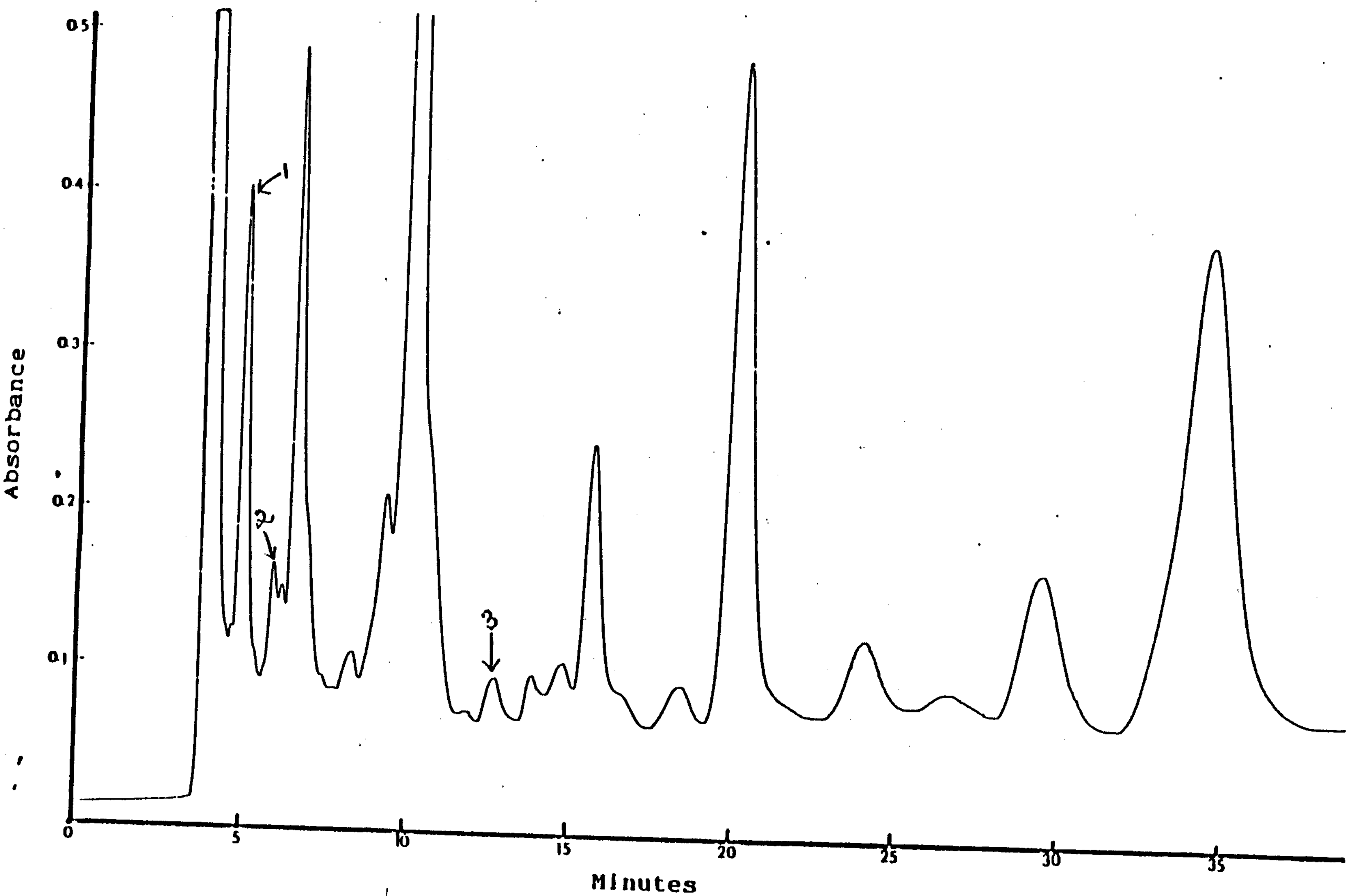


Fig.13 Urine chromatogram of a Homocystinuric patient (6) monitored at 205 nm
Peaks:1 citric acid,2 ascorbic acid, 3 pyroglutamic acid

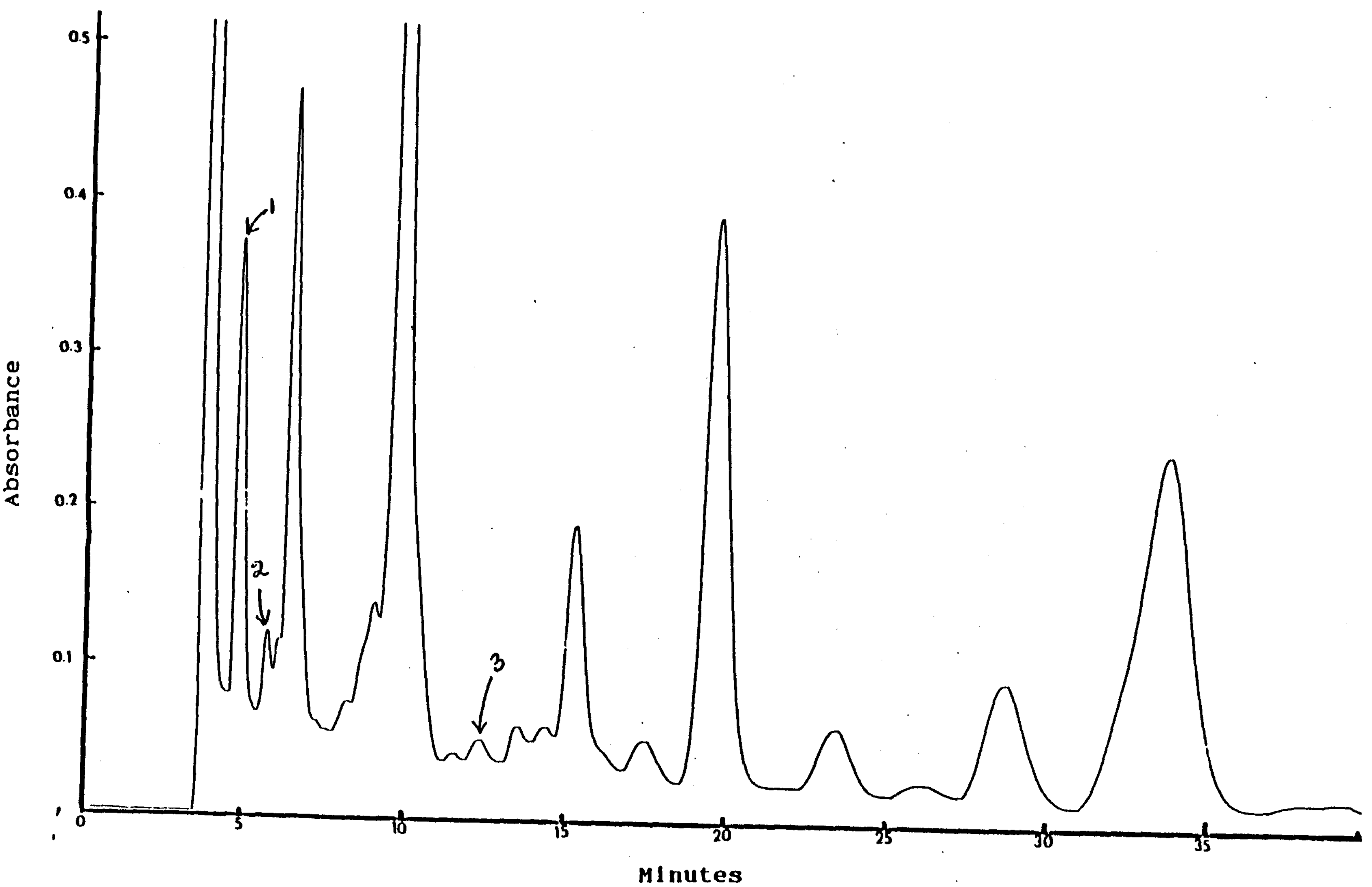


Fig.14 Urine chromatogram of a Homocystinuric patient (6) monitored at 210 nm
Peaks: 1 citric acid, 2 ascorbic acid, 3 pyroglutamic acid

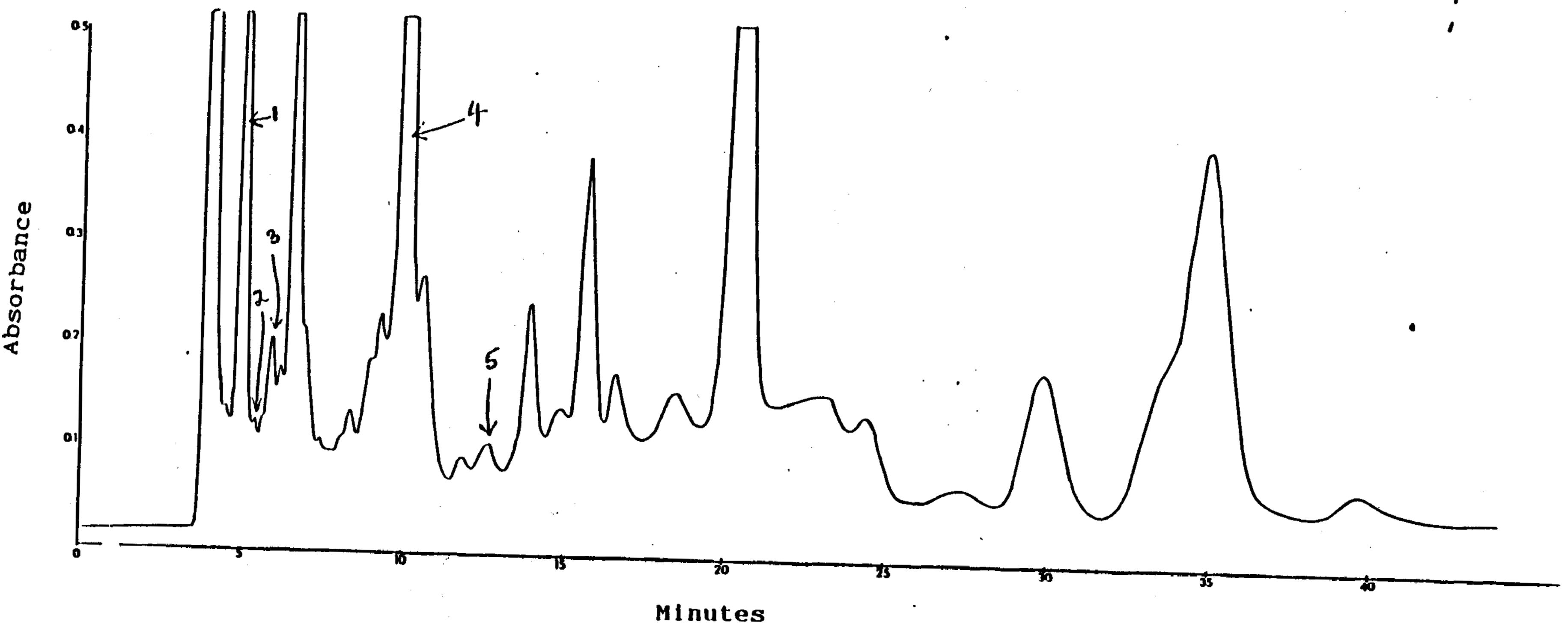


Fig.15 Urine chromatogram of a patient (7) from St.Elizabeth's Hospital monitored at 210 nm. Peaks:1 citric acid,2 ascorbic acid,3 succinic acid, 4 uric acid,5 pyroglutamic acid

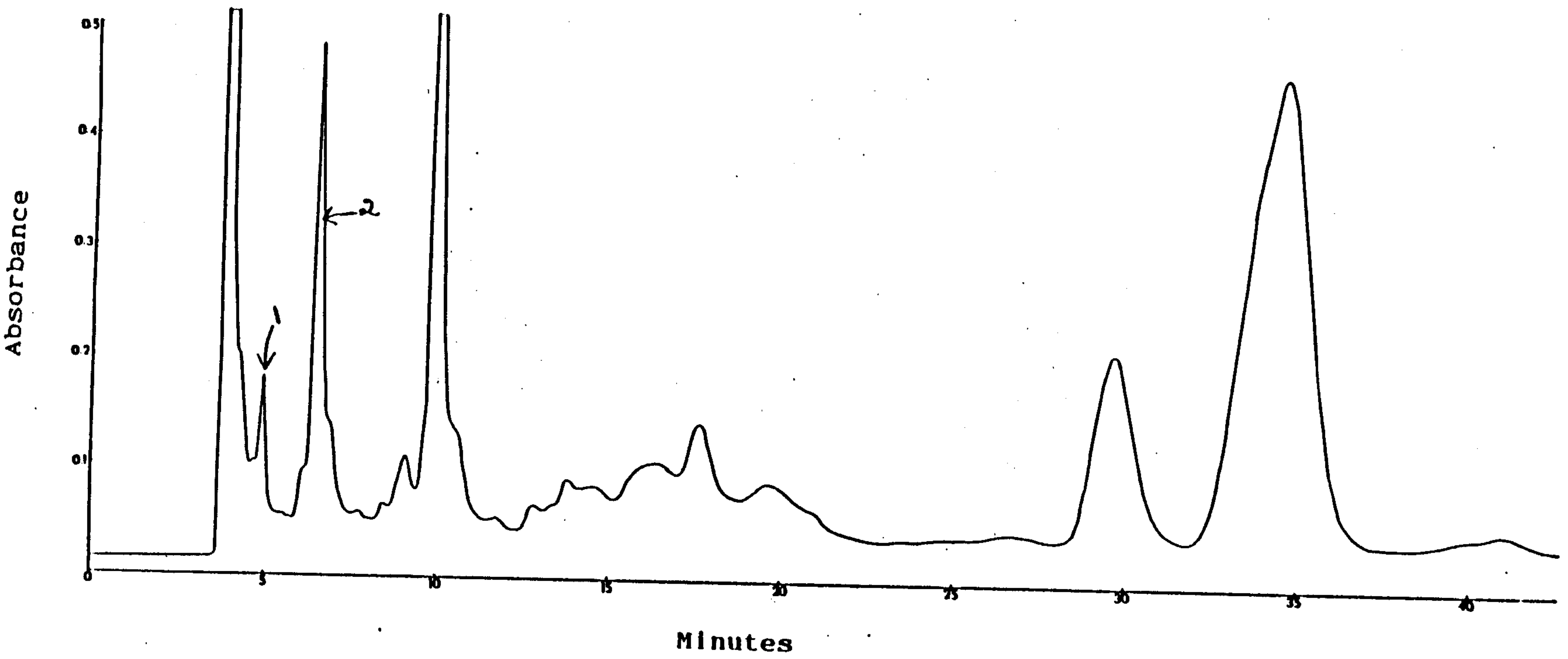


Fig.16 Urine chromatogram of a patient (8) from St.Elizabeth's hospital monitored at 210 nm.Peaks:1 citric acid,2 malic acid

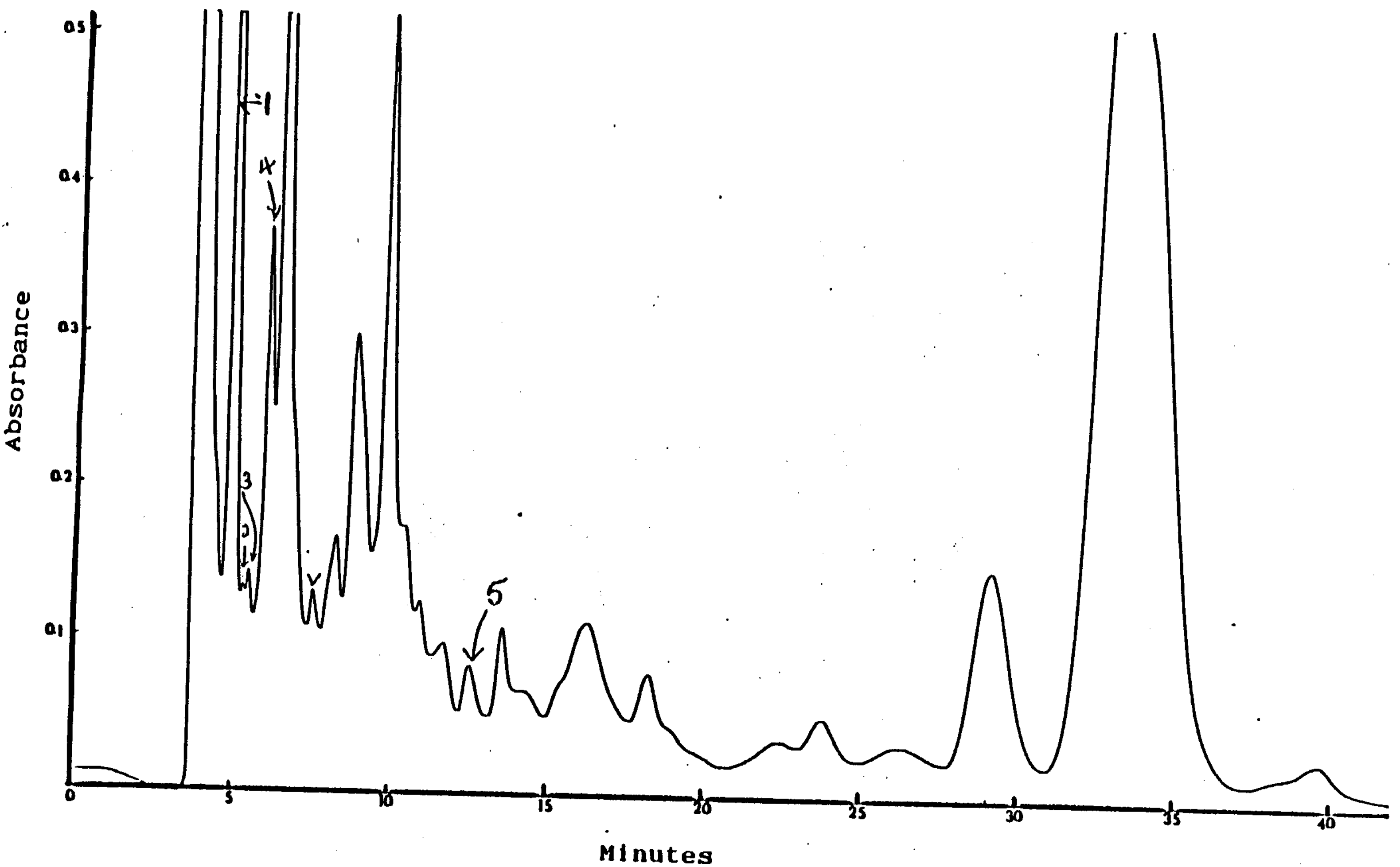


Fig.17 Urine chromatogram of a Homocystinuric patient (9) monitored at 210 nm
Peaks:1 citric acid,2 ascorbic acid,3 malic acid,4 succinic acid,5 pyroglutamic acid

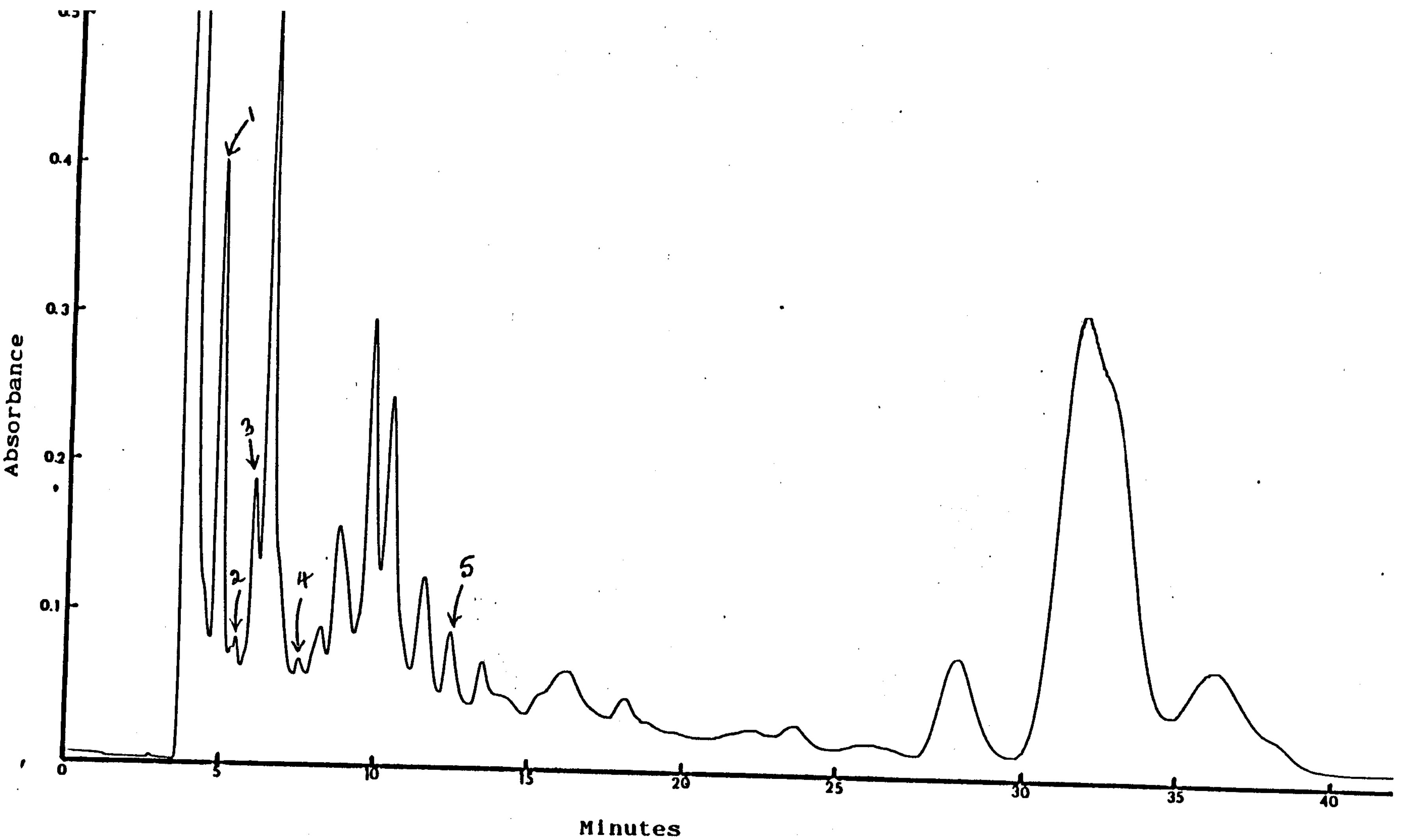


Fig.18 Urine chromatogram of a Homocystinuric patient (9) monitored at 205 nm
Peaks:1 citric acid,2 ascorbic acid,3 malic acid,4 succinic acid,5 pyroglutamic acid

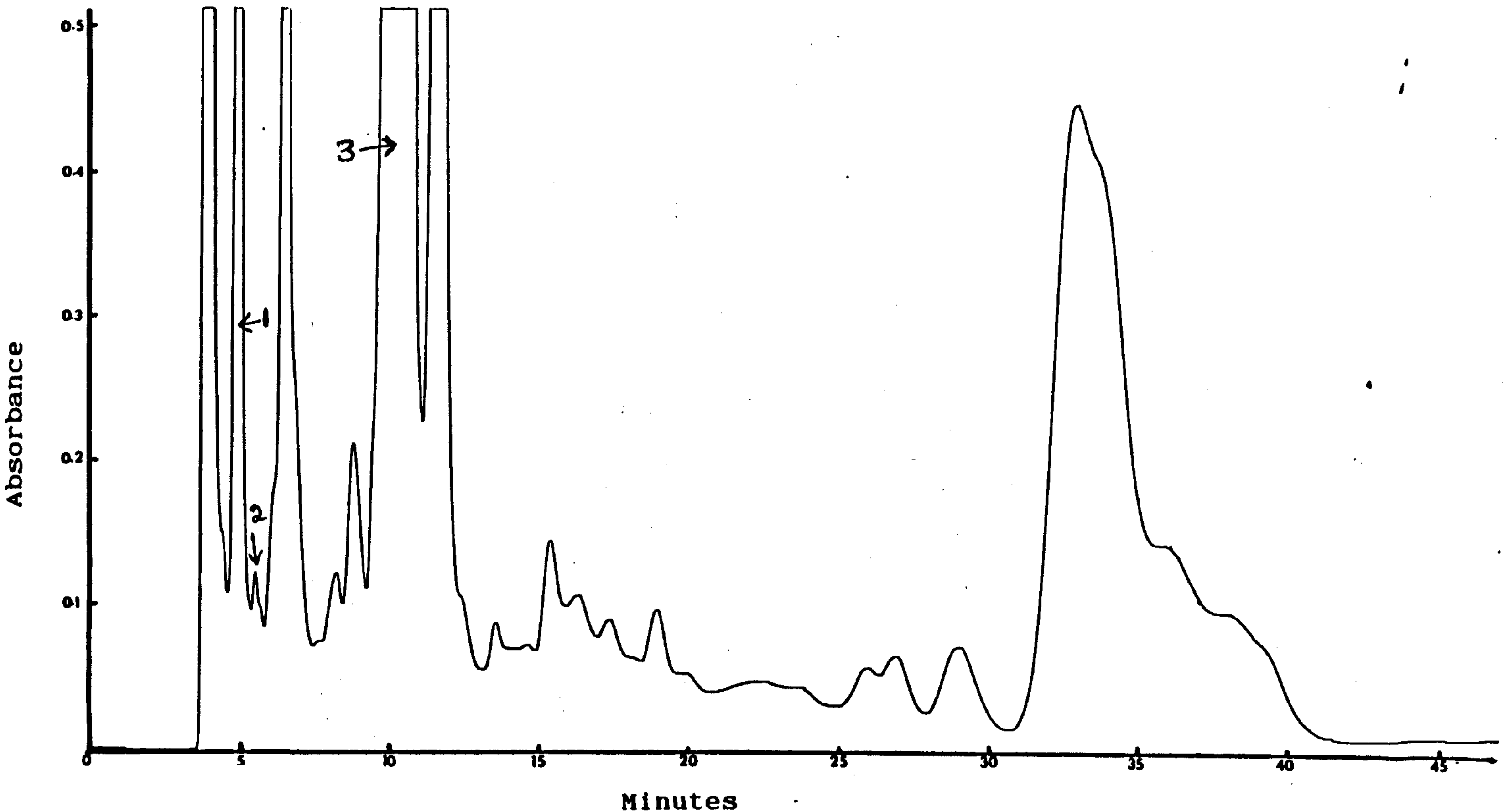


Fig.19 Urine chromatogram of a patient (10) with 3-hydroxy 3-methyl glutaryl CoA lyase deficiency.Peaks:1 citric acid,2 malic acid,3 uric acid

CHAPTER VI

CONCLUSIONS

This thesis has described a simple procedure for the quantitative determination of 5-oxoproline. A review of the literature shows that since the discovery of this disease - "5-oxoprolinuria or pyroglutamic aciduria" in 1970, much work has been done for its detection. Although a variety of techniques ranging from simple chromatography to refined instrumentation and computer analysis were employed, there seems to be an unusual importance given to chromatography even in the primitive stages. The result of these numerous attempts and modifications have led to the evolution of HPLC methods.

Thus by this simple technique of HPLC an amount as low as 2.0 nmoles of 5-oxoproline can be determined easily, which is practical enough for use in clincial labs. But for quantification of even lesser amounts, i.e., in the picomole range, derivatization of 5-oxoproline with 4-bromomethyl-6,7-dimethoxy coumarin is a very sensitive method (65).

The sensitivity of this method can be improved by the use of a better detector. The UV-detector used in this experiment, i.e., Hitachi model 100-10 is supposed to be the least sensitive one. The problems of a drifting base line and zero adjustments limited the use of a higher sensitive range. Hence we could work with an absorbance range of 0-0.5 AUFS only. This problem can be

overcome by the use of Hitachi model 100-20 or 100-40 or any comparable UV Spectrophometric detectors.

For further improvement of sensitivity in this method, other more sophisticated and modernized instruments specially computerized ones like the IBM LC/9533 could be used. With the choice of the instruments this method would be very easy and helpful in day-to-day work.

One of the most important steps in the treatment of a disease is the diagnosis. The diagnosis can be done only when sufficient knowledge of the disease is obtained, **i.e.,** normal values of metabolite concentrations in different body fluids, male vs female values; those of child vs adult; the geographical occurence of the disease and its inter-relationship with other diseases.

Some of the diseases inter-related with-5-oxoprolinuria are homocystinuria, hawkinsinuria, arthritis, hyperoxaluria, end stage renal disease etc. Cystathione -β-synthase deficiency is characterized by the presence of both hypermethioninemia and homocystinuria, consistent with a defect in the conversion of homocysteine to cystathionine. The elevated levels of homocysteine lead to an increase in resynthesis of methonine by remethylation. The clinical symptoms include mental retardation, osteoporosis and a characteristic eye defect known as ectopia lentis or

dislocation of the lens. The other less commonly observed clinical signs are myopia, scoliosis and other skeletal problems. Sometimes retinal detachement, cataracts, spasticity, and psychiatric disturbances are also found (69).

Postmortem examination on a homocystinuric patient revealed the existence of widespread fibrous thickening of the **intima** of arterial walls and increased amounts of collagen in the media. Hence, it is suggested that the normal collagen metabolism is disturbed by the excess of homocysteine and its interferance with cross-linking.

Hawkinsinuria, which is caused by a defect in **tyrosine** metabolism, is a dominantly inherited genetic defect. There is an excess accumulation of a toxic metabolite **"Hawkinsin".** A serious clinical effect is supposed to occur in heterozygotes. Excretion of ketones, metabolites of **tyrosine** and L-pyroglutamic acid are associated with it (59).

The **role** of glutathione in rheumatoid artliritis is not yet understood (62). However, since **5-**oxoprolinuria is an inborn error of glutathione metabolism, there may be an inter-relationship between **5-**oxoprolinuria and rheumatoid arthritis.

In conclusion, there is a real need of further research of this disorder. To determine a normal range for 5-oxoproline in the urine of healthy adults, 100

urines from healthy volunteers on a standardized diet should be analyzed.

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