EVALUATION OF COMMERCIAL IMMUNOLOGICAL PREGNANCY TESTS

by

Martha L. Gorman, B.A.

A THESIS

Presented to the Department of Clinical Pathology
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of
Master of Science

June, 1971

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

ACKNOWLEDGMENTS

I gratefully acknowledge the many hours of assistance graciously provided during the past two years by Dr. James L. Bramhall. Without this assistance and understanding, this thesis would not have been possible, and much of the knowledge gained would have been lacking. I also wish to thank Dr. Martha Hamilton, Dr. Bernard Pirofsky, Dr. Margaret Berroth, and Dr. Richard Thompson for help. Never have I found so many people so ready and willing to help at every turn. There are too many to enumerate separately.

I also wish to acknowledge scholarship funds provided by Ortho Diagnostics Inc. to aid in completing this course of study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES

INTRODUCTION

History

One of the earliest pregnancy tests recorded, according to Forbes (1), is found in Prescription 199 of the Berlin Papyrus, written between 1300 and 1100 B.C. It is thought to be the same test as that found in fragments of the earlier Carlsberg Papyrus (2000 to 1800 B.C.) (2). Directions call for the patient to package wheat and spelt (a hard grained variety of wheat) in separate "packs" with dates and sand, and water them with her urine. Germination of wheat indicated that the child would be a boy; of the spelt, a girl; and if neither grew, the patient was not pregnant. Schoeller and Goebel (3), Zollikofer (4), and Burkhardt (5), quoted by Forbes, showed that estrone, estradiol, and some hypophyseal hormones definitely stimulated the growth of corn, onions, and a meadow grass, "Poa". Henriksen (6), in 1941, tested fifty urines using the germination test, and found 75% correctly positive and 85% correctly negative. Thus, there may be some scientific basis for the empirical pregnancy tests involving plant growth.

In the hundreds of years since that time, many "strange and magical" tests for pregnancy have been used, many of which were tests on urine. In 1554, Rueff (7), stated that "a more certain experiment will be to bottle up the woman's urine in a glass for three days, at which time exactly, it is strained through pure muslin, and if she has conceived, minute animalcules looking like lice will appear." Another test (9), states "let the woman that supposes she has conceived, take a green nettle and put

it into her own urine, cover it close, and let it remain therein a whole night. If the woman be with child, it will be full of red spots on the morrow; but if she be not with child, it will be blackish." Earlier versions of a similar test refer to use of a needle rather than a nettle. Uroscopy, the diagnosis of disease by inspection of the urine, was practiced from the thirteenth to the eighteenth century (8). The urine of pregnancy was believed to have special features, particularly if it were stored in a glass container for a few days. By individual criteria, physicians pronounced the diagnosis and prognosis of pregnancy without aid of tests, or even examination of the patient. Some of their criteria may, however, have been valid. In 1486, Savonarola, according to Marshall (10), recorded observation of a cloud which forms in pregnancy urine. Later, workers noted that the particles which formed the cloud, given time, coalesced to form a pellicle. Nausche (11) in 1831, described the pearly white film resembling "fat on cooled broth", which formed on pregnancy urine, and called it Kyesteine, from the Greek word for conception. Pellicles were occasionally found to be formed by urines other than pregnancy urine, but they were of a different character. Tanchon (12) (1839), Bird (13) (1840), and Gull (reported by Bird) found very good correlation of Kyesteine test results with clinical conditions. In 1842, Elisha Kane (14) reported an extensive study of Kyesteine, describing a series of 85 pregnancy urines tested. Of these, 68 had typical pellicles, 11 had modified pellicles, still clearly recognizable, and 6 had none. In a series of 16 unknowns, he diagnosed all correctly, some contrary to presumptive

evidence based on history and physical examination. Pellicles were typically found before the second month of suspended menstruation. In 1845, Kleybolte (15) reported finding Kyesteine formed by a urine collected five days after conception. Gull reported finding a pellicle in the urine of a woman later delivered of a "hydatid mole". Because of its cheesy odor, the pellicle was thought to consist of a caseine precursor of milk. Kane found that it disappeared from the urine of nursing mothers, but reappeared upon interruption of nursing. He also found that moderate temperature was necessary for proper pellicle formation. Churchill (16), in 1851, briefly mentioned a French worker who found that the pellicle would not form in an atmosphere deficient in oxygen. Zimmerman (17) (1846) demonstrated that the pellicle consisted largely of bacteria. Following this discovery, the test lost much of its acceptance, but Tatta (18) (1934) reported the use of preparations of urinary pregnancy hormones to promote growth of certain bacteria. Marshall (10) (1948) believed that the careful work of Nausche, Kane, and others with the Kyesteine test deserved more than a casual dismissal as "Pure Magic". He stated that the hormones being tested by Ascheim were probably capable of promoting bacterial growth, and thereby, formation of the pearly white film. He thought that the Kyesteine test should be included among biological tests for pregnancy.

In 1927, Ascheim and Zondek (19) working with white mice, found a substance present only in the urine of pregnant mice, which induced maturation of ovaries when injected into immature mice. They quickly found the same system could be used to detect

the hormone in the urine of pregnant women. Thinking it to be a pituitary gonadotrophin, they called it "Prolan". Zondek, in 1929, noted the appearance of "Prolan" in the urine of four patients suffering from testicular carcinomas, according to Ferguson (20). Engle (21), that same year, demonstrated that the action of pregnancy urine on the ovaries of immature mice differed from that of the freshly implanted anterior pituitary gland. He found that although follicular stimulation was common to both, the pituitary gland promoted ovulation, while pregnancy urine induced hemorrhagic follicles and corpora lutea. Evans and Simpson (22), also in 1929, reported that increase in ovarian weight was directly proportional to the amount of hypophyseal tissue implanted into the animal, whereas a constant weight increase was caused by a wide dose range of hormone extracted from pregnancy urine.

In 1933, Hamburger (23) suggested that this effect might be explained by follicular stimulating action of the hypophyseal secretion which prepared follicles to be luteinized by urinary hormone. He further suggested that urinary pregnancy hormone was produced by chorionic tissue, and that it should be called "chorionic gonadotrophin". Evans (24), in 1935, concurred in this terminology and found it present in the spinal fluid of patients with hydatidiform mole and chorionepithelioma.

Comparable assays of hormone were facilitated by the establishment in 1939 of the first International Standard for Chorionic Gonadotrophin (25). The International Unit is described as follows: "The specific gonadotrophic activity of 0.1 mgrm. of the standard preparation shall be the international unit for recording

the activities of all gonadotrophic preparations of human urine of pregnancy, but only of such." This amount, under defined conditions will cause cornification of vaginal epithelium of the immature rat. Use of such a standard allowed comparison of biological tests for relative sensitivity as well as quantitation of hormone in unknown specimens. Currently, the second International Standard is used for comparison (26). (See Appendix I)

While some workers characterized chorionic gonadotrophic hormone, others established testing procedures to determine the presence of hormone in the urine of pregnancy. Animals utilized included many species, male and female, hypophysectomized and intact, mature and immature. The gonadotrophic effects of hormone present were measured by increase in weight of uterus, ovary, seminal vesicle, and ventral prostate; extrusion of ova and presence of sperm in the cloaca; ovarian hyperemia and ovarian ascorbic acid depletion; melanophore stimulation in the toad and in the Stichling fish; and increase in length of ovipositor in the Japanese bitterling. Time required for completion of the tests ranged from two hours to twelve days.

Still other workers preferred to demonstrate the biological test for pregnancy directly on the patient, rather than use a laboratory animal. Products related to pregnancy (such as placental tissue and colostrum) were injected into the skin and the reaction observed. Henriksen (6) in 1941, found the results of such skin tests disappointing in that results claimed by individual authors could seldom be achieved by others. Subjective errors of interpretation of the skin reaction were difficult to

avoid because of the vague nature of the reaction. Few of those interpreting the results were trained in reading "allergic" reactions; many patients were found to have nonspecific responses to the proteins injected, and many endocrine and metabolic disturbances modified and invalidated the results. Other tests performed on the patient, such as hormone withdrawal effects, vaginal changes and cytological studies as suggested by Papanicolaou are performed by the attending physician and associates and are more properly grouped as a part of the clinical examination of the patient. A summary of representative biological tests is found in Table I.

During the last decade, in vitro immunological tests have successfully challenged and almost entirely replaced biological tests in routine clinical laboratories. These tests for pregnancy had their beginning early in the twentieth century. Following the discovery of antibodies in 1889 by von Behring, and the description of precipitation reactions in 1897 by Kraus, in 1903 Liepmann (55) used a precipitin test in an attempt to prove that placental cells possessed specific differences from other body tissue. He used ground placental tissue injected subcutaneously, intravenously, and intraperitoneally into rabbits. Antisera were successfully produced from intravenous and intraperitoneal injections. He demonstrated specific precipitin reactions between his antiserum and placental tissue of two fetal cord sera, and a retroplacental serum. Negative reactions with nonpregnant female serum and male serum demonstrated the specificity of the reaction. Collip (56) in 1934, noticed that after repeated injections of such

hormones as thyrotropic hormone, growth hormone, and chorionic gonadotrophin, some animals became refractory to their effects. He described the cause as an "antihormone". The concept was widely accepted that for every hormone, there was an antagonist with opposite characteristics produced in response to excessive amounts of hormones. Under normal circumstances, the action was masked, but injection of excess hormone into the animals caused a rise in concentration of antihormone, which explained the difference in responsiveness of certain animals. The ratio of injected hormone to the amount of normal hormone produced by the animal determined the rate of production of the antihormone. Twombly (57) in 1936, stated that the "antihormone" described by Collip was probably an antibody. He produced a crude anti-HCG in rabbits and mice, using hormone from pregnancy urine concentrated by alcoholic precipitation. With this antiserum, he demonstrated a visible precipitation reaction with hormone concentrate; and he also demonstrated in vivo inhibition of the effects of the injected hormone.

Chase (58) in 1945, used a commercial preparation containing a combination of follicular stimulating hormone (FSH), and human chorionic gonadotrophin (HCG) from pregnancy urine as the antigen to produce antiserum. Because the antigens were weak, the antiserum was of very low titer. To increase the strength of the antiserum, Chase concentrated it by electrophoresis according to the method of Tiselius (59), and eluted the gamma globulin portion. Her procedure resulted in a threefold increase in activity of the antiserum. She adsorbed antigen onto collodion particles to in-

crease antigen surface for her test system. This test gave too many false positive results to be practical for routine use. Schuyler (60) in 1950, used a commercial preparation containing combined extracts of placental tissue and pregnancy urine to produce antiserum in rabbits. He performed a precipitin ring test by overlaying antiserum with antigen. This gave approximately six per cent false positive and six per cent false negative results. The accuracy could not yet compete with existing biological tests for pregnancy.

In 1960, four laboratories reported immunological tests for pregnancy which had greatly improved accuracy; one was a complement fixation test; one, a precipitin test; and two were hemagglutination inhibition tests. Brody and Carlstrom (61), in July of that year, reported production of a potent and specific antiserum. They used this antiserum to demonstrate the presence of HCG in a complement fixation test system, as originally described by Bordet and Gengou (62). The antiserum was produced by injecting rabbits with a crystalline, electrophoretically homogenous preparation of HCG combined with a modified Freund's adjuvant. The test was found to detect 24 I.U. of HCG/ml. Among twenty specimens tested (eight sera and three urines of non pregnant patients, and nine sera from women two to five months pregnant) no false results were found.

McKean (63) in September of the same year, published details of a precipitin test for diagnosis of pregnancy. The antigen he used for producing antiserum in rabbits was a commercial preparation of HCG further purified by adsorption of associated glycoproteins on anionic resin. The hormone had a final activity of 9,000

I.U./mg. This was combined with a modified Freund's adjuvant and injected into rabbits to produce antiserum. Urinary extracts were obtained by benzoic acid adsorption followed by alcohol precipitation. In the test system, antiserum was overlaid with extract to produce a ring test, which, to be considered positive, had to show a reaction in two hours. All but two of thirty nine specimens tested were confirmed by Friedman or Ascheim-Zondek tests. The exceptions were caused by defects of the biological tests. Two urines which were toxic to both mice and rabbits, gave immunological test results (one positive and one negative) which were clinically proven correct. He also reported forty additional known positive specimens and twenty five non pregnant specimens which all produced correct results with this antibody technique.

In August, 1960, Swierczynska and Samochowiec (64) published a hemagglutination inhibition test. This test is a modification of the hemagglutination test described by Salk (65) and later by Stavitsky (66). The modification involved the incubation of the antiserum with urine before the addition of the sensitized erythrocytes. Inhibition of agglutination by hormone in the specimen indicated a positive test. These workers used a commercial preparation of HCG and Freund's adjuvant (67) to produce antiserum in rabbits. Before use, the antiserum was inactivated at 56° C. for 30 minutes and then absorbed with sheep erythrocytes. Antigen coated cells were prepared by using bis-diazotized benzidine to couple HCG to erythrocytes, according to the method of Stavitsky and Arquilla (68). Urine to be tested was absorbed prior to use, with sheep erythrocytes. In their test system, antiserum was

added to absorbed urine sample and incubated for 60 minutes at 37° C. Coated erythrocytes were then added and the samples left at room temperature for another 60 minutes before reading. Of 78 pregnancy samples tested, 75 gave decisive positive results, while 3 from patients in the tenth month of pregnancy gave weak results. No positive reaction was noted in any of 60 negative controls. (Fifteen of these negative controls contained protein.)

In October of that year, Wide and Gemzell (69) published their test, also using hemagglutination inhibition. The two tests differed in their methods of coating the erythrocytes with hormone. While the earlier group used chemical coupling to coat the sheep erythrocytes, Wide and Gemzell used formalinized cells, tanned and coated according to the method of Boyden (70). Specific antiserum was produced by them using a commercial preparation of HCG and Ramon's adjuvant (72), They found that the relative time of addition of antiserum and erythrocytes to the sample produced no difference in final results. This test was able to detect 0.2-0.3 I.U. of HCG/ml. Three hundred six urines tested all gave correct results as determined by gynecological examination or confirmed by biological tests. Two hundred twelve urines gave inhibition of agglutination, indicating positive results, and the remaining 94 tests resulted in agglutination patterns, or negative results.

In 1961, Ortho Diagnostics used, as the particulate antigen, latex particles sensitized according to the method of Singer and

Plotz (73). This latex agglutination inhibition test had the same principle as the hemagglutination inhibition test, but the end point was visually different. Incubation of antiserum and urine specimen was followed by addition of sensitized latex particles. After one hour of further incubation, the tube was centrifuged at 1,000 G's for two minutes and compared to a standard solution. Agglutinated particles were sedimented by centrifugation, while non agglutinated particles were supposed to stay in suspension to give a turbid supernatant fluid. Twenty I.U. of HCG/ml. could be detected by the test. Overall accuracy of 3,000 tests in 14 laboratories was 79% (37). Because of inadequate sensitivity, and difficulty in controlling centrifugation, the test was soon replaced by a rapid slide test.

The first slide test required wax rings to be made on the slide. Urine or serum was combined with antiserum within the ring and rotated for four minutes. Latex suspension was added, and after mixing, the slide was covered and rotation continued for thirty minutes. Microscopic examination was made to detect agglutination. The sensitivity was 5-6 I.U./ml. (74). A recent simplification used a single drop of reagents on a prepared slide for a shorter time. One drop of antiserum was combined with one drop of urine on a commercially prepared slide and mixed with an applicator stick. After mixing, the slide was gently rocked for thirty seconds before the sensitized latex particles were added. A further two minute rocking ensued before macroscopic observation for agglutination. Agglutination, interpreted as a negative result, occurs when there is no inhibition of the antigen-antibody reaction.

A smooth suspension was interpreted as a positive test for pregnancy. Positive and negative specimens were run concurrently to facilitate evaluation of the end point. This rapid latex agglutination inhibition test is available from several commercial companies.

A commercial test using the results of McKean's precipitin reaction was produced, using an agar gel containing antiserum. This test still required an extract of urine to be made. A well in the agar plate was filled with the extract and the plate was incubated. Formation of a precipitin ring around the well after four hours indicated presence of hormone in the specimen. This test was supposed to detect 4 I.U. of HCG/ml. in the original specimen. In 497 normal pregnancies, accuracy was 63%; in 107 non pregnant patients, the accuracy was 94.3% (37). Once again, because of lack of sensitivity as well as the longer period of time required for testing, the test did not gain acceptance.

In 1966, Bagshawe, Wilde, and Orr (75) published a radioimmunoassay for human chorionic gonadotrophin. Their test depends on competition between luteinizing hormone (LH) and HCG in the unknown specimen, and I^{131} labelled, purified HCG, for binding sites on antibodies to HCG. A precipitating antibody against the first antiserum is used to cause aggregation of particles which are large enough to be separated by filtration from the rest of the system. The test is able to detect 0.007 I.U. of HCG/ml. This method lends itself readily to automation, but at present is not practical for routine pregnancy testing.

The Problem

The necessity of early and accurate diagnosis of pregnancy has become progressively more critical as society changes. The advent of new drugs on the market which may endanger the developing fetus (Thalidomide, antineoplastic drugs, etc); the increasing clinical use of radioisotopes; and the more liberal abortion laws, all contribute to this need. The need is unsatisfied for several reasons.

An ideal test would require a substance, unique to the conceptus, detectable from fertilization to delivery, and which is not mimicked by any maternal material. Placental hormone (human chorionic gonadotrophin), the material currently considered the indicator of pregnancy, is not produced during the initial period after conception, and other maternal substances mimic some of its actions. Some tumors, such as hydatidiform mole and chorionepithelioma also produce a hormone like chorionic gonadotrophin, which is antigenically identical. Thus, error in interpretation could occur through no fault in the HCG test system.

Some errors are inherent in antigen-antibody reaction systems. Temperature change, pH variation, and cross reacting substances can affect results. Luteinizing hormone is known to have an antigenic determinant in common with chorionic gonadotrophin. Conditions such as the post-menopausal state occur in which the secretion of luteinizing hormone is greatly increased, resulting in false positive pregnancy tests.

Commercial test systems produced in secrecy and under patents, imply variations do exist. These variations may affect the indi-

vidual responses under differing test conditions. Proteinuria and metabolites of certain drugs in urine have been shown to do this. The different sensitivities of the commercial systems will cause variable results in early pregnancy, where the actual amount of hormone present in the specimen varies not only with the duration of pregnancy, but also with the individual patient whose rate of hormone production is unique.

It is the purpose of this paper to examine those factors which could be found in clinical situations and which could interfere with immunological pregnancy tests; to compare the manner in which each of seven commercial tests is affected by these factors; and to suggest a practical method to improve diagnostic accuracy in those situations where pregnancy cannot be defined by clinical means.

MATERIALS AND METHODS

Hormone

Standard lyophilized HCG in urine* was supplied in three amounts which, when reconstituted with one ml of distilled water, produced solutions containing 5,000, 10,000, and 1,000,000 I.U./L.

A special diluent was provided by the same company for further dilutions, which is stated to minimize adsorption of hormone onto glassware. The composition of this solution is not given. To demonstrate the precision of calibration of the standards, dilutions of each were made to produce solutions of 1 I.U./ml. and these were compared using a pregnancy test** sensitive to this concentration. Identical results were found.

Specimens

Only urine specimens were used throughout the study. All were checked for specific gravity by means of a Goldberg Refractometer (See Appendix II) and for protein, pH, and glucose by means of Combistix (See Appendix III). Specimens were stored, after original testing, at -20° C.

The type of population used and method of sampling are detailed in the description of each experiment.

Kits

Six manufacturers of commercial pregnancy test kits were approached, and five agreed to have their products included in this study. They all furnished materials without cost. The sixth manufacturer produced numerous arguments against having their

---

* Supplied for this study by Organon, West Orange, N. J.
** UCG - Supplied by Wampole Laboratories, Stamford, Conn.

product included and would not supply material. Since it was a chemical test in a study primarily considering immunological tests, it was not considered important enough to pursue. Immunological tests used in this study were of three types.

Direct Agglutination Test

Latex carrier particles are coated with antiserum. Antigen, if present in the specimen, agglutinates the particles directly. One test of this type was used in this evaluation: DAP

Agglutination Inhibition Test

The test is performed in two stages. A defined amount of antiserum and specimen are allowed to react. If sufficient antigen is present, all antibody molecules are bound in the resulting complex, leaving no free antibody. The second step, to determine if free antibody is present, is performed by adding particles coated with antigen, which will agglutinate in the presence of free antibody. Thus, agglutination implies the patient is not pregnant. There are two types of agglutination inhibition tests; three examples of each are used in this study.

Hemagglutination Inhibition Tests

Pregnosticon Accuspheres

Pretel

UCG

Latex Agglutination Inhibition Tests

Planotest Dri-Slide

Gravindex

HCG

Appendix IV contains all descriptive details of the kits, and the manufacturers' recommended methods of use. These recommended methods were used throughout this study.

Glassware

Serological pipettes were used for this study after they had been shown to give sufficient accuracy. (See Appendix V). Pipettes, Erlenmeyer flasks, used for storing solutions, and volumetric flasks were borrowed from the routine Serology Section of the laboratory. All glassware was acid washed, rinsed thoroughly, and no detergent was used. The pH of the glassware was routinely spot checked, by adding bromthymol blue indicator solution to random pieces of glassware to be certain of adequate rinsing.

Slides used for latex tests were washed by coating with a thin film of non detergent cleansing powder in water and allowed to dry. They were polished with a clean towel or disposable tissue. The spreading pattern of the drop of serum or urine was always noted. If the drop did not spread (indicating improper cleaning) it was rejected and recleaned.

Distilled Water

Distilled water was prepared in a Barnstead Still (Sybron Corporation). After being distilled, the water was deionized in a "Deeminizer" (Crystalab Demineralizer). Each aliquot of water used was checked for purity and determined to have less than two parts per million contamination, with pH 6.0.

Saline

Saline was prepared by adding 8.5 g. sodium chloride* to

---

* Analytical reagent, Mallinckrodt Chemical Works, St. Louis, Mo.

1 liter distilled water. This was made in quantity by the Serology Section.

Filter Paper

Filter paper used to clarify urine specimens was Whatman No. 4**, which is recommended by Organon in their Pregnosticon Accusphere procedure as being least adsorptive.

Because the Results and Discussion of the main portion of this thesis involve comparison and evaluation of the results of several separate experiments, the individual experiments are described as complete units in the Materials and Methods section.

Factors Affecting Specimen Stability

A. Albumin

Procedure

A pool of pregnancy urine was made from the first morning specimens from two pregnant volunteers. This provided adequate volume for this experiment. The pool did not contain glucose or protein. It had a pH of 6.0, and a specific gravity of 1.017.

"Normal serum albumin, salt poor," processed by Hyland Laboratories for the American National Red Cross, was added to pooled pregnancy urine specimens to simulate abnormal concentrations of albumin which might appear in clinical specimens. The unit contained 25 grams in 100 ml. buffered diluent, stabilized with 0.02M sodium acetyltryptophanate and 0.02M sodium caprylate. A 50 ml volumetric

** W. & R. Balston, Ltd., England

flask was used to measure pooled urine. A serological pipette was used to withdraw 0.6 ml., which was replaced with 0.6 ml. of albumin solution to prepare a stock dilution of 300 mg.% final concentration. A 10 ml. aliquot was separated for use in this experiment. One ml. of the stock dilution was added to 9 ml. of pooled urine to give a second 10 ml. aliquot having 30 mg.% concentration of the stock dilution. Four ml. of the stock dilution were diluted to twelve with additional urine for 100 mg.% concentration. Ten mls. of this final dilution were used for the third sample. The three samples, tested with Combistix, gave 3+, 1+, and 2+ results, respectively.

The three 10 ml. portions containing added albumin and a fourth with nothing added, were each divided into two equal portions. One of these was stored at room temperature, and one at 4° C.

Twofold serial dilutions of each specimen being tested were made using 0.5 ml. of the diluent described above in each dilution. To this was added 0.5 ml. of urine, the sample mixed, and 0.5 ml. transferred to the next tube. Selected dilutions were tested with pregnancy tests. Once the titer had been determined, a dilution above, and one below, in addition to the predetermined dilution, were tested for each determination.

Titers of the specimens at given times were determined using hemagglutination inhibition tests. Because of limited materials available and the large number of tests being used,

UCG was used for the first half of the experiment and Pregnosticon Accuspheres for the second half. Parallel titrations were run at the time of changeover from one test to another, to assure consistent interpretation of the end point. The end point was considered to be complete inhibition, indicated by a heavy ring of cells settling to the bottom of the tube. Where partial inhibition occurred, giving less than optimal ring formation, results were interpolated between that dilution and the next lower, giving complete inhibition. Because the test detected 1 I.U./ml., the titer was taken as the reciprocal of the dilution in the tube giving the proper end point reaction. A one tube change in titer is not considered significant (76).

Results

Table II shows that there was no significant alteration in titer attributable to any concentration of albumin up to 300 mg.% at either refrigerator or room temperature, at any time up to seventy-two hours.

Wide (77) has shown that normal rabbit serum has a stabilizing effect on human chorionic gonadotrophin in solution. The effect of albumin might, therefore, be expected to be similar. A slight increase in titer shown by the specimen containing 300 mg% of albumin could (possibly) be interpreted as a synergistic effect upon the titer. It has been shown by many workers that the presence of "albumin" in urine specimens is sometimes responsible for false

positive results, but no effect upon positive specimens has, to my knowledge, been demonstrated previously.

B. Bacteria

Procedure

Two 10 ml. aliquots of the pregnancy urine pool used in the first section (Albumin) were used to determine the effects of bacteria at room temperature and at 4° C. upon the stability of specimen titer. A stock culture of Escherichia coli, and one of Staphylococcus albus, maintained by the Bacteriology Section of the Department of Clinical Pathology were grown separately in Trypticase Soy Broth* to match the McFarland 0.5 Standard Concentration. One tenth ml. of culture was then added to 1.4 ml. of broth to yield a concentration of approximately 10,000,000 organisms per ml. One tenth ml. of this dilution was added to 10 ml. of the urine pool, to produce a final urinary concentration of 100,000 organisms per ml., which is an index of clinical infection. Each specimen was divided into two 5 ml. aliquots, one of which was stored at 4° C. and the other at room temperature. HCG titers were determined using UCG and Accuspheres on each specimen at stated intervals. The results are shown in the upper portion of Table III.

This experiment was repeated in a modified form. Two urine specimens from pregnant patients and another from a

---

* BBL, Division of Bioquest, Cockeysville, Maryland

male patient were treated with the same bacteria in the same way. These specimens were cultured before and on completion of the experiment. They were stored at room temperature only during the test. HCG titers were determined at intervals using Pretel, and the results are recorded in the lower portion of Table III.

Results

All but one of the specimens to which bacteria were added showed a rise in titer of "HCG" occurring at 24-48 hours. The increase occurred with both Gram positive and Gram negative organisms, and was sufficient to cause a false positive result in a negative specimen. It occurred irrespective of the viability of the organisms after 48 hours in urine, as shown by the fact that when the three portions of one specimen were cultured at the conclusion of the experiment, only one to twelve colonies grew from each of the bacteria-containing portions, in contrast to profuse growth from the other specimens.

C. Storage Temperature

In the earlier two experiments, it was shown that using pooled pregnancy urine unchanged or with bacteria or protein added, there was no difference between room temperature storage and storage at 4° C.

Two additional pregnancy urine specimens, stored at room temperature, showed a difference with time. One maintained the same HCG titer for three days, while another, under the same conditions, demonstrated decreased hormone

titer.

Procedure

A further experiment was performed to show the effect of freezing upon the specimens. Three pregnant volunteers each provided a first morning specimen of urine. A portion of each was divided into 2 ml. aliquots which were stored frozen at -20° C. and the remainder was left at room temperature. At intervals up to 11 days, a new aliquot was removed from the -20° C. storage and its titer of HCG determined as described previously. The titer of the corresponding urine left at room temperature was determined at the same time.

Stability, over a longer period, was tested in an experiment in which five specimens, shown to have positive results in routine testing procedures, were frozen for six weeks. At the end of that time, all were retested by a different technologist, using the commercial kits being evaluated in this study, and found to have the same results.

Results

Table IV records the study results. In this study, five of the six urines showed no loss of activity irrespective of the temperature during the time of the study. The remaining specimen did show a drop in titer at room temperature.

These findings are compatible with those obtained by Wide (77) who found no loss of hormone activity in 11 of 15 specimens he tested. The remaining 4 specimens did show

a significant loss of activity which was unaltered by storage at 4° C. and only slightly retarded by freezing at -20° C. The reason for this deterioration is currently unknown. Noto and Miale (78) agree that more change occurs at room and refrigerator temperatures than at -18° C. Although they state that the loss is more rapid in specimens containing lower concentrations of hormone, their published data does not support the statement. Neither do the findings of Wide nor of this study support it.

Krieg and Henry (79) state that "prolonged standing at room temperature (longer than eight hours) may cause loss of HCG activity." Watson (80) also mentions that a "small loss of immunochemically reactive CG occurs...when the urine is stored at room temperature for more than a day."

These studies indicate that a small minority of urines do lose HCG activity on storage, and that storage at -18° C. will reduce the rate of deterioration. Storage of specimens in the refrigerator may give the worker a false sense of security since no demonstrable protective effect occurs. The recommendation that urines should be tested within eight hours or else stored frozen at -18° C. is supported.

Factors Affecting Test Systems

A. Sensitivity

Procedure

Twofold serial dilutions of "Standard HCG in urine"

were made by the classical method. Serological pipettes were used to place 0.5 ml. of special diluent in each titration tube. To the first was added 0.5 ml. of one concentration of standard containing 10,000 I.U./L., the material mixed, and 0.5 ml. transferred to the next tube. This process was repeated throughout the titration. A second titration was made using the standard containing 12,000 I.U./L. In this way, the final dilutions of 12.0, 10.0, 6.0, 5.0, 3.0, 2.5, 1.5, 1.25, 0.75, and 0.625 I.U. of HCG/ml. were obtained. With these dilutions, each kit was tested to determine the lowest concentration of hormone detectable. Sensitivity tests were repeated throughout the testing period to verify consistency of results. Where different lots of a commercial test were used, each was tested individually, and the lot number designated. See Table V for detectable concentration of hormone by each kit.

The relative sensitivities of the commercial tests were determined clinically. Four volunteers daily brought first morning urine specimens for testing by each of the commercial tests. The number of days elapsed between the first day of the last menstrual period (L.M.P.) and the first day each test became positive was used as an index of sensitivity.

Where multiple lots of the same product from the same manufacturer were tested (Planotest Dri-Slide, 3; Pregnosticon Accusphere, 3; HCG, 2; and Pretel, 2) the

earlier day was used in those few instances where a one day difference did occur. These results are shown in Table V.

Results

The hemagglutination inhibition tests as a group are more sensitive than any of the slide tests. Pregnosticon Accuspheres was the only test able to detect pregnancy from 31-35 days after the L.M.P. The less sensitive tests became positive as pregnancy progressed until the least sensitive test, HCG, gave a positive result 76 days after the last menstrual period. The close correlation between this method of determining the relative sensitivities and the actual sensitivities of the tests is apparent in Table V.

This study also shows that if all the tests had been performed at 41 days, as some of the test kits suggest, "Pregnosticon Accuspheres" and "Pretel" would have 100% accurate results; "UCG" and "DAP" 75%; "Planotest Dri-Slide" and "Gravindex" 50%; while "HCG" would be totally in error. These findings are supported by several workers using one or more of the tests used in this study.

In a series of pregnant patients no more advanced than 38 days from L.M.P., Sato and Greenblatt (81) found an accuracy of 40% for the slide test; 65% with the rat ovarian hyperemia test; and 85% with a hemagglutination inhibition test. Bermes and Isaacs (82) working with specimens from patients no more than 40 days from L.M.P. found

the hemagglutination test 75% accurate, and the latex agglutination inhibition slide test only 24% accurate. After 40 days, the accuracy of both tests improved to 98% and 96% respectively. Using a hemagglutination inhibition test, Whitelaw and Nola (83) found most tests positive at 20 to 22 days past ovulation as noted by rise in basal body temperature. Assuming this event to occur approximately midway in the cycle, this would compare with day 34 to 36 from L.M.P., and agree with the results of this study. Mayo and Thompson (84) showed that tests performed before 36 days, ranged in accuracy from 20% to 60%. From 36 to 49 days, tests ranged from 64% to 92% accuracy.

Fine, Morales, and Horn (85) emphasize the fact that of 34 false negative tests obtained, 17 had been performed earlier than 41 days, and when these were repeated three or more days later, all became positive.

Watson (80) states that women normally have a positive pregnancy test on day 38, while Krieg and Henry (79) say that false negative tests are common until day 48. Venning (86) explained some of the differences as she stressed the fact of individual variation in hormone secretion. She has diagrammed the hormone rise in several patients showing a variation in time when detectable concentration of hormone is attained from 25 to 45 days.

This fact has been shown in this study. While one patient at 35 days already had positive results with three tests, another did not have a positive test with the most

sensitive until day 36 and the next, not until day 43.

Accuracy of the slide tests increases rapidly after 41 days. Still, where early detection is required, or where abnormal results are suspected, the hemagglutination inhibition tests are superior.

B. Temperature

Procedure

A temperature of 80° C. for one hour (77) or 100° C. for 30 minutes (87) has been shown to have no effect on antigenic reactivity of Human Chorionic Gonadotrophin. Antibodies in serum have been shown not to lose reactivity readily on storage at 4° C. (88). Manufacturers all recommend storage of liquid reagents at refrigerator temperatures, 4°-8° C.

No mention has been found in the reviewed literature concerning the effect of temperature on the reaction between antiserum and hormone coated particles. This question has practical significance in view of the lack of regulation of ambient temperature in many Clinical Pathology Laboratories.

Commercial tests representative of each type of test, were used at three temperatures to determine whether or not the test systems were affected by ambient temperature change. The actual tests used were UCG for a hemagglutination tube test, HCG for a liquid latex reagent slide test, and Planotest Dri-Slide using dried reagents. "Standard HCG in urine" was used at the lowest concentrations

detectable by each of these test systems.

Results

Results are shown in Table VI. It will be seen that both slide tests became less sensitive at 37° C., while the tube test was unaffected. When dried reagents were taken to 37° C. and returned to room temperature before testing, no change was noted from the original sensitivity. The slide test using liquid reagents, when taken to 37° C. and allowed to return to room temperature before testing, showed some loss of reactivity which corresponded to that produced by testing at 37° C.

These differences in behavior between tube and slide tests are probably due to the effect of evaporation which is proportionately greater from the slide where the absolute volume of reagents is less and the surface area for evaporation increased.

C. Hydrogen Ion Concentration

Procedure

A solution of "standard HCG in urine" was diluted to the lowest concentration detectable by each test used in the study to investigate the effect of pH values considered possible in clinical situations. In addition, one positive and two negative patient specimens were tested in the same manner.

Hydrogen ion concentration was altered with concentrated HCl and NaOH introduced on a dampened applicator stick to minimize dilution of small specimen volume.

A fresh applicator stick was used each time.

Standard HCG in urine was also diluted with Universal Buffer (89), pH 2.0-12.0, to lowest detectable concentration for one hemagglutination inhibition test (UCG) and one latex agglutination inhibition test (Planotest Dri-Slide) to show changes due to pH extremes beyond the clinical range.

Values of pH from 4.5 to 7.5 were determined with a Beckman glass pH electrode and Heath pH recording Electrometer. Because all specimens had originally been tested between pH 5.5 and 6.5, with no variation found, these readings were omitted in this series. Because DAP was introduced into the testing series later, only one standard solution was tested with that kit. Pretel was introduced too late to be included in this study. For the second portion of the study, in which extremes of pH were tested, an Instrumentation Laboratory pH/mV Electrometer, Model No. 245 was used.

Results

Table VII shows the results of the clinical studies. It will be seen that all of the tests were affected adversely at some point outside the optimum range of 5.5 to 7.0. (HCG had one false result at pH 7.0.) Adjustment of pH resulted in no loss of titer. Denaturation of protein does not occur within the physiological pH range.

Wide (77) notes that there is no effect upon the hemagglutination inhibition test between 5.3 and 7.8. Only one difference was found in this study, when one specimen demonstrated a false negative result at pH 7.5 with UCG. Pregnosticon Accuspheres had no false results within this range. Fine, et al (85) showed no influence on the hemagglutination inhibition tests between pH 5.0 and 9.0. Below 3.5, they found non specific agglutination of erythrocytes which was reversed upon adjustment of the pH, just as was found in this experiment. He also found that decreased pH caused false positive results with a slide test (Gravindex).

Because there is an increase in false results caused by hydrogen ion concentration beyond the optimum range (pH 5.5 to 7.0) and because adjustment to pH 6.0 with indicator paper, using HCl or NaOH, is a simple procedure, all specimens outside the ideal range should be adjusted prior to testing for pregnancy. Many studies mention testing specimens for pH, but none have been found which advocate any adjustment. In addition, none of the manufacturer's directions suggest this modification.

D. Protein

Procedure

The effect of protein was tested in two ways.

a) Effect of naturally occurring protein.

Specimens submitted for routine urinalysis which had

been shown to contain protein were used. From this group, 23 urines from males, children, or women over sixty years of age were tested by each available pregnancy test according to the manufacturer's directions. The results are shown in Table VIII.

b) Effect of adding serum fractions to urine.

Procedure

A pool of male urine containing no glucose or albumin was prepared. "Normal serum albumin, salt poor"* was added to portions of the pool to produce solutions containing 1250 mg.%, 300 mg.%, 100 mg.%, and 30 mg.% of albumin.

"Immune Serum Globulin (Human) U.S.P."** containing 0.3M glycine and 1:10,000 Thimerosal N.F. was added to portions of pooled urine to yield solutions containing 1,650, 1,000, 500, 250, 125, and 62.5 mg.%.

Lyophilized Human Glycoprotein (Factor VI)*** was used similarly to give urine concentrations of 1,000, 500, 250, 125, 100, 50, and 25 mg.%.

Selected solutions were tested with each available pregnancy kit to determine the minimum concentration which caused a positive result. These concentrations are shown in Table IX.

---

* Hyland Laboratories, Costa Mesa, California

** Armour Pharmaceutical Company, Chicago, Illinois

*** Mann Research Laboratories, New York, New York

Results

The direct agglutination (DAP) and hemagglutination inhibition tests (UCG, Accuspheres, and Pretel) show no significant false positive results due to protein in the specimens tested, although Pretel was influenced by large amounts of glycoprotein.

The slide tests as a group show a highly significant (X^2 = 10.8, p = .01) protein effect in the urines tested, and this is supported by protein addition experiments.

When pure protein fractions were added to urine, it was noticed that far higher concentrations were required to produce positive test results than the naturally occurring protein concentration in the first experiment would indicate. This led to speculation that either a synergistic effect between different proteins was operating, or there was some other unknown factor present.

An experiment to ascertain presence or absence of a synergistic effect was performed. Aliquots of protein fractions containing 30 mg.% were combined using equal amounts of globulin, glycoprotein, and albumin. The ratios were then varied to 2:1:1, 1:2:1, 1:1:2 respectively. When none of these had any effect, concentration of the aliquots was changed to 100 mg.% with the same negative results. It was noted that this concentration of glycoprotein should have produced agglutination by itself, but none was observed.

E. Chloride

Procedure

The previous experiments showed that some factors other than protein must be influencing the results of the pregnancy tests. The possibility that ionic strength may be a factor was tested. The chloride ion content was determined using a Cotlove Buchler direct reading Chloridometer. Only ten of the previously tested urines were available.

Results

The results of these determinations are included in Table VIII. An analysis of the chloride effect on the pregnancy test result using low and high concentration with the boundary at 30 mEq., gives a Chi square of 4.5 which is significant ($p = 0.05$). If the chloride is classed as low, moderate, and high, with boundaries at 30 and 100 mEq., Chi square is 5.26, which is almost significant.

F. Drugs

Abuse Drugs

Procedure

Certain tranquilizers and antidepressants can cause false positive results with both immunological and biological pregnancy tests (97, 96). In spite of the variety of drugs investigated, no data concerning some of the common abuse drugs used by young adults today has been recorded.

Twenty five urine specimens known to contain one or more of the common abuse drugs were selected from those submitted by non pregnant participants in the Methadone Blockade Treatment Program of the Alcohol and Drug Section of the State of Oregon.

Identification of the drugs present was performed by the Toxicology Section of the Chemistry Division, using the extraction method and thin layer chromatography.

The specimens were tested with the available pregnancy test kits, using methods recommended by the manufacturers.

Results

The results are shown in Table X.

The presence of 12 false positive results in the 64 latex slide agglutination inhibition tests is highly significant. All three tests in this group were equally affected. Among hemagglutination inhibition tests, UCG and Accuspheres appear slightly better than Pretel.

Methadone contributes almost all the false positive results. The number of false positive results in the urines with a combination of other drugs with Methadone, showed no significant difference from that expected as a result of the drug acting alone. Morphine did cause one false positive result, but other factors not tested may be responsible for this isolated abnormal result.

Salicylates

Urines containing other drugs found in clinical

situations were also tested with pregnancy test kits available. These included seven patients taking therapeutic doses of salicylates. A significant number (8/42) of false positive results were found. Four volunteers took the frequently recommended dose of 10 gr. aspirin every four hours, and brought first morning urine specimens the following day. This group has a significantly smaller number of false positive results. ($X^2 = 5$, which is significant $p = .05$). Urine salicylate concentrations were found to be lower in the therapeutic group than those in the group taking aspirin for one day only.

Antineoplastic Drugs

Because of a report of a false positive pregnancy test result from Cytoxan (85), related drugs were investigated. Six urine specimens were collected from patients attending the Hematology Clinic who were known to be taking one of these drugs. The results are also included in Table X.

Miscellaneous Drugs

Urines from patients taking barbiturates, contraceptives, and tranquilizers were included in very small numbers to indicate whether any of these drugs might be contributing to false positive results.

Table X includes the results of tests on all the drugs studied.

G. Other Factors Affecting Test Systems

Technical facility can influence the choice of test

when other factors are similar. The worker subjectively ranked the tests on the ease of performance, and on clarity of end point. Because all tests are easy to perform, there is no great variation in the ranks on this criterion. UCG, requires an extra dilution prior to testing, which is the reason for its low rank. Additional factors such as cost and reduction or elimination of special glassware which must be cleaned and maintained were also considered. When evaluating time required for testing, large numbers of tests being performed make the tube test more economical. When very few tests are required, the slide test provides more rapid results. Ranking with regard to time was done according to total time required from setting up the test to reading the final results, with the test requiring the least time receiving the highest score.

Results are shown in Table XI.

RESULTS

Tables II, III, and IV all relate to the stability of the stored specimen. Table II documents the previously inferred assumption that albumin has no effect on the specimen from a pregnant patient. The effect of bacteria on the stability of the hormone-containing specimen is shown in Table III. One hundred thousand organisms per ml. did produce a rise in titer. This differs slightly from the findings of Fine, et al. (85) who suggested that only a slight effect was seen with 3×10^7 organisms. It also supports the manufacturers' caution that grossly contaminated specimens should be rejected when testing for pregnancy, but differs in that visually clear specimens containing 100,000 organisms per ml. were shown to have an effect upon the titer during storage. The common practice of storing specimens in the refrigerator to reduce bacterial growth has been shown to be valueless with respect to specimen storage for pregnancy testing. Table IV also shows that some urines are stable irrespective of how they have been stored, while in some urines, there is a deterioration which is only retarded by freezing at $-20^{\circ}C$. The cause of this deterioration is not known.

Tables V, VI, VII, VIII, IX, and X deal with factors affecting the test systems. The specific results of each factor is stated in its own section.

During this study, two tests were being modified, and because the earlier lots were experimental and will not be commercially available, they were not included in the final analysis.

To evaluate the results, Kendall's Coefficient of Concordance

(92) was used. For each factor, the tests were ranked with the lower percentage of false positive results given the higher rank. The numerical value of rank was assigned as the score for that test. When two tests proved equal, the ranks were averaged for the assigned value. Table XII presents these results.

For the four evaluations in which results for all tests are available, the coefficient of concordance, W, (0.724) is significant, ($p = 0.01$) showing that each method of evaluation has applied similar ranks to the tests. The pooled ranking, therefore, can serve as a legitimate "standard" for evaluation. The tests ranked in the following order: UCG; Pregnosticon Accuspheres; DAP; Pretel; Gravindex and Planotest Dri-Slide; and HCG. The remaining factors could not be analyzed because of missing values. However, the tube tests do appear better than the slide agglutination tests.

A critique of the individual tests follows:

UCG

Advantages:

1. Because of the dilution of specimens to measure 1.0 I.U./ml. of HCG, if they are used undiluted, measurement to a level of 0.3 I.U./ml. is easily accomplished when this is desirable. (Although cross reaction with LH makes this sensitivity undesirable for routine testing, when abnormal pregnancy is suspected, or treatment for choriocarcinoma is being followed, this may be highly desirable.
2. Use of a control tube allows detection of non-specific

agglutination of RBC which could give false negative results.

Disadvantages:

1. Prior dilution requires an extra operation.
2. Separate test tubes must be purchased when the larger kits are used.
3. Separate measuring pipettes are needed.

Pregnosticon Accuspheres

Advantages:

1. Premeasured lyophilized reagents are contained in the test unit. All that is needed is addition of urine and water.
2. The end point is very easily seen in the mirror of a well designed rack.
3. A ring, indicating a positive test, may usually be seen within one hour. Only negative tests usually require the two hour time.
4. Tests do not change if left longer than allotted time.
5. Appears more sensitive than routine UCG procedure.

Disadvantages:

1. Measuring pipettes are needed.

Pretel

Advantages:

1. No extra glassware is needed for this test.
2. Measured lyophilized reagents are contained in the test unit.
3. The end point, while not so easy to read as either UCG or Accuspheres, is readily seen in good light.

4. The test may be read in one hour.

5. Reagents may be stored at room temperature.

Disadvantages:

1. The test must be read at one hour. Rings appearing later are not to be interpreted as positive results.

DAP

Advantages:

1. As a direct test, there is only one step required, which gives this test the shortest time requirement.
2. A control of uncoated latex particles prevent reporting false positive results due to non-specific agglutination of latex particles.

Disadvantages:

1. The agglutination is difficult to read, it is finer than the others.
2. Many results are reported as equivocal due to agglutination in both control and test.
3. The possibility of false negative results due to non-specific interference with agglutination remains.

HCG and Gravindex

Advantages:

1. Agglutination is usually easy to read. (Some equivocal results are still difficult, however.)
2. Rapid results are possible.

Disadvantages:

1. Latex agglutination inhibition tests produce more false positive results than tube tests.

2. Sensitivity is higher than that of hemagglutination tests.

Planotest Dri-Slide

Advantages:

1. All reagents are on a disposable slide.
2. Only two minutes and a half are required.

Disadvantages:

1. Slick slide surface interferes with good mixing of urine and antiserum.
2. It is easy to confuse reagents wasting not only the total test but if not recognized also an invalid result.
3. The agglutination is difficult to read.

The reproducability of all tests is remarkable. Of the hundreds of tests performed, there were only three instances in which results were not found to be reproducible. This is supported by over five years experience in the clinical laboratory.

DISCUSSION

It is undesirable to perform surgery to remove a pregnancy in a non pregnant patient; yet unfortunately, this has occurred. One contributing factor is the unreliability of pregnancy tests in those critical instances where the diagnosis of pregnancy cannot be made on clinical grounds.

This thesis appears to be the first study to evaluate the presently available immunological pregnancy tests in this respect, and to determine what should be the most appropriate procedure for the routine clinical laboratory. Only one study (85) has been found which investigates several of the commonly occurring factors which influence pregnancy tests. It only compared two immunological tests.

The most common interfering factor is urinary protein. Hogan and Price (93) discussed this and found that 75% of 36 urine specimens containing 0.4 gm./ 100 ml. or greater of protein gave false positive reactions by the latex slide test (Gravindex). No false positive results were obtained with the UCG hemagglutination inhibition tests in the presence of urine protein concentrations as high as 3.6 gm./100 ml. This supports Fine, et al. (85) who found two false positive Gravindex tests in specimens from two males, containing 3+ and 4+ proteins. Godts and Mighorst (94) found albuminuria to be a significant cause of false positive reactions. They differed, however, in stating that these reactions did not depend on the concentration of protein.

The addition of serum fractions in this study showed that a higher concentration of protein had to be added to produce false

positive results than was present in patients' specimens containing protein. This phenomenon was also observed by others (85, 93, 95), and all agreed that the minimum concentrations of albumin needed was 1.2 gm./ 100 ml. and of globulin, 0.6 gm./ 100 ml. Bell (95) investigated glycoprotein and found that 250 mg./ 100 ml. were needed to produce positive results. The percentage of false positive results is correlated with the amount of protein present.

Since the Thalidomide tragedy of 1960-1962, drugs taken by pregnant women have been studied more closely. Drugs used in psychiatric disorders have been known to interfere with biological tests for over a decade. Phenothiazine, for example, gave up to 75% false positive results. Marks and Shackcloth (96) investigated the effects of tranquillizers on three commercial tests hoping the immunological test system would be less affected. They studied 158 non pregnant patients taking tranquillizers. The incidence of false positive results for Gravindex was 2.5%, for Pregnosticon, 5%, and only one false positive result was found with Prepuerin. Ravel, et al. (97), four years later, studied six tests from four different commercial companies. Seventy eight patients were tested. HCG consistently gave false positive results which were not dose related. Pregslide gave two false positive results, and Natatel, one. UCG, Gravindex, and DAP did not produce any. The urine specimens were devoid of protein. Fine, et al.(85) however, found it was impossible to incriminate the phenothiazines in his study using Gravindex and UCG. After the study, four false positive results were encountered with Gravindex. Three were in men with suspected choriocarcinoma, and one in a woman who had re-

ceived Librium. This study confirmed that some drugs can cause false positive results, and is the first study to mention Methadone. One commercial manufacturer, as a result of this, is now notifying physicians of this fact. Although salicylates have been incriminated, and mentioned by commercial manufacturers, this appears to be based on unpublished data. This study does document the effect.

That low chloride content is positively correlated with increased false positive results has also been shown by this study. The effect does not appear to have been discussed previously, in connection with pregnancy test systems, even though the effect of low ionic concentration on agglutination reactions is well documented.

Bacteria in urine can produce an increase in substances immunologically reacting as HCG in urine specimens, which could lead to false positive results in a sensitive test system. Fine, et al. (85) supports this with one doubtful Gravindex test found in a urine specimen containing 3×10^7 Pseudomonas organisms. The manufacturers, aware of this, recommend that grossly contaminated specimens should not be tested. Because of the possible change in titer on storage shown in this study, it is recommended that urine should be tested within a few hours of voiding.

Singer and Campbell (98) have shown the dissociation of antigen-antibody complexes at pH 3.5 using bovine serum albumin and antiserum to that protein. Fine, et al. (85) also showed that pH 3.5 was responsible for erroneous results, and that pH in excess of 9.0 similarly interfered with proper reactions. This study

shows that false results can occur with some tests at the extremes of the physiological range in urine. Correcting specimen pH to fall within the range of pH 5.5 to 7.0 can eliminate this source of error. This simple procedure should be incorporated into a routine testing procedure.

Evaporation from slide tests as a cause of false agglutination is well known in immunohematology and serology. No study, however, has been found to record this as a cause of erroneous results in pregnancy testing. In laboratories which are not temperature controlled, this problem should be considered in the selection of a testing system, and slide tests avoided in hot weather.

This study showed that hemagglutination inhibition tests had less false positive results than did the slide inhibition tests. Fine, et al. (85), however, quoted no false positive results for Gravindex, and 10 false positive results for UCG. The 10 false positive results were divided into two groups. Four occurred in women 47 to 55 years of age. All of these had doubtful test results with 1:3 dilutions of urine, which became positive when undiluted urine was used. This was postulated to be the result of cross reaction with pituitary gonadotrophin. The UCG test is not intended to be used with undiluted urine in routine testing. This evaluation, therefore, is questionable. In the second group of six patients 12 to 25 years of age, amenorrhea was present in all. Three patients admitted to abnormal uterine bleeding following the pregnancy tests. The 1:3 urine dilution gave positive results in three of these patients, and doubtful results in the other three. All gave positive results with undiluted urine. Since the possi-

bility of early pregnancy cannot be excluded, and the presence of substances which are known to produce false positive results was not commented upon, the validity of this observation is also questionable. Subsequent to this study, these workers reported seven false positive results with Gravindex.

The clinician often faces the problem of diagnosing pregnancy at an early date. It is important, therefore, to know which of the available test systems should be used to do this. The sensitivity of the commercial tests was found to be perfectly correlated with early diagnosis of pregnancy. The sensitivities determined generally support the claims of the manufacturers. A physician wishing to diagnose pregnancy early should use either Pregnosticon Accuspheres, or UCG testing undiluted urine. If this latter procedure is followed, the diagnosis should not be definitely stated until the 1:3 dilution also becomes positive. The first morning specimen, because it is more concentrated than later specimens, should be used when maximum sensitivity is needed.

The laboratory wishing to select the best test available needs to know the sensitivity, accuracy and precision, the clinical correlation, and the factors causing errors for each test. This study has shown the relative and actual sensitivities of the tests and that the hemagglutination inhibition tests are preferable. Accuracy and clinical correlation have been discussed in numerous large clinical studies, again with hemagglutination inhibition tests appearing superior to the slide tests. The actual percentage of accuracy stated varies with the population sampled. The ranking method used in this study cannot assign a numerical value

to the accuracy but does indicate which of the tests is more accurate with respect to factors known to cause errors. Again, the hemagglutination inhibition tests are superior. The remarkable precision of the whole group of immunological tests eliminates this as a differentiating criterion. The effect of known interfering substances was also shown to be least in hemagglutination inhibition tests, with UCG and Accuspheres slightly better than Pretel, as they were in the protein studies. The two new interfering substances, the drug Methadone, and the low chloride effect, were also shown to have less effect on the hemagglutination inhibition systems.

The current recommended protocol for a routine clinical laboratory to detect early pregnancy with least error includes a few new recommendations. A first morning specimen, corrected for pH if the value lies outside the range of pH 5.5 to 7.0, should be tested within a few hours of voiding using either Pregnosticon Accuspheres or UCG. If undiluted urine is used with the latter test, pregnancy should not be assumed as definitive until a 1:3 dilution of the patient's urine gives a positive result.

SUMMARY

Seven commercial immunological tests were studied. DAP was the sole member of the direct agglutination slide type. UCG, Pregnosticon Accuspheres, and Pretel were all members of the hemagglutination inhibition tests performed in tubes, and Gravindex, Planotest Dri-Slide, and HCG were the three tests in the latex inhibition tests.

A pool of urine from pregnant patients, and a control pool from non-pregnant patients were used to provide aliquots to study the stability of HCG containing specimens.

Differing amounts of albumin were added to aliquots. Final concentrations of 100,000 organisms per ml. were produced by adding E. coli or Staphylococci to aliquots. Samples of these and the controls were then stored at 4° C. and at room temperature. HCG titers were then determined at intervals during periods of up to eleven days.

These studies show that albumin has no effect on the stability of HCG in urine. Bacteria were shown to increase the content of some material which reacts in the test systems similar to HCG. It was also found that some urines were stable irrespective of the temperature at which they were stored, while others do deteriorate. The cause of this is unknown.

The sensitivity of each commercial test was determined and found to be: Accuspheres and UCG - 1-1.5; Pretel - 1.5-2; DAP and Planotest - 3-4; Gravindex and HCG - 4 I.U./ml. This was shown to correlate perfectly with the period from the first day of the last menstrual period on which the test became positive in four

patients who provided daily specimens throughout early pregnancy.

The factors which could cause false positive results were evaluated. Twenty three urines submitted to the routine laboratory from males, children, or women over 60 years of age all containing protein as determined by combistix, were tested with each of the commercial tests. The tests were ranked in order of the fewest false positive results as shown; UCG, DAP, Pregnosticon Accuspheres, Pretel, Gravindex, Planotest Dri-Slide and HCG.

Differing amounts of the serum protein fractions, albumin, gamma globulin, and glycoprotein were added to aliquots of a HCG free urine pool to determine the lowest concentration of added protein to cause a false positive result. Albumin at 1,250 mgms.% caused false positive results with Gravindex, HCG and Planotest. Glycoprotein at 50 mgs.% caused false positive results with HCG, while 250 mgs.% were needed for Pretel, Gravindex and Planotest. One hundred twenty five mgs.% of globulin was needed to produce false results in HCG and Gravindex. No false positive results were produced by Accuspheres, UCG or DAP at the highest concentrations tested, namely albumin 1250 mgs.%, glycoprotein 1000 mgs.% and globulin 1650 mgs.%.

Each test was shown to be affected at the extremes of the physiological pH range, but not with the optimum range for these tests, namely 5.5 to 7.

The chloride ion concentration was determined on 10 of the patient specimens used in the proteinuria study. It was shown that there is a significant correlation between low chloride ion concentrations and a higher incidence of false positives.

Twenty five urines from patients on the Methadone Blockade Program, known to contain abuse drugs, were tested with each of the commercial tests and of the 19 containing Methadone, Planotest had 6 false positive results, Gravindex had three, and Pretel had one. HCG showed 2 false positives in 13 of these urines. The other tests, Accuspheres, UCG, and DAP had none.

In the study with urines containing therapeutic drugs, salicylates were associated with false positive results in the tests Pretel, Accuspheres, and Planotest. False positive results were found with alkeran in the tests Gravindex and Planotest, and with the drug desimpramine, a false positive was seen in Pretel. The other drugs, cytoxan, barbiturates and Elavil were associated with false positive results only in the Planotest. Caution should be used in interpreting the effects of drugs since association only is shown and a causitive effect not definitively proved.

The ranking of the tests in the order UCG, Pregnosticon Accuspheres, DAP, Pretel, Gravindex and Planotest Dri-Slide, and finally HCG for both accuracy and sensitivity is supported by statistical analysis.

REFERENCES

1. Forbes, T.R. Early Pregnancy and Fertility Tests. Yale J. Biol. Med. 1957. 30, 16-29.

2. Iversen, Erik, Papyrus Carlsberg No. VII, With Some Remarks on the Egyptian Origin of Some Popular Birth Prognoses. Kobenhavn, Ejnar Munksgaard, 1939 pp. 1-31 (Cited by Forbes (1)).

3. Schoeller, W., and Goebel, H. Die Wirkung des Follikelhormons auf Pflanzen. Biochem. Z. 1931. 240, 1-11. (Cited by Forbes, (1)).

4. Zollikofer, C. Der Einfluss Tierischer Hormone auf Pflanzen. Ber. Schweiz. Botan. Gesell. 1939. 49, 427-428. (Cited by Forbes, (1)).

5. Burkhardt, A. Untersuchungen über die Wirksamkeit des Oestrons auf Pflanzen bie verschiedener Ernährung. Ber. Schweiz. Botan. Gesell. 1941. 51, 363-394. (Cited by Forbes, (1)).

6. Henricksen, E. Pregnancy Tests of the Past and the Present. W.J. of S. Ob. & Gyn. 1941. 49, 567-575.

7. Rueff, J. De Conceptu et Generatione Hominis Francofurtum ad Moenum 1580. Vol. 5, cap. V. (Cited by Forbes, (1)).

8. Bayon, H.P. Ancient Pregnancy Tests in the Light of Contemporary Knowledge. Proc. Roy. Soc. Med. 1939. 32, 1527-1538.

9. Aristotle's/Complete and Experienc'd/Midwife/In Two Parts, Made English by W--S--(London, 1st ed. 1700; Twelfth Edition n.d. 1775) University Library, Cambridge. (Cited by Bayon, (8)).

10. Marshall, M. The Kyesteine Pellicle. Bull. Hist. Med. 1948. 22, 178-195.

11. Nauche, M. Trans. Soc. de Méd Pratique. 1831. (Cited by Marshall, (10)

12. Tanchon and Equisier Recherches sur la kiesteine, substance signalée par M. Nauche dans l'urine des femmes encientes. Gaz de hop. Par. 1839, 2s I, 89-91. (Cited by Marshall (10)).

13. Bird, G. Observations on the existence of certain elements of milk in the urine during utero-gestation and on the application of this fact to the diagnosis of pregnancy. Guy. Hosp. Rep. London, 1840. V, 15-26. (Cited by Marshall (10))

14. Kane, E.K. Experiments on Kiesteine with observations on its application to the diagnosis of pregnancy. Amer. J. Med. Sci., Phila. 1842. n.s. IV, 13-38.

15. Kleybolte, Ueber das von Nauche im Urine der Schwangeren entdeckte Kystein. Wchnschr. f.d. ges. Heilk., Berl. 1845. (2s.)-XI, 269-272. (Cited by Marshall, (10)).

16. Churchill, The Science and Practice of Midwifery. (Cited by Marshall, (10).

17. Zimmerman, Ueber den Harn der Schwantern und das Kystein Wchnschr. f.d. ges. Heilk., Berl. 1846.(2.s.) XII, 347-362. (Cited by Marshall, (10)).

18. Tatta, G. Influenza degli ormoni gravidici sullo sviluppo die germi. Riv. Ital. de Ginecol. 1934. 17, 347-362. (Cited by Marshall, (10)).

19. Ascheim, S., and Zondek, B. Hypophysenvorderlappenhormon und Ovarialhormon im Harn von Schwangeren Klin. Wschr. 1927. 6, 1322.

20. Ferguson, R.S., Downes, H.R., Ellis, El, & Nicholson, M.E. Preliminary Note on a New Method of Differentiating the Testicular Tumors by Biological Means. Amer. J. Canc. 1931. 15, 835-843.

21. Engle, E.T. Ovarian Response to Urine from Pregnant Women and Implanted Anterior Lobe. J.A.M.A. 1929. 93, 276-277.

22. Evans, H.M., and Simpson, M.E. A Comparison of the ovarian changes produced in immature animals by implants of hypophyseal tissue and hormone from the urine of pregnant women. Amer. J. Physiol. 1929. 89, 381-387.

23. Hamburger, C. Studies on Gonadotrophic Hormones from the Hypophysis and Chorionic Tissue with Special Reference to Their Differences. Acta Path. Microbiol. Scand. 1933, Supp. 17, 9-184.

24. Evans, H.M. Clinical Manifestations of Dysfunction of Anterior Pituitary J.A.M.A. 1935. 104, 464-472.

25. The Department of Biological Standards, National Institute for Medical Research, Hampstead, London, N.W. 3 Memorandum on the International Standard for the Gonadotrophic Substance of Human Urine of Pregnancy (Chorionic Gonadotrophin) League of Nations, Bull. Hlth. Org. 1939. 7, 884-886.

26. Bangham, D.R., and Grab, B. The Second International Standard for Chorionic Gonadotrophin. Bull. WHO 1964. 31, 111-125.

27. Brouha, L., Hinglais, H. & Simonnet, H. Bull. Acad. de Med. (Paris) 1930. 103, 150. (Cited by Weisman, (33)).

28. Allen and Doisy, Investigated by Mazer, C. and Hoffman, J. The Three Hormone Tests for Early Pregnancy. J.A.M.A. 1931. 96, 19-23.

29. Rieprich, W. Ein Neue Schwangerschafts-Schnell-Reaktion Aus Den Harn. Klin. Wchnschr. 1933. 12, 1441-1444.

30. Fried, P.H. and Rakoff, A.E. Improvement of the Rat Ovarian Hyperemia Test for Pregnancy by Adding a Pituitary Synergist. J. Clin. Endocr. 1950. 10, 423-431.

31. Parlow, A.F. A Rapid Bioassay Method for Luteinizing Hormone and Factors Stimulating Luteinizing Hormone Secretion. Fed. Proc. 1958. 17, 402.

32. Friedman, M.H. and Lapham, M.E. A Simple Rapid Procedure for the Laboratory Diagnosis of Early Pregnancies. Amer. J. Obstet. Gynec. 1931. 21, 405-410.

33. Weisman, A.I. Recent Laboratory Tests for the Early Diagnosis of Pregnancy. Amer. J. Obstet. Gynec. 1938. 35, 354-364.

34. De Nito, G. Ueber ein neue Schnellmethode zur Fruhdiagnose der Schwangerschaft. München. Med. Wchnschr. 1936. 83, 1272-1273. (Cited by Weisman, (33)).

35. Kelly, G.L. and Florence, L. The effect of Serum from Pregnant Women on the Oestral Cycle of the Guinea Pig. Surg. Gynec. Obstet. 1930. 50, 435-437.

36. Gismondi, L. and Acevedo, B. Diagnostico Biologico Precoz del Embarazo. Semana Méd. 1935. 1, 1194-1200. (Cited by Weisman, (33)).

37. Hobson, B.M. Review, Pregnancy Diagnosis. J. Reprod. Fertil. 1966. 12, 33-48.

38. Hogben, L. Some Remarks on the Relation of the Pituitary Gland to Ovulation and Skin Secretion in Xenopus laevis. Proc. R. Soc. Afr. 1930. 5, 19. (Cited by Hobson, (37)).

39. Galli-Mainini, C. Pregnancy Test Using the Male Toad. J. Clin. Endocr. 1947. 7, 653-658.

40. Konsuloff, H. Schnelldiagnose der Schwangerschaft Durch die Melanophorenreaktion. Klin. Wchnscher. 1934. 13, 776-797.

41. Mandelstamm, A. Zbl. Gynaek. 1935. 59, 2737. (Cited by Weisman, (33)).

42. Kanter, A.E., Bauer, C.P. & Klawans, A.H. A New Biological Test for Hormones in Pregnancy Urine. J.A.M.A. 1934. 103, 2026-2027.

43. Kustallow, N. Zbl. Gynaek. 1937. 61, 269. (Cited by Weisman, (33)).

44. Lorrincz, investigated by Reeb. Diagnostic et Diagnostic Différentiel de la Grossesse Par Injection Intraveineuse D'Extrait Hypophysaire Postérieur Gynec. et Obstet. 1930. 21, 193-203.

45. Soskin, S., Wachtel, H. & Hechter, O. The Treatment of Delayed Menstruation with Prostigmine, a Therapeutic Test For Early Pregnancy. J.A.M.A. 1940. 114, 2090-2091.

46. Schwartz, H.A. A Simple, Accurate Pregnancy Test. Amer. J. Obstet. Gynec. 1950. 59, 213-216.

47. Bercovitz, Z. Studies on the pupillary reactions of pregnancy and non pregnant women and their practical applications to the diagnosis of pregnancy as a simple procedure. Amer. J. Obstet. Gynec. 1930. 19, 767-778.

48. Papanicolaou, G.N. The Diagnosis of Early Human Pregnancy by the Vaginal Smear Method. Proc. Soc. Exp. Biol. Med. 1925. 22, 436-437.

49. Smith, B.G. and Brunner, E.K. A Histologic Method for the Early Diagnosis of Pregnancy. Amer. J. Obstet. Gynec. 1937. 33, 404-414.

50. Bar, P. and Ecalle, G. De l'Utilisation pour le Diagnostic de la Grossesse des Recentes Decouvertes Biologiques. Arch. Mens. d'Obstet. et de Gynec. (Paris) 1919. viii, 372-400. (Cited by Henricksen (6)).

51. Gruskin, B. An Intradermal Test for Pregnancy. Amer. J. Surg. 1936. 31, 59-62.

52. Deutsch, A. Über die Verwendbarkeit der Porges-Pollatschek'schen Schwangerschaftsprobe. Zbl. Gynaek. 1929. 53, 2920-2921.

53. Gilfillen G.C. and Gregg, W.K. New, Rapid Economical Test for Pregnancy and Certain Gynecologic Conditions. Amer. J. Obstet Gynec. 1936. 32, 498-501.

54. Falls, F.H., Freda, V.C. and Cohen, H.H. A Skin Test for the Diagnosis of Pregnancy. Amer. J. Obstet. Gynec. 1941. 41, 431-438.

55. Liepmann, W. Ueber ein für Menschliche Placenta Spezifisches Serum. Deutsch. Med. Wschr. 1903. 5, 80-81.

56. Collip, J.B. Inhibitory Hormones and the Principle of Inverse Response. Ann. Intern. Med. 1934. 8, 10-13.

57. Twombly, G.H. Studies of the Nature of Antigonadotrophic Substances. Endocrinology. 1936. 20, 311-317.

58. Chase, J.H. Serological and Immunochemical Studies on the Gonadotrophic Hormones. Yale J. Biol. Med. 1945. 17, 517-538.

59. Tiselius, A. Electrophoresis of Serum Globulin. Biochem. J. 1937. 31, 313-317.

60. Schuyler, L.H., Anderson, K., Saslow, S., & Erickson, C.C. A Serological Study of Chorionic Gonadotrophin. Proc. Soc. Exp. Biol. 1950. 75, 552-555.

61. Brody, S. and Carlstrom, G. Estimation of Human Chorionic Gonadotrophin in Biological Fluids by Complement Fixation. Lancet. 1960. 2, 99

62. Bordet, J. and Gengou, O. Sur l'Existense de Substances Sensibilisatrices dans la Plupart des Serums Antimicrobiens. Ann. Inst. Pasteur 1901. XV, 289-302.

63. McKean, C.M. Preparation and Use of Antisera to Human Chorionic Gonadotrophin. Amer. J. Obstet. Gynec. 1960. 80, 596-600.

64. Świerczyńska, Z. and Samochowiec, E. Metoda Serologicznego Wykrywania Gonadotropiny Kosmówkowej w Moczu w Zastosowaniu do Rozpoznawania Ciazy.

65. Salk, J. A Simplified Procedure for Titrating Hemagglutinating Capacity of Influenza-virus and the Corresponding Antibody. J. Immun. 1944. 49, 87-98.

66. Stavitsky, A.B. Micromethods for the Study of Proteins and Antibodies, Part I. J. Immun. 1954. 72, 360-367.

67. Freund, J. and Mc Dermott, K. Sensitization to Horse Serum by Means of Adjuvants. Proc. Soc. Exp. Biol. (N.Y.) 1942. 49, 548-553.

68. Stavitsky, A.B. and Arquilla, E.R. Micromethods for the study of proteins and antibodies. III Procedure and applications of hemagglutination and hemagglutination-inhibition reactions with bis-diazotized benzidine and protein-conjugated red blood cells. J. Immun. 1955. 74, 306-312.

69. Wide, L. and Gemzell, C.Z. An Immunological Pregnancy Test. Acta Endocr. 1960. 35, 261-267.

70. Boyden, S.V. The Adsorption of Proteins on Erythrocytes Treated with Tannic Acid and Subsequent Hemagglutination by Antiprotein Sera. J. Exp. Med. 1951. 93, 107-120.

71. Wilson, G.S. and Miles, A.A. Topley and Wilson's Principles of Bacteriology and Immunity (5th ed.) Baltimore: The Williams and Wilkins Co., 1964. 2 vols. (Vol. 2, p. 1364.)

72. Ramon, G., Lametayer, E. & Richow, R. Rev. Immun. (Paris) 1935. 1, 199. (Cited by Wilson and Miles, (71)).

73. Singer, J.M. and Plotz, C.M. Latex Fixation Test. Amer. J. Med. 1956. 21, 888-892.

74. Kline, B.S. Slide Test of Urine and Blood for Pregnancy. Amer. J. Clin. Path. 1963. 40, 246-251.

75. Bagshawe, K.D., Wilde, C.E. & Orr, A.H. Radioimmunossay for Human Chorionic Gonadotrophin and Ruteinizing Hormone Lancet 1966, 1, 1118-1121.

76. Sparling, P.F. Diagnosis and Treatment of Syphilis. N.E.J.M. 1971. 284,643

77. Wide, L. An Immunological Method for Assay of Chorionic Gonadotrophin Acta Endocrinal 1962 Suppl. 70, 1-111.

78. Noto, T.A. and Miale, J.B. The Stability of Chorionic Gondotrophin in Urine and of Reagents for the Slide Immunologic Test for Pregnancy. Amer J. Clin. Path. 1965. 43, 311-313.

79. Krieg, A.F. and Henry, J.B. Pregnancy Tests, Part 2. Postgrad. Med 1967. 42, A48-A58.

80. Watson, D. Urinary Chorionic Gonadotrophin Determination. Clin. Chem. 1966. 12, 577-585.

81. Sato, T. and Greenblatt, R.B. Detection of Early Pregnancy Amer J. Obstet. Gynec 1965. 91, 31-36.

82. Berme, E.W. and Isaace, J.H. Evaluation of a Direct Agglutination Latex Particle Test for Human Chorionic Gonadotrophin Amer J. Obstet. Gynec 1969. 104, 865-890.

83. Whitelaw, M.J. and Nola, V.F. Accuracy of the Immunologic Pregnancy Test in Early Pregnancy and Abortion Obstet Gynec 1966, 27, 69-72.

84. Mayo, R.W. and Thompson, R.B. Comparison of Pregnancy Tests Obstet. Gynec 1965. 25, 699-704.

85. Fine, G. Morales, A.R. & Hom, R.C. Immunologic and Frog Pregnancy Tests Amer J. Clin Path 1966. 49, 171-182.

86. Venning, E. Pregnancy Tests. Obstet. Gynec. 1965. 26, 110-114.

87. Rao, S. S. and Shahani, S. K. The Antigenicity of Human Chorionic Gonadotrophin. 1961. 4, 1-12.

88. Gerlough, T. D. Some Immunological, Physical, and Chemical Properties of Purified Human Gamma Globulin Solution Stored for Prolonged Periods at Approximately 4° C. Vox Sang. (Basel). 1958. 3, 67.

89. Vogel, A. I. A Textbook of Quantitative Inorganic Analysis Theory and Practice. London: Longmans, Green and Co. 1953. (page 872).

90. Mulé, S. J. Narcotic Analgesics in Body Fluids. Anal. Chem. 36: 1907, 1964.

91. Dole, V. P.; Kim, W.K.; Eglitis, I: Detection of Narcotic Drugs Tranquilizers, Amphetamines and Barbiturates in Urine. J.A.M.A. 198: 349, 1966.

92. Edwards, Allen L. Statistical Methods (2nd Ed.) New York: Holt, Rinehart and Winston, Inc., 1967 (pages 344-351).

93. Hogan, W. J. and Price, J. W.: Proteinuria as a Cause of False Positive Results in Pregnancy Tests. Obstet. Gynec. 4: 585-588.

94. Godts, P. and Mighorst, J. C. A.: Comparative Examination of a Serologic Pregnancy Test and the Frog Test. Amer. J. Obstet. Gynec. 89: 590-594.

95. Bell, J. L.: Comparative Study of Immunological Tests for Pregnancy Diagnosis. J. Clin. Path. 22: 79-83, 1969.

96. Marks, V. and Shackcloth, P.: Diagnostic Pregnancy Tests in Patients Treated with Tranquilizers. Brit. Med. J. 1: 517-519, 1966.

97. Ravel, R.; Riekers, H. G.; and Goldstein, B. J.: Effects of Certain Psychotropic Drugs on Immunologic Pregnancy Tests. Am. J. Obstet. Gynec. 105: 1222, 1969.

98. Singer, S. J. and Campbell, D. H.: Influence of pH on Antigen-Antibody Equilibria. J. Am. Chem. Soc. 76: 4052-4055, 1954.

99. Katzman, P. A.; Gotfeid, M.; Cain, C. K.; and Doisy, E. A.: The Preparation of Chorionic Gonadotrophin by Chromatographic Adsorption. J. Biol. Chem. 148: 501, 1943.

100. Instructions for Use and Care of the TS Meter. A. O. Instrument Company.

101. Kark, R. M.; et al: A Primer of Urinalysis. Harper & Row Publishers Incorporated. March, 1964.

TABLE I

Biological Tests Used for Determination of Human Pregnancy

Tests for Pregnancy, Using Laboratory Animals

Date	Worker(s)	Procedure[1,2,3]	Criterion for Pregnancy	Time Required
1927	Ascheim & Zondek(19)	female mice[1]	hemorrhagic follicles corpora lutea	5 days
1930	Brouha(27)	male mice[1]	seminal vesicle weight	4 days
1931	Allen & Doisy(28)	female mice, rats[1]	vaginal desquamation	4 days
1933	Rieprich(29)	female rat[1]	ovarian hyperemia	24-36 hours
1950	Fried & Rakoff(30)	female rat[1]	ovarian hyperemia (augmentation test)[4]	24 hours
1958	Parlow(31)	female rat[1]	ovarian ascorbic acid depletion	10 da. prep.[5] 1 hr. post inj.
1929	Friedman & Lapham(32)	female rabbit[1]	hemorrhagic follicles corpora lutea	36-48 hours
1936	DeNito(34)	rabbit[1]	decrease in leucocyte count	2 minutes
1930	Kelly & Florence(35)	female guinea pig[1]	vaginal desquamation	12 days
1931	Gismondi & Acevedo(36)	male guinea pig[1]	hypertrophy of gonads	48 hours
1930	Hogben(38)	female toad[1]	ova extrusion	6-9 hours
1947	Galli-Mainini(39)	male toad[1]	sperm in cloaca	4-6 hours

Table I (Continued)

Date	Worker(s)	Procedure	Criterion for Pregnancy	Time Required
1934	Konsulov(40)	hypophysectomized frog[1]	melanophore stimulation	2 hours
1935	Mandelstamm(41)	Stichling fish[2]	melanophore stimulation	2 hours
1934	Kanter, Bauer, & Klawans(42)	female Japanese bitterling	lengthening of ovipositor	24-72 hours
1937	Kustallow(43)	protozoa	inhibition of motion	2 minutes
Tests for Pregnancy, on Patient				
1928	Lorrincz(44)	posterior pituitary extract injected I.V.	contraction of uterus (by palpation)	immediate
1940	Soskin, Wachtel, & Hechter (45)	neostigmine bromide injection x 3	no uterine bleeding	app. one week
1950	Schwartz(46)	estrogen-progesterone injection x 3	no uterine bleeding	10 days
1930	Bercovitz(47)	serum instilled in eye	pupillary reaction (contraction, dilation)	2 minutes
1925	Papanicolaou(48)	vaginal smears	navicular and disintegrated cells	2 hours
1937	Smith & Brunner(49)	vaginal biopsy	vacuolization of cells greatly increased	variable

Table I (concluded)

Date	Worker(s)	Procedures	Criterion for Pregnancy	Time Required
1919	Bar & Ecalle(50)	placental peptones[3]	wheal with pseudopodia and erythema	10 minutes
1936	Gruskin(51)	placental extract[3]	wheal with pseudopodia and erythema	10 minutes
1929	Deutsch(52)	antuitrin-S[3]	no reaction	30 minutes
1936	Gilfillen & Gregg(53)	antuitrin-S[3]	no reaction	30 minutes
1941	Falls, Freda, & Cohen(54)	colostrum[3]	no reaction	30 minutes

1. Urine, usually concentrated, is injected into lymph sacs, intraperitoneally, or intravenously.
2. Urine is added to surrounding water
3. Intradermal injections are made using these materials. The last three cause reactions in non pregnant individuals.
4. Addition of pituitary synergist (FSH) and 15 I.U. of chorionic gonadotrophin to sample for injection, reduced false negative reactions.
5. Rats are injected with pregnant mare serum, and 56 hours later, with 25 I.U. HCG. Material to be assayed is injected 5-9 days later, immediately after removal of one ovary. One hour after injection, the second ovary is removed, and both are assayed for ascorbic acid.

TABLE II

Effect of Albumin on Stability of Specimens
(Titers expressed in I.U./ml.)

Final Concentration of Albumin	Storage Time Before Testing Temp.	3 hrs.	5 hrs.	8 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.
30 mg.%	R.T.[1]	48	48	48	64	64	64	64
	4°C.	--	--	48	48	64	64	64
100 mg.%	R.T.	48	48	48	64	64	64	64
	4°C.	--	--	48	64	64	64	64
300 mg.%	R.T.	96	64	64	64	64	64	64
	4°C.	--	--	64	64	64	64	64
Nothing added	R.T.	48	48	48	64	64	64	64
	4°C.	--	--	56	48	64	64	64

1:64 dilution made on specimen containing 100 mg% albumin at 12 hours was still positive at 72 hours.

1. Room Temperature (R.T.)

TABLE III

Effect of Bacteria on the Stability of the Specimen

Specimen	Temp.	Storage Time Before Testing: 0 hrs.	3 hrs.	18 hrs.	24 hrs.	36 hrs.	48 hrs.	64 hrs.	72 hrs.
Preg. Pool[1]	R.T.	--	48	64	64	--	64	--	64
	4°C.	--	--	--	64	--	64	--	64
Staph.	R.T.	--	48	64	128	96	--	64	--
	4 C.	--	--	64	128	128	--	64	--
E. coli	R.T.	--	48	64	128	96	--	96	--
	4°C.	--	--	64	128	96	--	96	--
Preg. Spec.a[2]	R.T.	16	--	--	16	--	16	--	--
Staph.	R.T.	18	--	--	20	--	20	--	--
E. coli	R.T.	16	--	--	20	--	22	--	--
Preg. Spec.b	R.T.	14	--	--	10	--	10**	--	--
Staph.	R.T.	14	--	--	18	--	18	--	--
E. coli	R.T.	14	--	--	18	--	18	--	--
Neg. Spec.[3]	R.T.	--	--	0	0	--	1	--	--
Staph.	R.T.	--	--	0	0	--	0	--	0
E. coli	R.T.	--	--	Pos.	4	--	4	--	4

1. Two specimens from pregnant patients pooled, bacteria added
2. One specimen from pregnant patient, bacteria added
3. One specimen from male patient, bacteria added

* Specimens in second portion of experiment were cultured at this time

** This specimen inhibited bacterial growth

TABLE IV

Effect of Storage Temperature on Specimen Stability

		Storage Time Before Testing				
Spec. No.	Temp.	0 hrs.	24 hrs.	48 hrs.	72 hrs.	264 hrs.
1	R.T.	4	4		4	4
	-20°C.		4		4	
2	R.T.	40	40		40	40
	-20°C.		40		40	
3	R.T.	60	60		60	60
	-20°C.		60		60	60
4	R.T.	16	16	16		
5	R.T.	14	10	10		
6	R.T.		64		64	
	4°C.		64		64	

TABLE V

Comparative Sensitivity of Immunoassay Methods

for Human Chorionic Gonadotrophin

Method	Technique	Range (I.U./ml.)	Interval	(Days)[1] (Range)
Pregnosticon Accusphere #1	Hemagglutination Inhibition	1.0-1.2	34.2	(31-37)
#2		1.0-1.5		
#3		1.0-1.5		
UCG	Hemagglutination Inhibition	1.0-1.5	38	(35-43)
Pretel #1	Hemagglutination Inhibition	1.5-2.0	37.6	(35-41)
#2		2.0		
DAP	Direct Latex Agglutination	3.4-4.0	39	(36-43)
Planotest Dri-Slide #1	Latex Agglutination Inhibition	4.0-6.0		
#2		4.0-5.0	42.7	(34-50)
#3		3.0-3.5		
Gravindex	Latex Agglutination Inhibition	4.0-5.0	45.7	(41-52)
HCG #1	Latex Agglutination Inhibition	4.0-10.0	56	(43-76)
#2		5.0		

1. Number of days past the first day of the last normal menstrual period when each test became positive. HCG and Pretel figures are based on three determinations; the rest, on four.

TABLE VI

Effect of Temperature Upon Commercial Test Systems
(Lowest detectable titers of HCG in I.U./ml.)

Test	4°C.	23°C.	37°C.	returned to 23°C.
HCG	8.0	10.0	10.0	10.0
#2 Planotest Dri-Slide	4.0	4.0	6.0	4.0
UCG	1.0	1.0	1.0	--

TABLE VII

Effect of pH on Immunological Pregnancy Test Systems

Specimen	Test	pH 4.5	5.0	5.5	7.0	7.5
Pos. #1	HCG	=	=	+	+	+
	Gravindex	=	+		+	+
	Accuspheres	=	+	+	+	+
	Planotest	+	+		+	+
	UCG	+	+	+	+	=
Neg. #2	HCG	=	=	=	=	=
	Gravindex	+	±		=	=
	Accuspheres	=	=	=	=	=
	Planotest	±	±		=	=
	UCG	=	=	=	=	=
Std.	HCG	=	+	+	=	=
	Gravindex	=	=	+	+	+
	Accuspheres	+	+	+	+	+
	Planotest	+	+	+	+	+
	UCG	+	+		+	+
	DAP	+	+	+	+	+

The second negative specimen test was not adversely affected at any pH with any of the tests.

TABLE VIII

Effect of Naturally Occurring Proteins in Non Pregnant Urines

No.	D	U	A_3	P_e	P_1	G	H_2	P_2	P_3	H_1	False Positives Total	False Positives Slide	Protein	Cl^- (mEq.)
1	=	=			+	+		+			3/5	3/3	3+	
2	=	=	=	=	+	+	+	+	+		5/9	5/5	3+	12
3	=	=			=	+		=			1/5	1/3	3+	
4		=		=	=		=		±	+	2/6	2/4	3+	
											11/25	11/15		
5	=	=		+	+	+	+	+	+		6/8	5/5	2+	
6	=	=			+	+		+		+	4/6	4/4	2+	
7*	=	=	=	+	+	+	±	+	+	+	7/10	6/6	2+	
8	=	=			=	+		=		+	2/6	2/4	2+	
9	=	=	=	=		=	+	±	±	=	3/9	3/5	2+	58
10	=	=	=	=		=	+	=	+		2/8	2/4	2+	86
11	=	=			=	=		+		=	1/6	1/4	2+	155
12		=		=	=		=		=	±	1/6	1/4	2+	126
13	=	=		=		=	=	=	=		0/7	0/4	2+	15
14	=	=				=		=		=	0/5	0/3	2+	
											26/71	24/43		
15	=	=	+	+	=	±	=	+	+	+	6/10	4/6	1+	16
16	=	=	=	=	=	=	=	+	=	+	2/10	2/6	1+	73
17	=	=				=		=		+	1/5	1/3	1+	
18	+	=	=	=	=	=	=	=	=		1/9	0/5	1+	
19	=	=				=		=			0/4	0/2	1+	
20	=	=		=	=	=	=	=	=		0/8	0/5	1+	
21	=	=				=		=			0/4	0/2	1+	
22	=	=	=	=	=	=	=	=	=	=	0/10	0/6	1+	49
23	=	=		=	=	=	=	=			0/7	0/4	1+	138
											10/67	7/39		
±	1	0	1	3	5	8	5	9	7	8				
Tot.	21	23	8	14	16	21	14	21	13	12	47/163	42/97		

* Specimen contained blood

D	DAP Test	G	Gravindex
U	UCG Test	H_2	HCG Test, Lot 2
A_3	Accuspheres, Lot 3	P_2	Planotest, Lot 2
P_e	Pretel Test	P_3	Planotest, Lot 3
P_1	Planotest, Lot 1	H_1	HCG Test, Lot 1

TABLE IX

Effect of Adding Serum Fractions to Urine

Minimum Concentration (mg.%) Found to Cause Positive Test Results

	DAP	UCG	Acc_3	Pre	Pl_1	Gra	Pl_2	Pl_3	HCG_1
Albumin									
1250	=	=	=	=	=	+	+	±	+
300						=	+	=	=
100							+		
30							=		
Globulin									
1650	=	=	=	=	*		=	=	
250						+			
125						±			+
62.5						=			=
Glycoprotein									
1000	=	=	=		=	+			
250				+		+		+	
125				=		=	+	=	
50							=		+
25									=

* Antiserum agglutinated with globulin mixture, before addition of latex particles.

TABLE X

Effect of Abuse Drugs on Non Pregnant Specimens

No.	1	2	3	4	DAP	UCG	Acc$_3$*	Pre	Pl$_3$	Gra	HCG$_2$
1	x				=	=	=	=	=	=	
2	x					=	=	=	=	=	
3	x					=	=	=	=	=	
4	x				=	=	=	=	=	+	=
5		x				=	=	=	=	=	
6											
7				x		=	=	+	=	=	
8				x		=	=	=	+	±	+
9				x	=	=	=	=	+	=	
10				x	=	=	=	=	=	=	
11				x	=	=	=	=	=	=	=
12				x	=	=	=	=	=	=	=
13				x	=	=	=	=	=	=	=
14				x	=	=	=	=	=	=	=
15			x	x	=	=	=	=	+	+	=
16			x	x	=	=	=	=	+	+	=
17		x		x		=	=	=	=	=	=
18		x		x	=	=	=	=	=	=	
19	x			x	=	=	=	=	=	=	=
20	x			x		=	=	=	=	=	
21	x			x		=	=	=	=	=	
22	x			x	=	=	=	=	+	=	=
23	x	x		x	=	=	=	=	=	=	=
24	x	x		x	=	=	=	=	=	=	
25	x	x		x	=	=	=	=	+	=	+
					0/16	0/24	0/24	1/24	6/24	4/24	2/13

1. Morphine
2. Codeine
3. Ritalin
4. Methadone

* Earlier lots gave positive results, but these were all negative when retested with lot 3.

Table X (continued)

Effect of Salicylates on Non Pregnant Specimens

No.	Therapeutic Regimen	UCG	Acc_3	Pre	Gra	HCG_2	Pl_3
1		=	+	=	=	=	=
2		=	+	=	=	=	+
3		=	=	=	=	=	=
4		=		=	=	=	=
5		=	=	+	=	=	±
6		=	=	+		=	+
7		=	=	=		=	=
8		=	=	+		=	=
	Maximum Dose, 1 Day						
1		=	=	=	=	=	=
2		=		=	=	=	=
3		=		=	=	=	=
4		=	=	=	=	=	=

Effect of Antineoplastic Drugs on Non Pregnant Specimens

No.	Drug	UCG	Acc_3	Pre	Gra	HCG_2	Pl_3
1	Leukeran	=*	=	=	=	=	=
2	Alkeran	=	D	=	+	=	+
3	Alkeran	=*	=	=	=	=	+
4	Cytoxan	=*	=	=	=	=	=
5	Cytoxan	=*	=	=	=	=	=
6	Cytoxan	=	=	=	=	=	+

* RBC pattern settled out irregularly and later than usual.

Effect of Miscellaneous Drugs on Non Pregnant Specimens

No.	Drug	UCG	Acc_3	Pre	Gra	HCG_2	Pl_3
1	Librium	=	=			=	=
2	Valium	=	=	=	=	=	=
3	Valium	=	=	=	=	=	=
4	Ritalin	=	=	=		=	=
5	Contraceptive	=	=			=	=
6	Contraceptive	=	=	=		=	=
7	Barbiturates	=		=	=		
8	Barbiturates	=	=	=	=		+
9	Desimpramine		=	+	=		=
10	Elavil		=	=	=		
11	Elavil				=		+

TABLE XI

Other Factors Affecting Test Systems

Criterion	DAP	Acc_3	UCG	Pre	Pl_3	Gra	HCG
Ease of setting up the test	3	2	1	7	4	5.5	5.5
Ease of reading end point	1.5	7	6	5	1.5	3	4
Cost	4	2	1	-	-	3	6
Glassware	4	2	1	6.5	6.5	4	4
Total time required	7	2	1	3	4	4	4

TABLE XII

Ranking Scores for the Tests

Criterion	DAP	UCG	Acc_3	Pre	Gra	Pl_3	HCG	Significance
Table V, Sensitivity	4	5.5	7	5.5	2	3	1	
Table VIII, Natural Protein	6	7	5	4	3	2	1	
Table IX, Serum Fractions Added Protein	6	6	6	4	2	3	1	
Table X, Abuse Drugs	6	6	6	4	2	1	3	W = 0.724, p = 0.01
Table VI, Temperature		3				2	1	
Table VII, pH		4	4		1.5	1.5	4	
Table X, Other Drugs		5.5	3	2	4	1	5.5	

APPENDIX I

FIRST INTERNATIONAL STANDARD FOR HUMAN CHORIONIC GONADOTROPHIN

At the third Conference on the Standardization of Hormones, held in Geneva in August, 1938, under the auspices of the Permanent Commission on Biological Standardization, results of preliminary assays of six contributions toward the world standard were considered. Based on the results, the Conference was able to formulate details of the manner in which the contributions should be mixed. Subject to approval by members of the Conference after the mixture had been assayed, they defined a unit of activity as "the specific gonadotrophic activity of 0.1 mgrm. of the standard preparation". This International Unit is an amount of activity "very similar to that required under the conditions used by many workers, to cause cornification of the vaginal epithelium of the immature rat". The contributions were subsequently mixed as arranged, diluted with lactose if they had not already been diluted, dried over phosphorus pentoxide, made into tablets, and packaged in evacuated ampules. A specimen was approved by each member of the Conference and the standard was established on April 1, 1939. This standard was kept by the Department of Biological Standards, National Institute for Medical Research, London, N.W.3.

SECOND INTERNATIONAL STANDARD FOR HUMAN CHORIONIC GONADOTROPHIN

In 1960, the Expert Committee on Biological Standardization of the World Health Organization, when informed that the stock of standard was low, authorized the National Institute for Medical Research, London, to arrange for its replacement.

Ten grams of a single batch of hormone of medium potency

(highly purified material tends to be unstable) were provided by N. V. Organon (Oss, Netherlands). The material was prepared from human urine of the first trimester of pregnancy, and purification was carried out according to the method of Katzman (99). This included adsorption on permutit, elution with ammonium acetate in alcohol, followed by precipitation with excess alcohol, and removal of pyrogens with calcium phosphate. The material was washed with ether and dried in vacuo. Upon receipt by the National Institute, the glass bottle containing the hormone preparation was placed in a polyethylene bag with anhydrous silica gel and stored at -10° C. Two months later, it was diluted with glass distilled water containing lactose; aliquots were distributed into ampules, and freeze dried. Check assays revealed potency of 4998 I.U. in 6.78 mg/ampule. Nine laboratories in six countries participated in a collaborative assay of the material, based on the first International Standard. Each group used at least two recognized methods of assay. Raw data were returned and statistically analyzed. Six assays were rejected as invalid on the grounds of lack of significant regression, lack of parallelism, or because of curvature. The geometric mean potency varied from 4524 I.U./ampule by the seminal vesicle weight method to 5966 I.U./ampule by the vaginal smear method. Six immunological assays submitted by one laboratory had a mean potency of 5664 I.U./ampule and the smallest variance of any method. The final value agreed upon by all participants in the assay was 5300 I.U./ampule. One I.U., therefore, equals 0.001279 mg. of the Second International Standard for Human Chorionic Gonadotrophin.

APPENDIX II

GOLDBERG REFRACTOMETER
(American Optical Company, Total Solids Meter, Model No. 10406)

This instrument indicates the specific gravity of a urine specimen by determining its refractive index.

The instrument was developed in close cooperation with Dr. A. V. Wolf, Head, Department of Physiology, University of Illinois College of Medicine, Chicago, Illinois. It uses a special hollow glass prism, filled with a stable liquid. The refractive index of the liquid changes with temperature, and accurately compensates for changes in the refractive index of an aqueous solution in the range 60 - 100° F. The manufacturer will provide correction tables for non aqueous solutions on request. The small sample volume (0.02 ml) used rapidly assumes the temperature of the instrument. Scales for direct reading of the specific gravity of urine facilitate reading. The necessary experimental data for calibrating the scale was developed under the direction of Dr. Wolf. The zero point was checked by measuring the refractive index of distilled water in the temperature range 70 - 85° F. The manufacturer states that within the range 1.001 to 1.035 the greatest error is 0.1% and this occurs at the upper extremes of the range. Above 1.035, there is poor correlation between refractive index and specific gravity. Measurements are made in 0.001 intervals.

To measure specific gravity using this instrument, a drop of specimen is placed at the top or bottom of the chamber and allowed to diffuse under the plastic plate on top of the prism. Gentle pressure is exerted on the plastic plate with the finger to hold

the specimen in place while the instrument is tilted toward a light source. Reading is made on the proper scale at the point of sharp contrast between dark and light. Only 0.02 ml of specimen is required for measurement, and about ten seconds to read the results. (100)

APPENDIX III

COMBISTIX REAGENT STRIPS
(Ames Company, Division of Miles Laboratories, Inc.
Elkhart, Indiana)

At one end of a plastic strip are three cellulose rectangles impregnated with reagents designed to develop or change color in the presence of glucose, protein, and variations in pH of the solution into which the strip is momentarily dipped and shaken free of excess solution.

Hydrogen ion concentration is measured by use of the indicators methyl red and bromthymol blue giving a range of pH from 5 to 9 with a series of distinct colors from orange to blue. This measures with an accuracy of $\pm$ 0.5 units and may be compared immediately to standard colors.

The test for protein utilizes tetrabromphenol blue and citrate buffer. It is based on the well-known phenomenon of "protein error of indicators"--the fact that at a fixed pH certain indicators will have one color in the presence of protein and another color in the absence of protein. The citrate buffer in the protein test area of Combistix provides a hydrogen ion concentration of approximately pH 3. At pH 3, tetrabromphenol blue has a yellow color when no protein is present. At the same pH, the indicator will change to yellowish green, green, and blue with increasing amounts of protein. Colors are matched to standards representing trace, 30, 100, 300, and over 1000 mg. per 100 ml. urine, as trace, +, ++, +++, and ++++ respectively. The only condition noted which may cause a false test for protein under these test conditions, according to the manufacturer, is highly buffered alkaline urine.

This same condition may cause false negative tests with sulfosalicylic acid or nitric acid tests. The color may be compared immediately to standard colors.

The area measuring glucose is impregnated with glucose oxidase, peroxidase, and orthotolidine. Glucose oxidase reacts with glucose in the urine to remove two hydrogen ions, forming gluconolactone which is promptly hydrated to gluconic acid. The removed hydrogen ion is then combined with atmospheric oxygen to form hydrogen peroxide. The hydrogen peroxide, in the presence of peroxidase, oxidizes orthotolidine which in its oxidized state turns blue. After ten seconds reaction time, this color is matched with color standards. If glucose is present, the red area on the stick changes to purple. Generally, a light color corresponds to a small amount of glucose and a dark color to a larger amount, but quantitation is not accurate. Enzyme dipstick tests are much more sensitive than copper reduction tests and will sometimes give a positive reaction with small amounts of glucose in the urine when the Clinitest is negative. (101)

APPENDIX IV

PREGNANCY TEST KITS

NOTE: No positive or negative controls are supplied in test kits.

HCG (Hyland, Division Travenol Laboratories, Inc., Costa Mesa, Calif.)

Latex Agglutination Inhibition Test

Contents of Kit:

Polystyrene latex particles sensitized with HCG; 0.1% sodium azide, preservative

Anti-HCG, derived from rabbit serum; 0.1% sodium azide, preservative

Capillary tubes for delivery of specimen to slide

Black glass slide marked for three tests

Procedure:

Bring reagents to room temperature

Place one drop antiserum on slide, from bottle dropper, held vertically

Add one drop urine from capillary tube, held vertically

Mix with applicator stick

Rock gently thirty seconds

Add one drop latex reagent from bottle dropper, held vertically

Mix with applicator stick

Rock gently two minutes longer

Observe for macroscopic agglutination, using a good light source

Agglutination indicates a negative test; a smooth suspension is interpreted as a positive test.

HCG (continued)

Precautions:

Incomplete mixing may cause incorrect readings

Turbid specimens should be filtered or centrifuged

High protein may cause false results

Reagents are stored at 4° C.

Pretel (Ames Co., Division of Miles Laboratories, Inc., Elkhart, Ind.)

Hemagglutination Inhibition Test

Contents of Kit:

Plastic test units containing lyophilized rabbit antiserum in lower portion and lyophilized erythrocytes with chemically coupled HCG in the covering portion. The closed container forms a miniaturized test tube.

Disposable droppers

Mirrored Rack

Procedure:

Open test unit carefully

Using disposable dropper, add one drop of water to bottom well

After expelling excess water, add one drop urine to well

Close cover carefully

Shake thoroughly to dissolve reagents

Place unit on mirrored rack, and leave undisturbed for one hour

Observe hemagglutination pattern in mirror at base of rack

Any ring at one hour is a positive result

Precautions:

Specimens should be brought to room temperature

Specimens should be free of turbidity

Test units are stored at room temperature

Jarring tests during incubation can cause erroneous results

Test material is hygroscopic and should not be used if color change is noted

Pregnosticon Accuspheres (Organon Inc., West Orange, New Jersey)

Hemagglutination Inhibition Test

Contents of Kit:

Buffer for quantitative tests

Diluent for quantitative tests

Screw cap glass test units each containing one lyophilized sphere of antiserum and one of sheep erythrocytes coated with HCG

Mirrored Rack

Procedure:

Using a measuring pipette, add 0.1 ml. urine to test unit

With a second pipette, add 0.4 ml. distilled water

Shake to insure thorough mixing

Place in mirrored rack

Allow to stand undisturbed for two hours (Positive reactions may usually be read by one hour, but negative results should not be reported before two hours.)

Definite brown ring in bottom of tube indicates positive test (read in mirror at base of rack)

Smooth mat indicates negative result

Precautions:

Specimen should be filtered or centrifuged if turbid

An irregular light brown ring indicates that sedimentation has been disturbed and the test should be repeated

Reagents are stored at 4^{o} C.

Accuspheres are very hygroscopic. Don't use if color is changed

DAP (Wampole Laboratories, Division Denver Chemical Mfg. Co., Stamford, Conn.)

Direct Agglutination Test

Contents of Kit:

Latex particles coated with HCG antibody

Latex particles, uncoated

Filter paper

Blue glass slide, marked for three tests

Calibrated capillary tubes with rubber bulb

Procedure:

Bring reagents to room temperature

Insert capillary tube into rubber bulb so that inside flap is lifted

Allow tube to fill by capillary action with urine to calibration line

Place finger over hole in bulb and dispense sample onto slide by squeezing bulb

Repeat the procedure to place a second drop on slide for control

Add one drop of uncoated control latex particles to first drop

Add one drop of coated latex particles to second drop of urine

Mix both with wooden applicator sticks

Rock slide gently for two minutes

Read under good light source for macroscopic agglutination

Agglutination in test, with smooth control, indicates positive results

Agglutination in both test and control indicates nonspecific

DAP (continued)

interference

Smooth suspension in both test and control indicates a negative test

Precautions:

Urine should be filtered

Specimens grossly contaminated by bacteria should be rejected

Reagents are stored at 4° C.

Gravindex (Ortho Diagnostics, Raritan, New Jersey)

Latex Agglutination Inhibition Test

Contents of Kit:

Latex particles coated with HCG

Rabbit antiserum to HCG; 1:10,000 thimerosal, preservative

Plastic dropper tubes with a rubber bulb for dispensing urine

Glass slide marked for two tests

Wooden sticks for mixing reagents

Procedure:

Bring reagents to room temperature

Add one drop urine to slide from plastic dropper, held vertically

Mix with wooden stick

Rock gently thirty seconds

Add two drops latex reagent from bottle dropper, held vertically

Mix with wooden stick

Rock slowly and gently

Observe, under a good light source, for macroscopic agglutination during the next two minutes

Agglutination indicates a negative test

A smooth suspension is interpreted as a positive test

Precautions:

Improper mixing and drying of reagents can give false results

Specimen bottles and slides must be free of detergents

Specimens with specific gravity greater than 1.015 show increased accuracy

Gravindex (continued)

Test specimens within twelve hours; use no preservatives

Centrifugation is not necessary

Specimens containing bacteria or blood should be rejected

Specimens containing more than 100 mg.% protein should be rejected

Negative tests prior to 41 days should be confirmed by a further test after that time

Reagents are stored at 4° C

Planotest Dri-Slide (Organon Inc., West Orange, New Jersey)

Latex Agglutination Inhibition Test

Contents of Kit:

Heavy paper slide with sensitized latex particles and antiserum dried in two separate "spots" on the slide

Plastic Dispensitrs

Distilled Water

Procedure:

One drop distilled water (or tap water) added to latex "spot" from bottle dropper, held vertically

One drop urine from dispenstir, held vertically, added to antiserum "spot" on the slide

Spatulate end of dispenstir is used to mix urine and antiserum

Slide gently rocked, being careful not to disturb drop of water

Mix in latex and water with urine and antiserum

Rock gently for two minutes longer

Read under good light source for macroscopic agglutination

Agglutination indicates a negative test

Smooth suspension indicates a positive test

Precautions:

Turbid specimens should be filtered with Whatman #4 filter paper (least adsorptive)

Reagent slides are stored at 4^{o} C.

APPENDIX V

PIPETTE CALIBRATION

Procedure

Accuracy and precision of volumetric pipettes of various types were determined by replicate gravimetric technique using both distilled water and mercury. Pfieffer and Oswald Folin volumetric pipettes, and serological graduated pipettes were checked for total delivery using water. Graduated serological pipettes were also tested for point to point delivery using water. Dispenstirs used in the "Planotest Dri-Slide" were used as directed to produce a drop of water which was weighed. Ultrapets were tested with mercury.

Results

Pipette	Mean Value	Deviation	
1 cc. volumetric (Pfieffer)	1.0085	± 0.00832	0.2 -1.7 %
1 cc. Oswald-Folin	0.9969	± 0.005	0.2 -0.9 %
1 cc. serological (repeated)	0.9939	± 0.0044	0.17-1.05%
1 cc. serological (different)	0.9952	± 0.0064	0.1 -1.12%
1 cc. serological (to 0.5 ml.)	0.5009	± 0.0042	0.6 -1.0 %
1 cc. serological (to 0.1 ml.)	0.1026	± 0.0031	0.5 -5.0 %
Ultrapets (combination, 500λ)	0.4944	± 0.0029	0.4 -1.6 %
Dispenstirs	0.0303	± 0.0024	7.0 -9.0 %

Because small volumes of standard were diluted to many different concentrations in serial fashion, the relative accuracy of the different dilution schemes was compared using a solution of sodium I^{125} iodide. The dilutions were then counted using a dual channel gamma ray spectrometer and scaler (Nuclear Chicago).

Results

Radioisotope dilution scheme using combinations of serological and ultrapets	% error calculated by comparing final count with proper fraction of original count
1) Serial dilutions, varying amounts	0.1-2.4 %
2) Each dilution made directly from same standard dilution	0.4-3.7 %
3) Serial dilutions, same amounts	0.7-5.5 %

Discussion

All errors are in the same range, with the exception of Dispenstirs, with 5% maximum error from pipettes.

At 750 I.U. HCG, this would give a range of 712.5-787.5

1,000	950-1050
2,000	1900-2100
4,000	3800-4200

In no instance would this overlap with dilutions being made, to cause significant interference with results.

The first dilution scheme, used because of a shortage of material, compared very favorably, and was used for the rest of the experiments.