Title

Development of a column chromatography-gas chromatography method for urinary placental estriol

Author

Gotelli, George,

Publication Date

1976

Peer reviewed|Thesis/dissertation

Development of a Column Chromatography-Gas Chromatography Method for Urinary Placental Estriol

by
George R. Gotelli
B.A., University of California, Berkeley, 1960

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF CLINICAL LABORATORY SCIENCE

in

Clinical Laboratory Science

in the

GRADUATE DIVISION

[San Francisco]

of the

UNIVERSITY OF CALIFORNIA

[redacted]

Date Librarian

Degree Conferred: . . . . . SEP 22 1976 . . . . . .

Table of Contents

ACKNOWLEDGEMENT

I would like to express my sincere appreciation and gratitude to Laurence J. Marton, M.D., for his patience and assistance in drafting this thesis. I would also like to gratefully thank Pokar M. Kabra, Ph.D., for his guidance and direction throughout the development of this work. I am also indebted to Sigfried K. Nussenbaum, Ph.D., for his guidance, assistance and friendship during my graduate studies.

August, 1976
GRG

INTRODUCTION AND LITERATURE REVIEW

Over 3000 years ago the Egyptians recorded that urine from pregnant women contained substances that were not found in the urine of non-pregnant women. In 1933 Smith and Smith (1) reported that there was a progressive rise in urinary estrogens during normal pregnancy until term. However, the sources of these estrogens was unknown until Steuart (2), in 1951 found that they originated in the placenta. Subsequent publications (3,4) have shown that in cases of fetal insufficiency urinary estrogens decrease prior to the onset of clinical signs and symptoms. These studies showed the first correlation between urinary estrogen excretion and fetal well-being. In 1959, Cassmer (5) showed that the fetus played an important but unknown role in the production of placental estrogens. It has since become well established that the fetus and the placenta combine to produce the estrogens of pregnancy.

During the last decade there has been a better understanding of estrogen metabolism in pregnancy. Diczfalusy (6), MacDonald and Siiteri (7,8) have shown that maternal estrogens result primarily from the placental conversion of dehydroepiandrosterone to estradiol. In addition, they have shown that both estradiol and estriol, produced in the placenta, are not derived from maternal precursors.

Bolte (9) et al. have formulated a metabolic scheme for estrogen production in pregnancy which involves both the fetal liver and the placenta. The non-estrogenic steroid precursor, dehydroepiandrosterone, is produced in the fetal adrenal cortex, reduced and hydroxylated in the fetal liver, and then, through a series of conjugations and hydrolysis reactions,

carried out in both the fetus and in the placenta, is converted into estrogens. These estrogens are finally excreted in the maternal urine (10) (Figure 1). A large number of estrogens are known to be excreted in the urine of pregnant women. These estrogens occur as conjugates of glucuronic acid or sulfuric acid. In the third trimester of pregnancy estriol conjugates account for about 95% of the total urinary estrogens (11), about 95% of which is conjugated with glucuronic acid (11,12). The study of urinary estriol as a measure of fetal well-being is now well documented (13,14).

In 1931, Kober (15) published his observation that estrogens form colored compounds when heated with concentrated sulfuric acid. This observation formed the basis of the first laboratory methods for the measurement of urinary estrogens. Cohen (16), in 1933, and Brown (17), in 1955, published the first colorimetric methods for estimating urinary estrogens. Both of these methods utilized the Kober reaction. Ittrich (18), in 1958, described a purification step involving the extraction of the Kober chromogen into cold chloroform containing p-nitrophenol. In this way, the nonspecific portion of the Kober chromogens are eliminated. Today, methods using the Kober-Ittrich reaction are still widely used.

The method of Brown (17), measured the three estrogens, estriol, estrodiol and estrone. This method utilizes acid hydrolysis followed by solvent extraction. The estrogens are separated from the neutral steroids by solvent partition and aqueous sodium hydroxide extraction. The estrogens are then methylated and further purified by chromatography on alumina columns. Quantitation is by colorimetric measurement using the

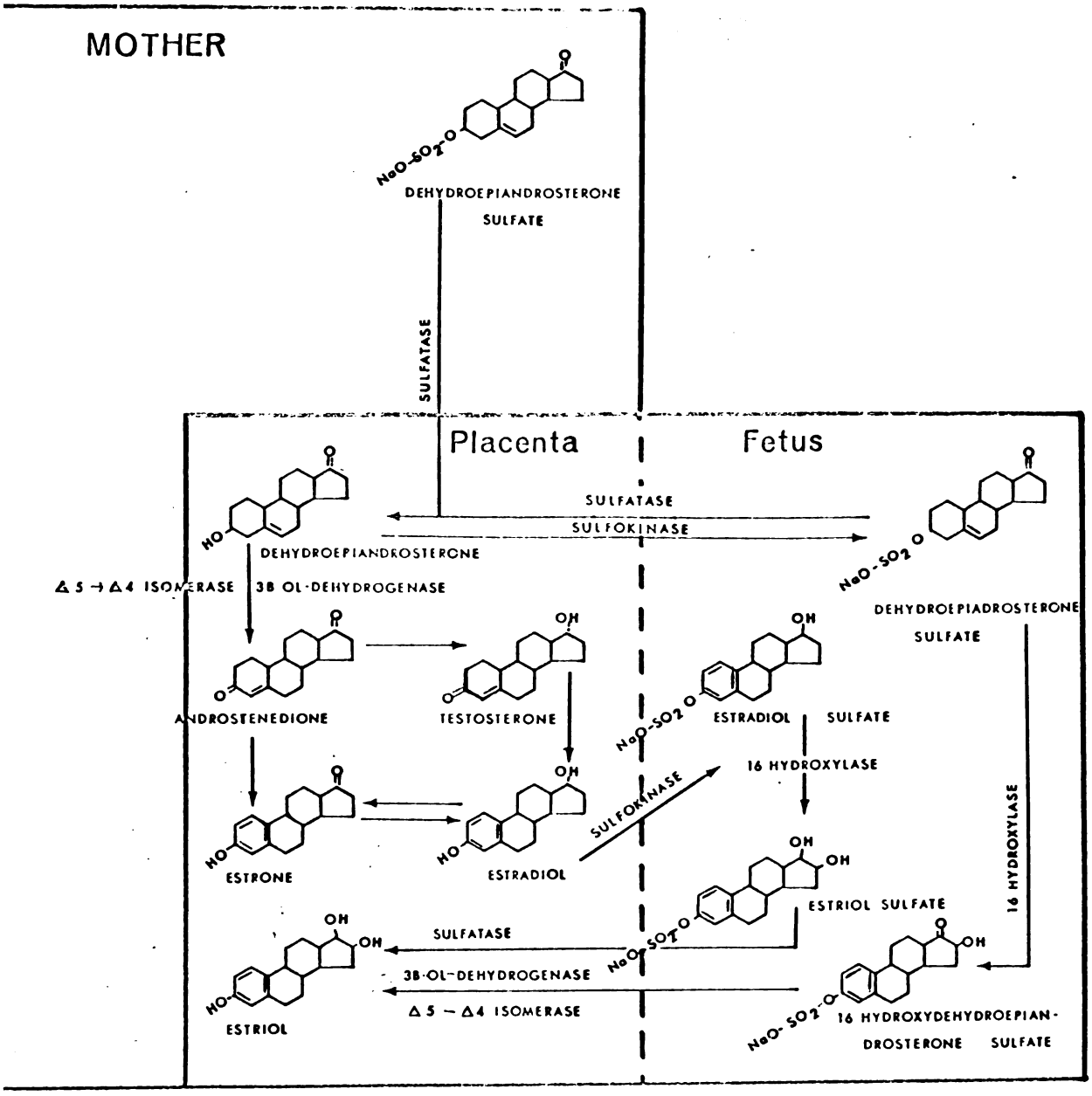


FIGURE 1

ESTRIOL SYNTHESIS IN PREGNANCY

Kober reaction. Bauld (19) modified the method of Brown in 1956 by adding a column chromatographic purification using celite. Quantitation was again achieved by means of the Kober reaction, but included a spectrophotometric correction for interfering chromogenic material. Cohen (20), in 1966, introduced a method utilizing ammonium sulfate precipitation of the unhydrolyzed estrogen conjugates. Quantitation was by the Kober-Ittrich colorimetric reaction.

Most older methods are modifications of the original method of Brown (17). More recently, emphasis has been directed toward optimizing conditions for the measurement of estriol only (21,22,23), as there is considerable evidence that estriol is a valid index of fetal well-being (13,14).

The method of Brown (17) and almost all subsequent methods for estrogen determinations can be condensed into four basic steps.

1. HYDROLYSIS - Estrogens are excreted in the urine as water soluble conjugates of glucuronic acid and sulfuric acid. It is necessary to hydrolyze these conjugates so that the free estrogen molecule can be extracted into organic solvents. Hydrolysis takes two forms, acid hydrolysis and enzymatic hydrolysis.
2. EXTRACTION - Following hydrolysis, the free estrogenic steroids become highly soluble in organic solvents and are easily removed from the urine matrix by solvent partition. The neutral steroids are also removed from the urine matrix by this process.
3. PURIFICATION - After extraction from the urine matrix, the estrogens are separated from the neutral steroids. Because of

the acidic nature of the phenolic group, estrogens are readily extracted from organic solvents by aqueous sodium hydroxide, forming the water soluble phenolic salt of the estrogen.

4. QUANTITATION - Following purification, the estrogens are measured, most commonly by colorimetric or fluorometric spectrophotometry.

The techniques used to accomplish these basic steps are briefly reviewed.

HYDROLYSIS

The techniques of hydrolysis have been a controversial subject and continues to be an unsolved issue. Brown (17) reported that acid hydrolysis results in about 20% loss of estrogens. Other investigators (24,25) have reported that the loss of estriol during acid hydrolysis is due to substances present in the urine which in some manner reduce the recovery of estriol from the urine. The detrimental effect of glucose (25,26), methenamine mandalate (27), phenolphthalein (25), and hydrochlorothiazide (28) on estriol during acid hydrolysis has been reported. However, acid hydrolysis remains a commonly used technique due to its simplicity and efficiency. It is apparent, however, that under the extreme conditions used during acid hydrolysis (generally 2-6 M hydrochloric acid at 100^{o}C for 30 to 60 minutes) that many organic reactions occur which produce numerous by-products, and that these conditions are destructive to estrogens (29).

Enzymatic hydrolysis is considered to be a much milder technique, which causes no destruction of estrogens and produces few, if any, by-products. A major objection to enzymatic hydrolysis is the time required to accomplish complete hydrolysis, reported to be from 12-48 hours.

Recently, enzymatic hydrolysis methods have been introduced which use relatively high temperature and high enzyme concentrations. These conditions allow for relatively short periods of time to achieve complete hydrolysis. Dray et al. (30), using a highly active β-glucuronidase of bacterial origin, found that this preparation liberated in 2 hours the same quantity of steroids as was liberated by the enzyme preparation of *Helix pomatia* in 18 hours. Crowley and Rosser (31) described a method using this bacterial enzyme preparation, in which hydrolysis was completed in 15 minutes at 37°C.

EXTRACTION

Extraction techniques generally utilize organic solvent partition for the recovery of free estrogens from hydrolyzed urine. These are generally time consuming and can result in solvent loss of the estrogens. Recently, newer methods have been introduced for the recovery of urinary steroids which do not utilize solvent extraction. Amberlite XAD-2 resin[R] has been shown to be useful in extracting free or conjugated estrogens from urine. First introduced by Bradlow (32) in 1968, there have been subsequent methods for urinary estrogen determinations using Amberlite XAD-2 resin (33,34,35).

PURIFICATION

The purification step separates the phenolic estrogens from the neutral steroids. This step involves the formation of a water soluble salt at the phenolic group of the estrogen molecule. The neutral steroids do not form salts, and remain in the organic phase. This method has

R) Rohm and Haas Co., Philadelphia, PA. 19105

remained a common tool for the separation of estrogens from neutral steroids.

QUANTITATION

Colorimetric spectrophotometry or fluorometry of the Kober-Ittrich chromogen are still the most commonly used methods of quantitation.

In early 1960, gas-liquid chromatography was shown to be of great value in the separation of steroids, including estrogens. However, quantitative urinary estrogen methods which appeared at this early date were generally not suitable for routine clinical use (36,37,38). These early methods involved long preparatory procedures, and in addition, gas liquid chromatography equipment was generally scarce at the time. More recently, gas-liquid chromatography has become a common tool in clinical laboratories.

Numerous gas-liquid chromatographic methods which are suitable for routine clinical use have been reported (22,39,40,41,42,43). However, all these methods lack true internal standardization. Some methods use an external standardization technique (39,40). Others use (22,41,42) an internal standardization technique, but the internal standard is not added until after the extraction has been completed. In these procedures, therefore, correction for analytical variables are not accomplished. Thus, the internal standard is used only for peak quantitation. Smith and Stitch (43) use phenolphthalein as an internal standard, and it is added early in the procedure so correction for analytical variation is accomplished. However, phenolpthalein is commonly used as a laxative, and thus, can find its way into maternal urine, a fact noted by Smith

and Stitch (43). Furthermore, Sims et al. (44) and Bolognese et al. (25) have reported that phenolphthalein can cause destruction of estrogens during acid hydrolysis, thus precluding its addition prior to acid hydrolysis. Richardson (42) uses a unique method of internal standardization using estrone. However, it is added at the end of the procedure, and thus, does not correct for analytical variations.

It is appreciated that accuracy in gas-liquid chromatographic quantitation may be impaired by using the wrong method of standardization, and that internal standardization is the method of choice. The criteria for an ideal internal standard are:

a) A chemical structure similar to the unknown.

b) A retention time close to the retention time of the unknown.

c) Elution in an area where other substances are not found.

d) A compound not found in biological samples.

e) A compound added sufficiently early in the procedure so as to correct for analytical variables.

f) A compound with a recovery similar to the recovery of the unknown.

This paper presents a method utilizing rapid enzymatic hydrolysis, XAD-2 resin extraction, and quantitation by gas-liquid chromatography. An estrogenic internal standard meeting the criteria outlines above is added early in the procedure to correct for procedural losses.

METHOD DEVELOPMENT AND RESULTS

I. INTERNAL STANDARD

The compounds investigated for their potential as internal standards were all of the estrogen series (Figure 2). These were:

a) Ethinyl estradiol

b) Equilenin

c) Equilin

d) Estradiol cyclopentane propionate

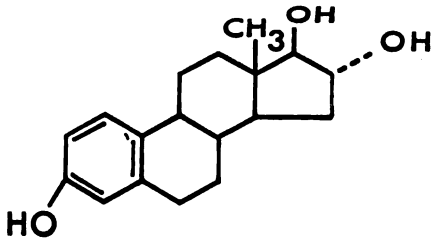
The four possible internal standards, and estrone, estradiol and estriol were silylated and chromatographed. Each estrogen formed a single symmetrical peak indicating silylation was complete. Equilenin eluted in an area close to, but completely separated from estriol. Neither estradiol or estrone interfered with the elution of equilenin. Its response was about 70% that of estriol (Figure 3). Ethinyl estradiol and equilin co-eluted with estrone and were eliminated from the study, in as much as estrone is present in all maternal urines. Estradiol cyclopentane propionate required twenty minutes for elution, a time period too long for this study. Equilenin was chosen for further study since it had fulfilled the following criteria for a true internal standard:

a) The chemical structure was similar to estriol.

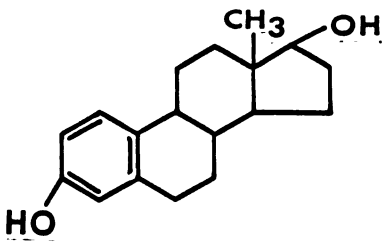
b) It eluted from the chromatographic column close to estriol.

c) It is not normally found in maternal urine.

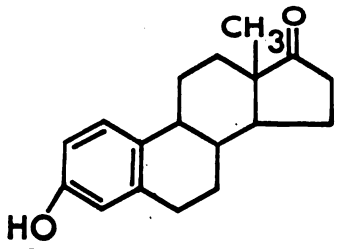
Equilenin was continued through the study to determine if it would fulfill the remaining criteria for an internal standard:



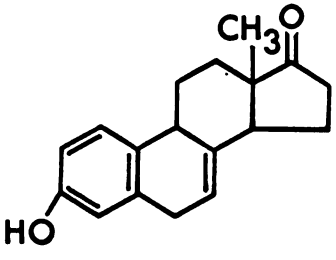
ESTRIOL



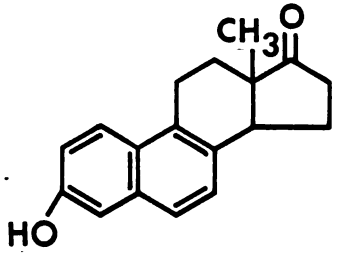
ESTRADIOL



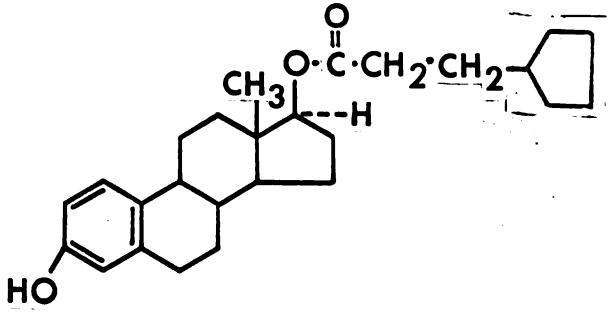
ESTRONE



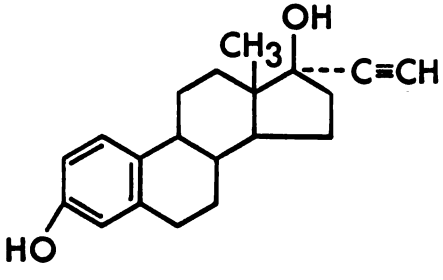
EQUILIN



EQUILENIN



ESTRADIOL
CYCLOPENTANE PROPIONATE



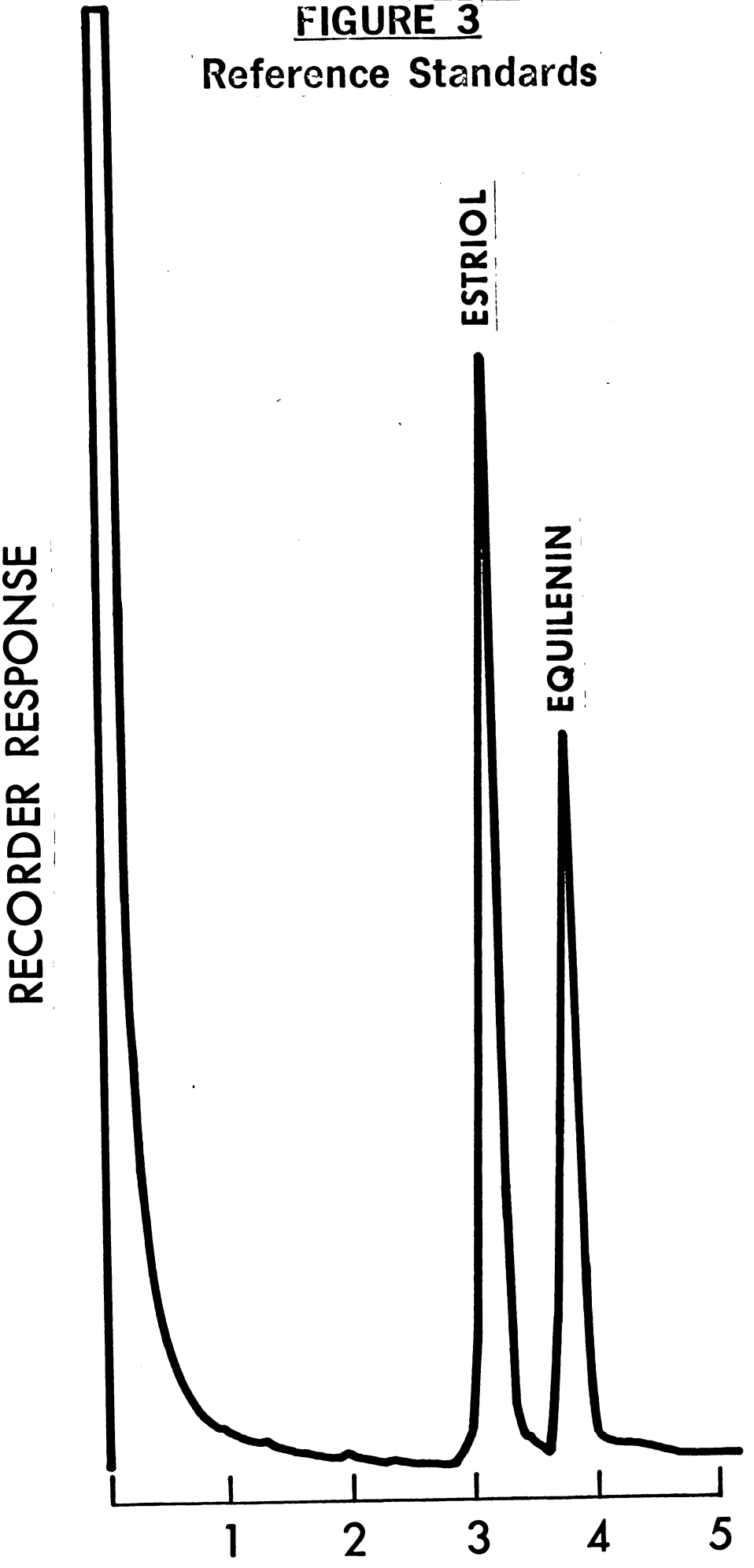
ETHYNYL ESTRADIOL

FIGURE 2

Selected Estrogens

FIGURE 3

Reference Standards



a) That it eluted in an area free from normal substances in urine.

b) That it could be added sufficiently early in the procedure so as to correct for analytical variation.

c) That its recovery from urine was similar to the recovery of estriol.

II. HYDROLYSIS

The enzyme chosen was β-glucuronidase derived from *Escherichia Coli*. The activity of the enzyme was expressed in Sigma-Fishman units. One Sigma-Fishman unit will release 1 microgram of phenolphthalein from phenolphthalein glucuronide per hour at 37°C in 0.075 M phosphate buffer at a pH 6.8-7.0. The activity of the enzyme used was about 52,000 units per gram without chloroform. When chloroform was added the activity increased to about 170,000 units per gram of enzyme. The increased enzyme activity in the presence of chloroform is probably due to the solubilization of some component in the enzyme preparation by the chloroform, or by traces of alcohols in the chloroform.

All hydrolysis reactions were performed using 5.0 ml of urine and 1.0 ml of 1 M phosphate buffer at pH 7.0 (25°C). Two drops of chloroform were added to each urine sample before hydrolysis.

A. OPTIMAL TEMPERATURE

5.0 ml aliquots of a 24 hour urine from a normal pregnant woman were adjusted to pH 7.0. Seventeen hundred units of β-glucuronidase and 2 drops of chloroform were added to each aliquot and incubated at 25, 37,45,55,60,65 and 70°C for 30 minutes. After incubation, the hydrolysates

were adjusted to pH 2 and partitioned with ethyl ether to extract the free estriol. The ethyl ether was back extracted with aqueous 10% sodium hydroxide which removed the estriol as the water soluble sodium salt. The sodium hydroxide was acidified to pH 2 and the free estriol was extracted into ethyl ether. The ethyl ether was evaporated to dryness and 125 micrograms of the internal standard, equilenin, was added to each. (In the final method, the internal standard is added at the beginning of the procedure). The samples were silylated and chromatographed. Results of this experiment were as follows:

TEMPERATURE	ESTRIOL - mg/l
25°C	9.1
37°C	14.5
45°C	19.0
55°C	20.1
60°C	21.6
65°C	21.2
70°C	7.2

It was concluded that 60°C achieved complete hydrolysis and at 70°C the enzyme was inhibited.

B. OPTIMAL INCUBATION TIME

Using the above mentioned procedure, a series of 5.0 ml aliquots of urine were incubated at 60°C for 15,20,30,40,50 and 60 minutes. Results were as follows:

INCUBATION TIME AT 60°C	ESTRIOL - mg/l
15 min	24.6
20 min	28.7
30 min	28.0
40 min	28.2
50 min	29.0
60 min	28.5

It was concluded that 30 minutes incubation at 60°C would achieve complete hydrolysis.

C. OPTIMAL ENZYME CONCENTRATION

A similar procedure was again used. 5.0 ml aliquots of urine were incubated at 60°C for 30 minutes using, 260, 520, 1040, 1560, 2080, 2600 and 3120 units* of β-glucuronidase per aliquot. Results were as follows:

UNITS OF β-GLUCURONIDASE	ESTRIOL mg/l
260	16.8
520	20.1
1040	27.1
1600	28.2
2080	28.6
2600	28.1
3120	28.9

From the above three experiments it was concluded that 1600 units of β-glucuronidase per 5.0 ml of urine incubated for 30 minutes at 60°C would complete hydrolysis of the estriol glucuronides in maternal urines. The following experiment was performed to verify that the conditions established were optimal.

Estriol 16 α glucuronide was added to a male urine at a concentration of 47.2 mg estriol per liter of urine. This urine and known dilutions were hydrolyzed using the above conditions. The estriol levels were determined using the final procedure described. Results were as follows:

KNOWN ESTRIOL mg/l	ESTRIOL DETERMINED mg/l	% ESTRIOL % RECOVERED
5.9	6.1	103
11.8	11.7	99
17.7	17.0	96
29.5	27.0	92
41.3	38.7	94
47.2	44.7	95

It was concluded that the established conditions were adequate for complete hydrolysis.

* *Based on 52,000 units per gram of enzyme*

Estriol glucuronides comprise approximately 95% of the total estriol conjugates in maternal urine, and the remaining estriol is conjugated as sulfates. A comparison was made between β-glucuronidase alone and an enzyme preparation containing β-glucuronidase and sulphatase. The enzymatic hydrolysis described by Van de Calseyde (45) et al. was utilized. This method uses 10,000 units of β-glucuronidase and 80,000 units of sulphatase per milliliter of urine buffered in 0.1 ml of 2 N acetate buffer at pH 4.6. Incubation is for 30 minutes at 62°C. The source of the enzyme used by Van de Calseyde et al. is from the digestive juices of *Helix pomatia*. The estriol levels following hydrolysis were determined using the final procedure described. Results of the linear regression analysis of the two methods of hydrolysis were as follows:

Slope = 1.059

Intercept = -0.92

Correlation coefficient = .951

N = 20

It was concluded that the two methods of hydrolysis were comparable.

III. COLUMN EXTRACTION

Amberlite XAD-2 resin[R] is a polymeric absorbant bead (.30-.45 mm) diameter) of crosslinked polystyrene. It has high porosity, a nonionic structure and a large surface area. These characteristics, and the hydrophobic nature of the resin, allows selective adsorbtion of organic molecules through the attachment of the hydrophobic portion of the organic molecules to the resin. Amberlite XAD-2 resin has been used to effectively adsorb estrogen conjugates from urine (32,33). Bradlow (32) has also shown that

R) Rohm and Haas Co., Philadelphia, PA. 19105

the free estrogens can similarly be adsorbed from urine following hydrolysis, and that the adsorbtion is independent of pH. This has been verified by Osawa and Slaunwhite (33).

Small organic molecules like glucose and urea, and inorganic ions are not retained by the resin.

A. BINDING CAPACITY OF COLUMN

A known amount of free estriol was added to a male urine at levels of 5, 10, 20, 30, 40 and 50 mg/l. Five ml aliquots of these urines were adjusted to pH 7.0, hydrolyzed, and the hydrolysates poured through the columns. The hydrolysis tubes were rinsed with 5 ml of distilled water and the rinses also poured through their respective columns. The combined urine and water effluent from the columns were collected to determine if any estriol was present. These effluents were acidified, extracted into ether, the ether evaporated to dryness, and the residues silylated and chromatographed. The effluents, up to and including the 40 mg/l estriol urine, contained no estriol, the 50 mg/l effluent contained only an insignificant amount of estriol, while the 60 mg/l effluent contained a significant amount of estriol. It was concluded that the column would effectively bind up to .25 mg of estriol.

B. ELUTION OF BOUND ESTRIOL FROM THE RESIN

Experiments were carried out comparing methanol, ethanol, ethyl acetate, chloroform and ethyl ether as eluting solvents. Ethyl ether was the most efficient eluting solvent. However, the flow characteristics of ether through the column were poor due to bubble formation within the resin bed which frequently stopped the flow of ether. Elution of the

bound estriol from the XAD-2 resin was accomplished most efficiently by removing the resin from the column, placing it into a wide-mouth screw cap polypropylene tube, and shaking the resin with acidified ether. The resin was removed from the column with gentle air pressure followed by an ether rinse. Ether was then added to the polypropylene tube to the 25 ml mark. The ether resin mixture was acidified with 2 drops of concentrated hydrochloric acid and shaken on a mechanical shaker for 10 minutes. After shaking the ether was easily poured off. Centrifugation to pack the resin was not necessary.

Using the above elution technique, a series of recovery experiments were conducted to determine the efficiency of this technique. Five milliliter aliquots of a male urine containing known amounts of estriol were hydrolyzed and poured through the columns as described. The resin was removed from each column and extracted into acidified ether, following the addition of 125 micrograms of the internal standard, equilenin. The ether was poured off into glass tubes and evaporated to dryness. The residue was silylated and chromatographed. Results were as follows:

KNOWN ESTRIOL	RECOVERED ESTRIOL	% RECOVERY
5 mg/l	5.0 mg/l	100
10	9.5	95
20	18.7	94
30	28.6	94
40	42.0	105
50	49.0	98

This technique leads to the efficient removal of estriol from the XAD-2 resin.

IV. PURIFICATION

Following hydrolysis, the urine contains both neutral and phenolic steroids which bind indiscriminately to the XAD-2 resin. When the urinary steroids, which are bound to the resin, are extracted, silylated and chromatographed, the resulting chromatogram contains numerous peaks, some of which interfere with estriol and the internal standard. Thus, a separation of the estrogens from the neutral steroids by solvent partition is essential. This separation is problematic in that estriol is a polar estrogen relative to the internal standard, equilenin, which has a polarity similar to estrone. This difference in polarity can be noted when estriol and equilenin are dissolved in ether and partitioned with 10% sodium hydroxide. After shaking, the estriol is completely transferred into the sodium hydroxide, while the equilenin remains partially in the ether fraction. Brown (17) reported that only through tedious and exhaustive extractions was estrone and estradiol removed from benzene by sodium hydroxide. In this respect, equilenin is similar to estrone. Because of this fact, a simple partioning of the ether extract of the XAD-2 resin with 10% sodium hydroxide would not be sufficient to remove all of the internal standard from the ether. If the solubility of equilenin in ether could be decreased there would be an increased solubility of the equilenin in the sodium hydroxide. This could be achieved by adding petroleum ether to the ethyl ether at a ratio of 3 parts petroleum ether to 1 part ethyl ether. When estriol and equilenin were partitioned with 10% sodium hydroxide under these conditions, both the estriol and the equilenin were completely transferred into the sodium hydroxide.

A second approach was to form the phenolic salts of the estrogens in a manner which did not involve extraction into sodium hydroxide. This was accomplished by evaporating the ether extract of the XAD-2 resin and then adding 10% sodium hydroxide to the residue. The soluble phenolic estrogen salts can then be separated from the insoluble neutral steroids by solvent partition. The solvent used to remove the neutral steroids was ether-petroleum ether at a ratio of 1 to 3 parts. This solvent effectively removed the majority of the interfering neutral steroids while the phenolic estrogen salts remained in the sodium hydroxide. Utilizing this technique, interfering peaks were kept at a minimum and the only major peaks were estriol and the internal standard (Figure 4, 5).

V. INTERFERING COMPOUNDS

A. OTHER NATURALLY OCCURRING ESTROGENS

The interference that might occur from other polar estrogens which would partition similar to estrogen was investigated. Other naturally occurring estrogens found in maternal urine are estrone, estradiol, 16 epiestriol, 17 epiestriol, 16-17 epiestriol and 6 α hydroxyestriol. These pure compounds were silylated and chromatographed. Estrone, estradiol and 16 - 17 epiestriol eluted well before estriol and the internal standard. The two positional isomers of estriol, 17 epiestriol and 16 epiestriol eluted as a front shoulder and a back shoulder respectively on the estriol peak. 6 α hydroxyestriol co-eluted with 16 epiestriol. Thus, these three estrogens could potentially interfere. However, these three estrogens are present in late pregnancy urines at average levels reported to be as follows (46):

FIGURE 4

Unknown urine processed as described in final procedure

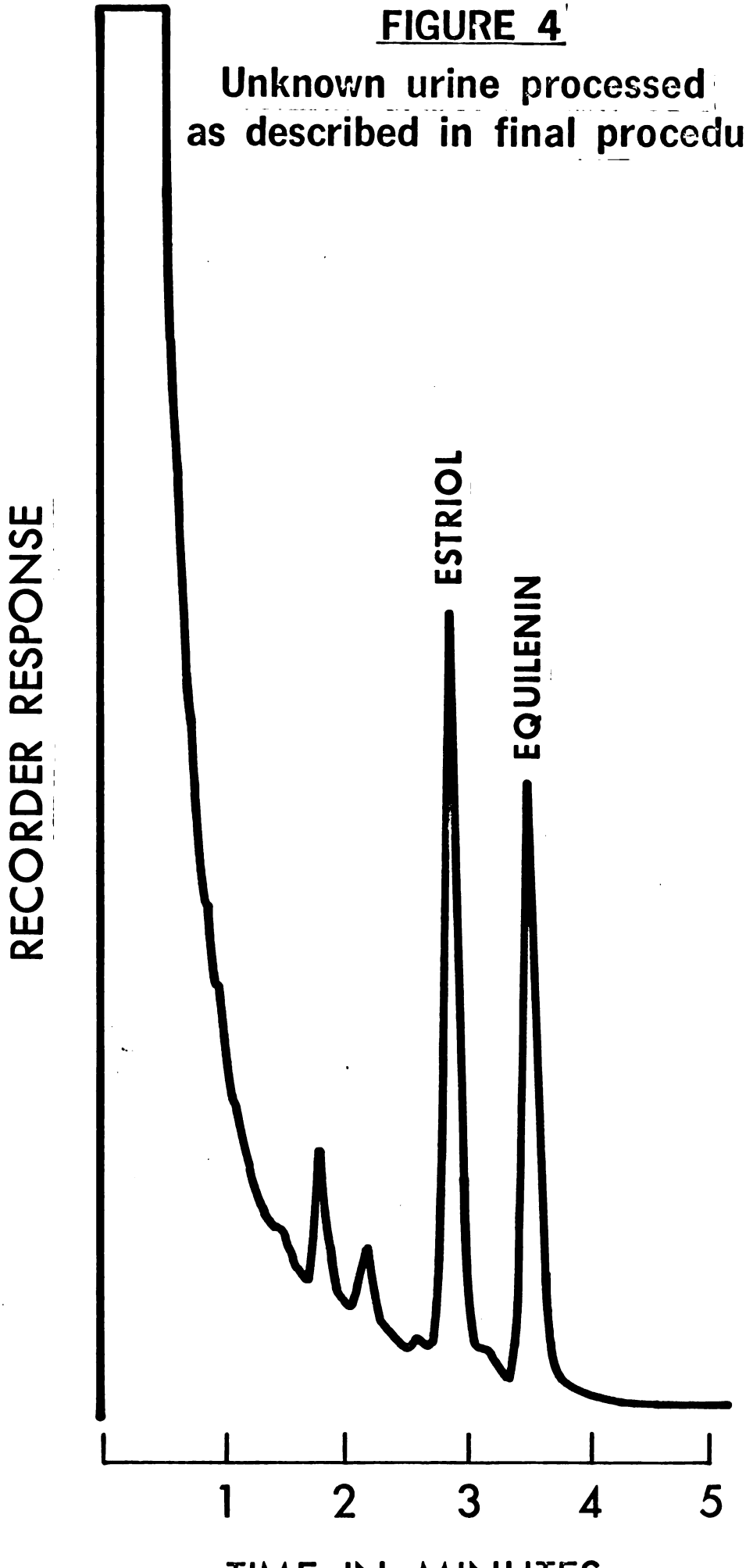
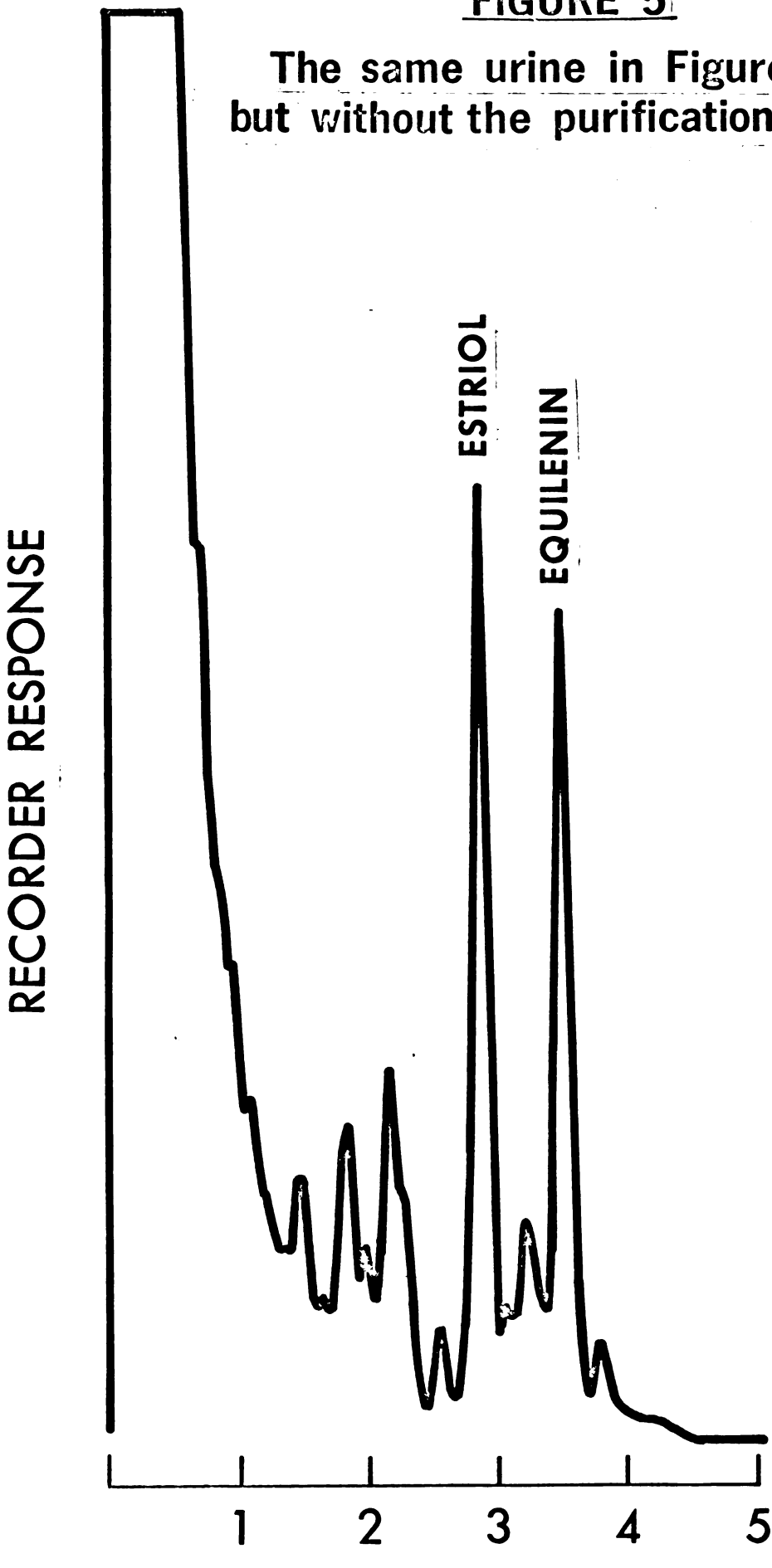


FIGURE 5

The same urine in Figure 4 but without the purification step



16 epiestriol	0.8 mg/l
17 epiestriol	0.1 mg/l
6α hydroxyestriol	1.0 mg/l

Thus, these levels present in maternal urine are relatively insignificant compared to estriol levels seen in pregnancy.

B. DRUGS AND OTHER NATURAL SUBSTANCES

The interference caused by drugs or drug metabolites on the estimation or urinary estriol has been reported (25,27,28,44). These interferences are seen most commonly in methods using acid hydrolysis.

The influence of glucose on the decreased recovery of estriol following acid hydrolysis is well known and has been reported (25,26). Hydrochlorothiazide has also been reported to cause decreased yields of estriol following acid hydrolysis (28). Phenolphthalein has been reported to decrease estriol values in both acid and enzymatic hydrolysis methods (25,44). Methenamine mandelate (Mandelamine^{R}), a urinary tract antibacterial agent, has been reported to cause destructuion of estrogens during acid hydrolysis (27). Salicylate (aspirin) has also been reported to effect estriol methods using enzymatic hydrolysis (47).

To determine the effect of the above substances on the method described, the following experiments were conducted.

GLUCOSE

Glucose was added to a normal maternal urine at levels of 4, 6, 10, and 20 grams per liter. The glucose free urine and those with the added glucose were carried through the entire procedure. Results were as follows:

R) Warner/Chilcott, Morrisplanes, N.J. 07950

URINE	ESTRIOL mg/l
Without Glucose	27.3
4 gm/l Glucose	27.9
6 gm/l Glucose	28.0
10 gm/l Glucose	27.1
20 gm/l Glucose	27.0

It was concluded that glucose did not interfere with this method.

HYDROCHLOROTHIAZIDE

This diuretic is excreted unchanged in the urine following the usual dose of 25 to 100 mg per day.

Hydrochlorothiazide was added to a normal maternal urine at levels of 25, 50 and 100 mg/l and were processed through the described procedure. Results were as follows:

URINE	ESTRIOL mg/l
Without hydrochlorothiazide	15.4
25 mg/l hydrochlorothiazide	15.9
50 mg/l hydrochlorothiazide	15.5
100 mg/l hydrochlorothiazide	16.0

The conclusion was that hydrochlorothiazide did not interfere.

PHENOLPHTHALEIN

Phenolphthalein is found in many laxatives. The average dose of 100 mg per day is poorly absorbed in the intestinal tract. Approximately 15% is excreted in the urine predominately as the glucuronide. Free phenolphthalein was added to a normal maternal urine at levels of 20, 33 and 100 mg/liter and the urines were processed as described. Results on two different samples were as follows:

URINE	ESTRIOL mg/l URINE # 1	URINE #2
Without phenolphthalein	14.9	18.7
20 mg/l phenolphthalein	14.4	19.0
33 mg/l phenolphthalein	15.3	18.4
100 mg/l phenolphthalein	11.9	13.3

It can be seen that only when the free phenolphthalein in the urine reaches a level of 100 mg/l does interference occur. Free phenolphthalein at levels of 100 mg/l would be unlikely, considering the average dose and the poor absorbtion of phenolphthalein. However, phenolphthalein is easily detected since it will turn pink in the sodium hydroxide during the processing.

Because phenolphthalein is excreted as the glucuronide it may interfere with enzymatic hydrolysis by substrate competition. Phenolphthalein glucuronide was added to a normal maternal urine at levels of 20, 50, and 100 mg/l, and the urines were processed. Results were as follows:

URINE	ESTRIOL mg/l
Without phenolphthalein Glucuronide	10.9
20 mg/l phenolphthalein Glucuronide	11.0
50 mg/l phenolphthalein Glucuronide	11.3
100 mg/l phenolphthalein Glucuronide	6.6

It is apparent that substrate competition does not occur up to 50 mg/l, but that competition becomes evident at a level of 100 mg/l of phenolphthalein glucuronide. It would be very unlikely to achieve urinary levels above 50 mg/l of phenolphthalein glucuronide considering the average dosage and the poor intestinal absorbtion of phenolphthalein.

Phenolphthalein carries through the entire procedure and forms a silyl derivative which elutes as a peak on the final chromatogram. However, it elutes well after estriol and equilenin, and does not interfere.

METHENAMINE MANDELATE (MANDELAMINE)

This antibacterial agent is hydrolyzed in vivo at an acid pH to form ammonia and formaldehyde. The parent compound or the metabolite, formaldehyde, is the interfering compound in acid hydrolysis methods. It has been reported that formaldehyde interferes with acid hydrolysis methods (26). To determine the effects of formaldehyde on this method, formaldehyde was added to a normal maternal urine at a level of 20, 50, 100, and 1000 mg/liter, and the urines were then processed. Results were as follows:

URINE	ESTRIOL mg/l
Without formaldehyde	15.6
20 mg/l formaldehyde	16.0
50 mg/l formaldehyde	15.0
100 mg/l formaldehyde	15.9
1000 mg/l formaldehyde	15.1

It was concluded that formaldehyde did not interfere with this method.

To determine the effect of the parent compound, methenamine mandelate was added to a normal urine at levels of 500 and 1000 mg/liter and the urines were processed. Results were as follows:

URINE	ESTRIOL mg/l
Without mandelate	9.8
500 mg/l mandelamine	9.3
1000 mg/l mandelamine	10.0

It was thus concluded that the parent compound did not interfere.

SALICYLATES

Ingested acetylsalicylic acid is excreted in the urine as a glucuronide. This has been reported to interfere with enzymatic hydrolysis by substrate competition (47).

A male volunteer ingested 2 grams of aspirin over a 6 hour time period. Urine was collected for 12 hours. The salicylate level of the urine was 1.5 grams in the total volume of 600 ml collected. Four normal maternal urines were diluted two-fold with the salicylate containing urine. The same maternal urines were diluted two-fold with a normal urine collected from the same male volunteer. These urines were processed. Results were as follows:

URINE	ESTRIOL mg/l			
	#1	#2	#3	#4
without Salicylate	11.3	10.9	11.0	24.0
with Salicylate (125 mg/l)	9.8	8.5	9.8	20.8

These results suggest that substrate competition occurs in the presence of salicylate glucuronides. This was verified by performing additional experiments on specimen #2 as follows:

a) The salicylate concentration was reduced in half (to 62.5 mg/l).

b) The β-glucuronidase added for hydrolysis was increased two-fold (to 3200 units).

Results were as follows:

URINE #2	ESTRIOL mg/l
without Salicylate (1600 units β-glucuronidase	10.9
125 mg/l Salicylate	8.5
62.5 mg/l Salicylate	9.5
125 mg/l Salicylate (3200 units β-glucuronidase	10.0

These results confirm that substrate competition occurs and that a two-fold increase in enzyme concentration during hydrolysis will effectively eliminate this substrate competition.

C. URINE BACKGROUND

To determine if any normally occurring substance found in a maternal urine would interfere with the internal standard, a series of maternal urines were processed through the procedure as described. The internal standard was not added. Only insignificant small peaks eluted in the area of the internal standard. A peak common to all urines eluted just beyond, but separate from the internal standard. This peak was small enough not to cause any interference (Figure 6).

ACCURACY AND PRECISION

I. ACCURACY

The accuracy of this method was evaluated by determining the recovery of known amounts of free estriol and estriol glucuronide added to an estriol free urine.

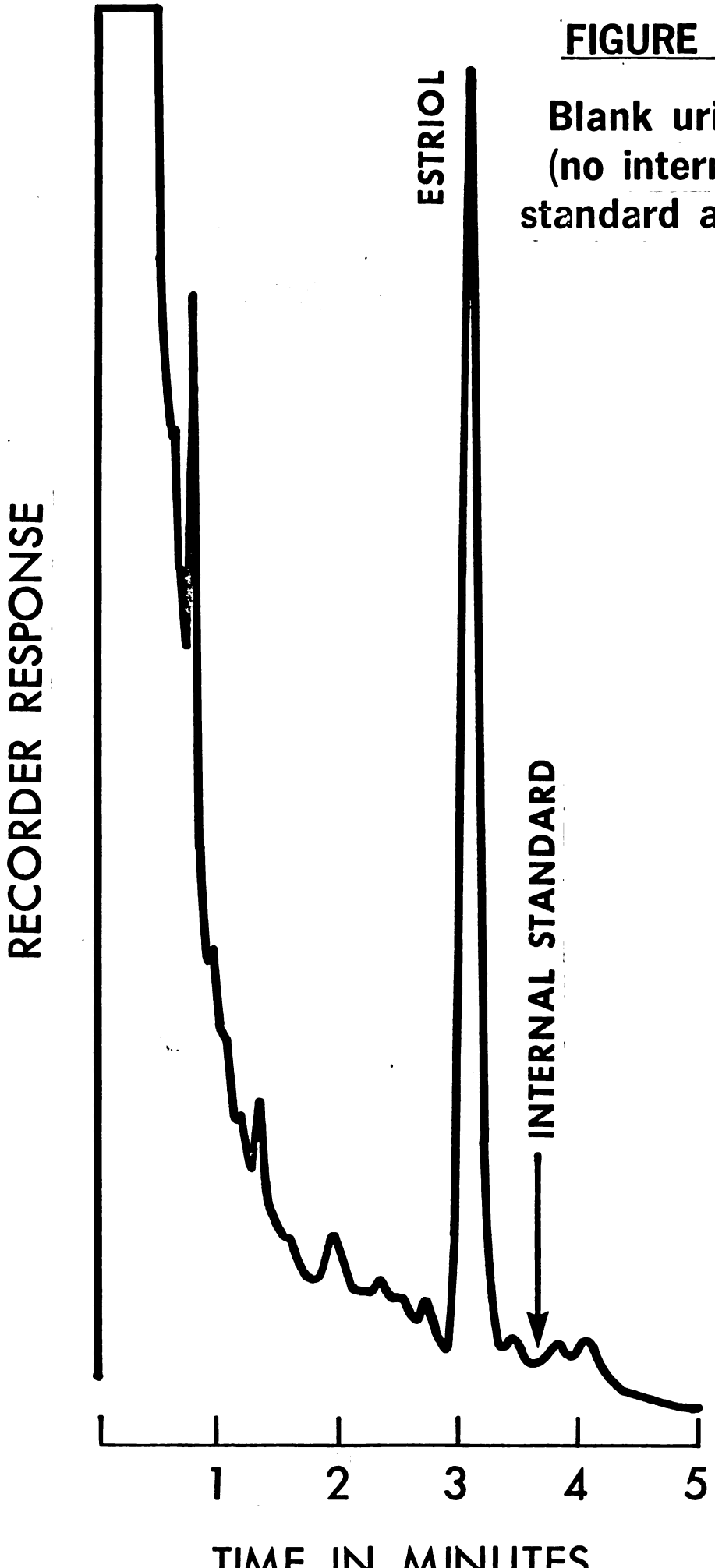
A. RECOVERY OF ADDED FREE ESTRIOL

Free estriol was added to a male urine at levels of 10, 20, 30, 40, and 50 mg/l. Each of these levels were determined five times, carrying the urine through the entire procedure. Results were as follows:

KNOWN ESTRIOL LEVEL mg/l	AVERAGE ESTRIOL RECOVERED mg/l	AVERAGE % RECOVERED	RANGE OF RECOVERY
10	10.2	102	94-108
20	19.9	99	94-104
30	29.9	99	93-106
40	38.4	96	92-100
50	49.8	99	92-110

FIGURE 6

Blank urine (no internal standard added)



B. RECOVERY OF ESTRIOL 16 α GLUCURONIDE

As stated previously, the recovery of estriol 16α glucuronide ranged from 92-103% (see HYDROLYSIS).

II. PRECISION

The precision of this method was evaluated by determining replicate aliquots of a maternal urine. Both within run, and day-to-day precision were evaluated.

A. WITHIN RUN PRECISION - all replicate aliquots were run at the same time.

LOW LEVEL ESTRIOL mg/l		HIGH LEVEL ESTRIOL mg/l	
7.3		37.2	
7.3		37.2	
6.9		37.0	
6.7		37.1	
7.3		37.6	
6.9	N=15	37.1	N=15
6.7	Mean=7.03	36.9	Mean=36.9
7.1	S.D.=.235	37.0	S.D.=.565
7.3	C.V.=3.3%	35.9	C.V.=1.6%
6.8		36.0	
7.2		37.5	
7.0		37.4	
6.9		36.3	
7.3		36.7	
6.8		37.9	

B. DAY-TO-DAY PRECISION - all replicate aliquots were run once per day for 15 consecutive days.

LOW LEVEL ESTRIOL mg/l		MID LEVEL ESTRIOL mg/l		HIGH LEVEL ESTRIOL mg/l	
9.0		12.0		21.2	
7.9		12.1		21.0	
8.0		10.6		23.4	
8.5		12.7		21.6	
9.0		12.4		22.5	
8.8		10.1		19.9	
9.3		11.5		19.7	
8.5	N=16	12.7	N=17	20.2	N=15
7.8	Mean=8.5	12.2	Mean=11.9	20.0	Mean=21.3
8.4	S.D.=0.4	11.6	S.D.=0.75	22.3	S.D.=1.4
8.5	C.V.=5.2%	11.7	C.V.=6.4%	21.2	C.V.=6.6%
8.1		12.1		22.8	
8.7		12.9		23.6	
8.4		12.5		20.3	
8.6		11.8		19.0	
9.1		11.3			
		11.9			

LINEARITY

There was a linear relationship between the ratio of the peak heights of estriol to the internal standard and the concentration of estriol over a range of 0-50 mg/l estriol (Figure 7).

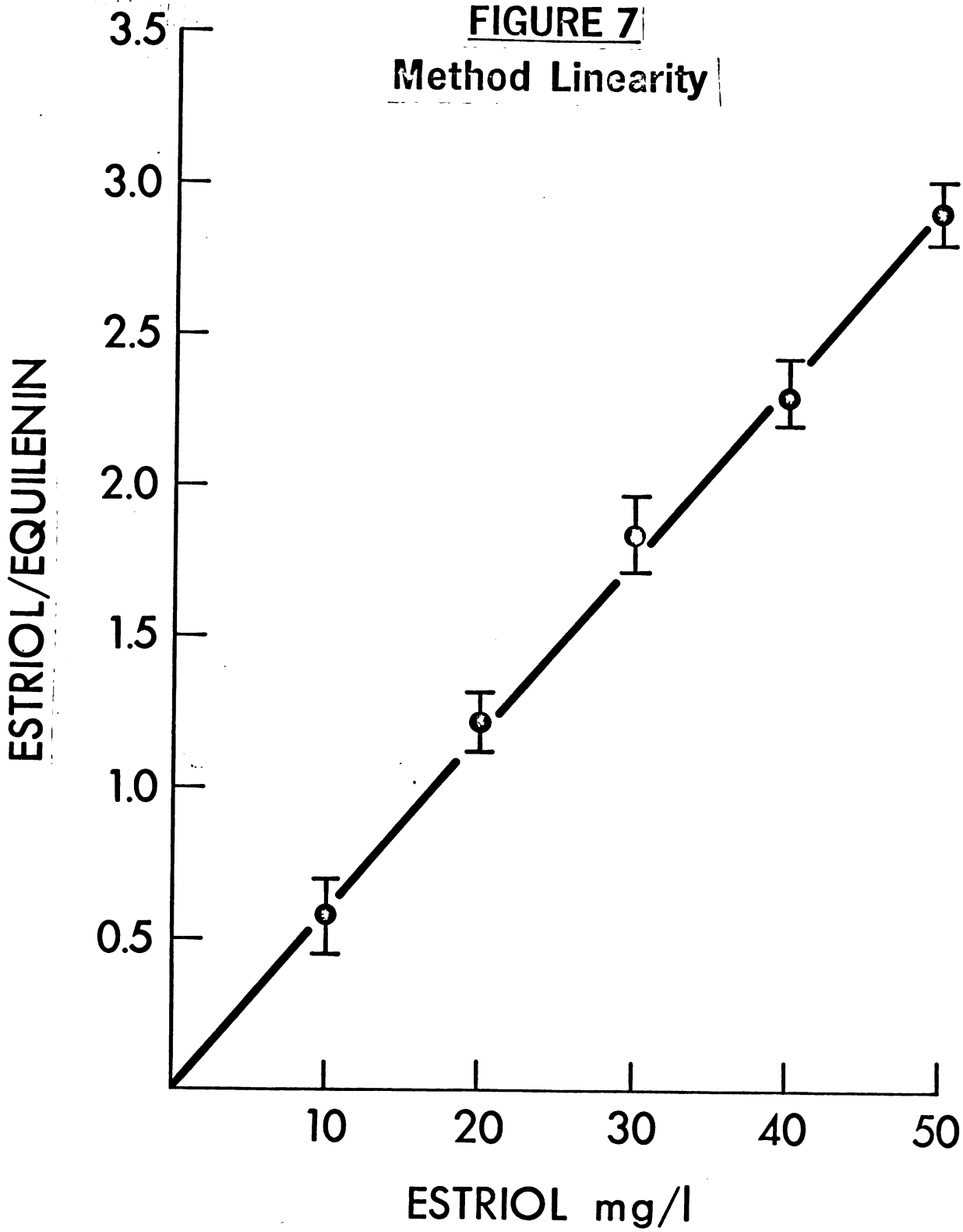
DISCUSSION

Of the large number of published assay methods for placental estriol, few methods correct for analytical losses by incorporating a valid internal standard. This paper describes a method which incorporates the use of a true internal standard which is added early in the procedure.

The use of Amberlite XAD-2 resin extraction is an efficient and simple method which eliminates the necessity of washing the ether extract with alkaline bicarbonate, a procedure which is common to most estriol assay methods. This alkaline bicarbonate wash is intended to remove many strongly acidic, potentially interfering compounds. Coincidentally, the alkalinity of this bicarbonate solution also removes some polar estrogens,

FIGURE 7

Method Linearity



such as estriol. This method does not require centrifugation, as phase separation is rapid and complete. There is no emulsion formation, a common occurance in most acid hydrolysis procedures and some enzymatic procedures. The lack of emulsion formation eliminates a potential source of estriol loss during processing.

Rapid enzymatic hydrolysis of the conjugated estrogen eliminates the decreased recovery of estriol which occurs with acid hydrolysis. The decreased recovery of estriol caused by glucose, mandelamine, phenolphthalein, hydrochlothiazide and salicylates does not occur when using this method of hydrolysis. Hydrolysis time is comparable to acid hydrolysis with the added advantage that enzymatic hydrolysis is less destructive, causing virtually no estriol loss.

The purification step which separates the phenolic estrogen from the potentially interfering neutral steroids helps create a more specific method. The short additional time needed to accomplish this separation is well worth the effort. The elimination of this purification step will result in a very rapid method if necessary, although, it will be less specific and less accurate.

In conclusion, this method is an accurate, precise and specific assay for urinary placental estriol. A single sample can be processed in about two hours and multiple samples can easily be batched in such a way that about 30 samples can be processed in a 8 hour day.

FINAL METHOD

1. To 5.0 ml of urine add 1.0 ml of buffer-enzyme.

   BUFFER ENZYME - 1600 units of β-Glucuronidase/ml of pH 7.0 1 M phosphate buffer.

2. Add 2 drops of chloroform, mix and incubate at 60°C for 30 minutes.
3. Pour cooled hydrolysate through hydrated column, rinse hydrolysate tube with 5.0 ml distilled water and pour through column. Allow column to drain.
4. Expel resin into wide mouth tube with gentle air pressure rinsing column with ether.
5. Add ether up to 25 ml, add 2 drops of concentrated hydrochloric acid, and 0.5 ml working internal standard
6. Shake the tube 10 minutes, then pour ether into glass tube and evaporate ether to dryness.
7. Dissolve the residue in 5 ml of 10% sodium hydroxide and add 15 ml of ether: petroleum ether (1:3).
8. Shake 5 minutes and aspirate off the ether layer.
9. Add 1.5 ml of conc. hydrochloric acid and 15 ml ether to the sodium hydroxide. Shake 5 minutes.
10. Remove ether layer, dry ether with sodium sulfate and evaporate ether to dryness.
11. Evaporate 0.4 ml of reference standards to dryness.
12. To residues add 50 µl Dimethyl foramide and 120 µl µf BSTFA, incubate at 70°C for 30 minutes. 1-2 µl of the silylated products are used for analysis.

MATERIALS

1. β-Glucuronidase Bacterial powder approximately 50,000 sigma units per gram. (Sigma Chemical Co., St. Louis, MO. 63178).
2. INTERNAL STANDARD (d-EQUILENIN)(3-hydroxyestriol-1,3,5 (10) 6,8-pentaen-17-one) Sigma Chemical Co. A working standard of 25 mg/dl methanol was used.
3. ESTRIOL (Δ 1,3,5 (10) ESTRATRIEN - 3, 16∝ 17 β-triol) Sigma Chemical Co.
4. ETHYNYL ESTRADIOL (17 ETHYNYL Δ 1,3,5 ESTRATRIEN-3, 17 β-triol) Sigma Chemical Co.
5. EQUILIN (Δ 1,3,5 7-ESTRATRIEN-3, 16 17 β-triol) Sigma Chemical Co.
6. 16 EPIESTRIOL (Δ 1,3,5 (10)-ESTRATRIEN-3, 16β, 17β-triol; 16β-hydroxy-17β- estradiol) Sigma Chemical Co.
7. 17 EPIESTRIOL (Δ 1,3,5 (10) ESTRATRIEN-3, 16∝, 17∝ triol, 16∝ hydroxy-17∝ ESTRADIOL) Sigma Chemical Co.
8. 16,17 EPIESTRIOL (Δ 1,3,5,(10) ESTRATRIEN -3, 16β, 17∝ triol; 16β-hydroxy-17∝ estradiol) Sigma Chemical Co.
9. ESTRADIOL CYCLO PENTANE PROPIONATE K & K Laboratories, Plainview, N.Y.
10. ESTRONE (Δ 1,3,5 (10)-ESTRATRIEN-3,16∝ 17β triol) Sigma Chemical Co.
11. ESTRADIOL (Δ 1,3,5, (10) ESTRATRIEN-3, 17β diol) Sigma Chemical Co.
12. REFERENCE STANDARDS a working standard of 25 mg/dl of estriol and equilenin in methanol was used.
13. BSTFA (N, O-Bis(Trimethylsilyl)trifluoroacetamide) Pierce Chemical Co., Rockford, ILL. 61105.
14. AMBERLITE XAD-2 Columns Brinkman Drug Screen Columns. Brinkman Instrument Co., Westbury, N.Y. 11590.

A Varian gas chromatograph, model 1200 equipped with a flame ionization detector was used (Varian Aerograph, Walnut Creek, CA. 94598). A 2 meter glass coil column, 2 MM I.D. was packed with 3% OV-17 on chromosorb W H/P, 100/200 mesh size (Varian Aerograph).
Gas flow rates were: hydrogen 20 ml/min., air 360 ml/min., and nitrogen 30 ml/min. The injector and detector temperatures were maintained at 295^{o}C. The column temperature was maintained at 280^{o}C.

REFERENCES

1. Smith, G. V., Smith, O. W.: *Proc. Soc. Exper. Biol. and Med.* 30:918, 1933.
2. Stewart, H. L.: *Amer. J. Obstet. Gynec.* 61:990-1000, 1951.
3. Smith, G. V., Smith, O. W.: *J. Clin. Endocrin.* 1:470, 1941.
4. Smith, G. V., Smith, O. W.: *Physiol. Rev.* 1:28, 1948.
5. Cassmer, O.: *Acta Endocrin. Suppl.* 45: 1959.
6. Diczfalusy, E., Dion, R., Schwers, J.: *Arch. Micr. Anat.* 54:67, 1965.
7. Siiteri, P. K., MacDonald, P. C.: *Steroids* 2:713, 1963.
8. Siiteri, P. K., MacDonald, P. C.: *J. Clin. Endocrin.* 26:751, 1966.
9. Bolte, E., Mancuso, S., Eriksson, G., Diczfalusy, E.: *Acta Endocrin.* 45:535-559, 1964.
10. Cummings, R. V., Rourke, J. E., Shelley, T. F.: *Amer. J. Obstet. Gynec.* 104:1047-1052, 1969.
11. Goebelsmann, U., Jaffe, R. B.: *Acta Endocrin.* 66:679-693, 1971.
12. Goebelsmann, U., Wiquist, N., Diczfalusy, E.: *Acta Endocrin. Metabol.* 59:595, 1968.
13. Goebelsmann, U., Freeman, R. K., Mestman, J. H., Nakamura, R. M., Woodling, B. A.: *Amer. J. Obstet. Gynecol.* 115:795-802, 1973.
14. Beischer, N. A., Brown, J. B.: *Obstet. Gynecol. Surv.* 27:303, 1972.
15. Kober, S.: *Biochem. Z.* 239:209-212, 1931.
16. Cohen, S. L., Marrian, G. F.: *Biochem. J.* 28:1603-1614, 1934.
17. Brown, J. B.: *Biochem. J.* 60:185-193, 1955.
18. Ittrich, G.: *Hoppe-Seylers Z.* 312:1-14, 1958.

19. Bauld, W. S.: *Biochem. J.* 63:488-495, 1956.

20. Cohen, S. L.: *J. Clin. Endocrin.* 26:994-1004, 1966.

21. Goebelsmann, U.: *Clin. Chim. Acta* 23:469-474, 1969.

22. Adessi, G., Eichenberger, D., Goutte, C., Jayle, M. F.: *Clin. Chim. Acta* 45:369-374, 1973.

23. Anderson, D. W., Goebelsmann, U.: *Clin. Chem.* 22, 5, 611-615, 1976.

24. Schindler, A. E., Ratanasopa, V., Herrmann, W. L.: *Clin. Chem.* 13, 3, 186-195, 1967.

25. Bolognese, R. J., Corson, S. L., Touchstone, J. C.: *Clin. Chem.* 39: 2, 683-687, 1972.

26. Ryan, M., Gray B. C.: *Clin. Chem. Acta* 60:197-204, 1975.

27. Touchstone, J. C., Stojkewycz, M., Smith, K.: *Clin. Chem.* 11:1019, 1965.

28. Rosenthal, A. F., Tomson, M. R.: *Clin. Chem.* 18, 5:471-472, 1972.

29. Bramhall, J., Britten, A. Z.: *Clin. Chim. Acta* 68:203-213, 1976.

30. Dray, J., Tillier, F., Dray, F., Ullmann, A.: *Ann. Inst. Pasteur* 123:853-857, 1972.

31. Crowley, M. F., Rosser, A.: *Clin. Chim. Acta* 49:115-122, 1973.

32. Bradlow, H. L.: *Steroids* 11:265-272, 1968.

33. Osawa, Y., Slaunwhite, W. R.: *Steroids* 15:73, 1970.

34. Chu, T. M., Osawa, Y., Reynoso, G.: *Clin. Chem.* 17:5, 1971.

35. Beckloff, S., Faucette, W., Cawley, L. P.: *Clin. Chem.* 16:532, 1970.

36. Adlercreutz, H., Herrmann, N. L.: *IN: Gas Chromatography of Steroids in Biological Fluids.* page 215, 1965.

37. Fishman, J., Brown, J. B.: *J. Chromato.* 8:21-24, 1962.

38. Wotiz, H. H., Martin, H. F.: *J. Biol. Chem.* 236, 5:1312-1317, 1961.

39. Baynton, R. B., Campbell, D. J.: *Clin. Chem.* 15, 3:190-196, 1969.

40. Touchstone, J. C., Murawec, T., O'Leary, D. K., Luha, L., Mantell, G.: *Clin. Chem.* 17, 2:119-121, 1971.

41. Rosenthal, A. F., Yaseen, A.: *Clin. Chem. Acta* 31:171-174, 1971.

42. Richardson, S. J.: *Clin. Chim. Acta* 29:473-480, 1970.

43. Smith, P. D., Stitch, S. R.: *Clin. Chim. Acta* 36:439-451, 1972.

44. Sims, F. H., Giesbrecht, E., Taylor, J.: *A.J.C.P.* 62, 6:759-765, 1974.

45. Van de Calseyde, J. F., Scholtis, R.J.H., Schmidt, N. A., Kuypers, A.M.J.: *Clin. Chim. Acta* 25:345-349, 1969.

46. Estrogen Assay in Clinical Medicine. *University of Washington Press*, page 251, 1965.

47. Stempfel, R. S., Jr., Sidbury, J. B., Jr., Migeon, C. J.: *J. Clin. Endocrin. Metab.* 20:814, 1960.