A method for the determination of human chorionic gonadotrophin in urine extracts

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SYNOPSIS A method is described for the preparation in rabbits of a specific antiserum to human chorionic gonadotrophin. The ability of this antiserum to fix complement in the presence of urinary human chorionic gonadotrophin is demonstrated. The standardization of antiserum against the international standard preparation of human chorionic gonadotrophin, the determination of antiserum specificity using appropriate controls, and the value of antiserum in the diagnosis of pregnancy and conditions associated with high urinary levels of human chorionic gonadotrophin is emphasized.

By the complement-fixation test it is possible to detect a minimum of 1·5 international units of human chorionic gonadotrophin contained in 0·1 ml. of a 1 in 2 dilution of urine extract. In the Hogben pregnancy test, using 2·5 ml. of urine extract per animal, a minimum concentration of 70 international units of human chorionic gonadotrophin is required to give a positive response. The ease and economy of the complement-fixation test are described and results are compared with those of the Hogben test.

PREPARATION OF HUMAN CHORIONIC GONADOTROPHIN ANTISERUM

Human chorionic gonadotrophin is a mucopolysaccharide of molecular weight 100,000 (Gurin, Bachman, and Wilson, 1940) and might, therefore, be suitably antigenic. At the present time no chemically pure preparation of this hormone exists, a fact which must obviously complicate the issue from an immunological standpoint. Two commercial preparations of human chorionic gonadotrophin having a high degree of chemical purity were selected as antigens. These preparations were Pregnyl (Organon) and H.C.G. (Leo). Female rabbits weighing approximately 2,000 to 3,000 g. were used as test animals.

Various immunizing procedures were adopted (Table I). Either Pregnyl or H.C.G. (Leo) was used as antigen admixed with Ramon's adjuvant or Freund's complete adjuvant. Aluminium phosphate was used as adjuvant on four occasions but was found to be unsatisfactory. One thousand five hundred international units of antigen (H.C.G.) well mixed with adjuvant were administered subcutaneously at fortnightly or monthly intervals. The optimum number of injections required to produce antiserum varied in most cases from two to six. In six rabbits, antigen was also administered by the intravenous route. This procedure did not appear to confer any

advantage over the subcutaneous route alone and the intravenous route was subsequently abandoned.¹

TITRATION OF HUMAN CHORIONIC GONADOTROPHIN ANTISERUM

The test rabbits were bled from an ear vein at weekly intervals commencing two weeks after the first injection of antigen (H.C.G.). Test bleeds should not be undertaken earlier than six days after any injection of antigen (H.C.G.). The sera were separated from the clotted blood samples and each serum was heated at 56°C. for 30 minutes to inactivate complement. Doubling dilutions of test sera were made in normal saline and 0·1 ml. volumes of these dilutions were titrated against 0·1 ml. amounts of a human chorionic gonadotrophin solution (Department of Biological Standards) used at a strength of 1·5 international units in 0·1 ml. normal saline. Fresh pooled guinea-pig serum was used as a source of complement. Throughout the test, complement was used at 3 minimal haemolytic doses (3.M.H.D.) strength and 0·1 ml. volumes of this dilution of complement were used. A 2% suspension of sensitized sheep red cells was used as the haemolytic system. The antisera under test, complement, and antigen were mixed and incubated at 37°C. for one hour. At the end of this time

¹Since the submission of this article for publication we have obtained satisfactory human chorionic gonadotrophin antiserum using the intramuscular technique of Fulthorpe *et al.* (1963).

0.2 ml. volumes of the 2% sensitized sheep red cell suspension were added to each tube and after further incubation at 37°C. for 30 minutes readings were made. Any serum yielding a titre of 1 in 8 or more was considered to be satisfactory. It is most important that those animals giving satisfactory antiserum titres should be bled out on the same day as the test bleed. After anaesthetization, blood was collected by sterile cardiac puncture from suitable rabbits. Our experience is that undue delay between the test bleed and the final bleeding out can result in complete loss of antibody or occasionally in the development of sheep red cell haemolysins. Blood obtained from rabbits showing satisfactory anti-human chorionic gonadotrophin titres was allowed to clot and serum was separated from the resulting clot by centrifugation. The serum was heated at 56°C. for 30 minutes to inactivate complement and was then divided into 1 ml. aliquots and stored without preservative in sealed hard glass ampoules at -20°C. It is necessary to store the samples in this way for the following reasons:—1 It has been established that too frequent freezing and thawing of large volumes of antiserum causes marked deterioration in antibody potency. 2 Only small quantities of antiserum are required for each batch of tests. 3 If frozen antiserum is kept in screw-cap containers, CO₂ used for the purposes of refrigeration or air from the atmosphere is able to seep through the junction of cap and bottle. This has been shown to result in progressive deterioration in the potency of the sample.

PROPERTIES OF A SATISFACTORY ANTI-HUMAN CHORIONIC GONADOTROPHIN SERUM A satisfactory anti-human chorionic gonadotrophin serum for use in complement-fixation tests involving pregnancy urine extracts prepared by the Scott technique (1940) must fulfil the following conditions.

1 It must be able to fix a minimum of 1.5 international units of human chorionic gonadotrophin per 0.1 ml. urine extract. The reason for this is twofold. First, in the Hogben test it is customary to inject 2.5 ml. of urine extract prepared by the Scott technique into a *Xenopus laevis* toad weighing 60 to 100 g. It has been repeatedly found that a satisfactory animal of this weight will respond to a minimum of 70 international units of human chorionic gonadotrophin, i.e., 2.8 international units per 0.1 ml. urine extract. In the second instance it was found preferable to dilute the urine extracts 1 in 2 with barbitone complement-fixation test diluent¹ pH 7.2 before examination by the complement-fixation test, as many of the undiluted urine extracts had anticomplementary properties. In most cases the anticomplementary effects were abolished at the 1 in 2 dilution.

2 It must give no complement-fixation with urine extracts prepared by the Scott technique from human adult males or from non-pregnant, non-menopausal adult females.

3 It must have no anticomplementary properties.

4 It must neither agglutinate nor haemolyse sensitized sheep red cells.

5 It must give a titre of at least 1 in 8 when titrated against the international standard preparation of human chorionic gonadotrophin used at a strength of 1.5 international units per 0.1 ml. normal saline.

6 It must remain stable when sealed in hard glass ampoules¹ in 1 ml. amounts at a temperature of -20°C.

RESULTS OF IMMUNIZING PROCEDURES IN THE PREPARATION OF HUMAN CHORIONIC GONADOTROPHIN ANTISERUM

Twenty-four rabbits in the series were inoculated with antigen and adjuvant as described in the text. The results obtained are set out in Table I. Reference to Table I shows the difficulties of preparing a specific anti-human chorionic gonadotrophin serum of suitable potency. In view of the low molecular weight and of the impurities in commercial preparations this is not surprising.

Failure to obtain satisfactory antisera from rabbits 16 and 21 was due to delay between the test bleed and the final bleeding out. During the period of delay, a powerful sheep red cell haemolysin had developed in both antisera. As already emphasized, it is most important, therefore, to bleed out any animal whose serum shows a satisfactory titre on the same day and within a few hours of the test bleed.

A method for the further chemical purification of commercial preparations of human chorionic gonadotrophin and of urine extracts has been described by Butt and Round (1958). Further purification of human chorionic gonadotrophin used for immunization or of urine extracts containing human chorionic gonadotrophin was not adopted in the present work. The antigenicity of human chorionic gonadotrophin might be improved if it were possible to conjugate it to a suitable protein. This procedure, however, could well result in the production of interfering antibodies in the test animals and complex absorption experiments would then be required for the removal of these non-specific components.

ESTIMATION OF URINARY HUMAN CHORIONIC GONADOTROPHIN

In the diagnosis of pregnancy, an early morning specimen of urine, of at least 100 ml. by volume, is extracted by the Scott technique. The resulting extract is carefully neutralized and 0.1 ml. is diluted 1 in 2 with barbitone complement-fixation test diluent² at pH 7.2. Volumes, each of 0.1 ml., of the diluted extract are placed into each of two Wassermann reaction 3 × $\frac{3}{8}$ in. tubes. To one tube is then added 0.1 ml. 3M.H.D. complement plus 0.1 ml. anti-human chorionic gonadotrophin serum used at its predetermined optimum titre. To the other tube is added

¹Hard glass ampoules for storing small volumes of antisera can be obtained from Henry Bush and Co. Ltd., Waddon Factory Estate, Croydon, Surrey.

²Barbitone C.F.T. diluent can be obtained from Oxo Ltd., London.

TABLE I

RESULTS OF IMMUNIZING RABBITS WITH HUMAN CHORIONIC GONADOTROPHIN

Female Rabbit (3 kg.)	Antigen (H.C.G.)	Adjuvant	No. of Injections of Antigen (H.C.G.)		Total Dose of Antigen Administered (i.u.) ¹	Final Titre of Anti-Human Chorionic Gonadotrophin Serum	Development of Sheep Red Cell Haemolysin	Anti-serum Specific for Human Chorionic Gonadotrophin (H.C.G.)	Interval from Start of Immunization to Development of Final Titre (days)
			Intravenous	Sub-cutaneous					
1	Pregnyl	Ramon	3	5	4,000	Nil			
2	Pregnyl	Ramon	2	3	2,500	Nil			
3	Pregnyl	Ramon	8	2	4,000	1/8 ≡ 12 i.u. per 0.1 ml.		Yes	25
4	Pregnyl	Ramon	1	23	17,000	1/16 ≡ 6 i.u. per 0.1 ml.	Yes	Yes	269
5	Pregnyl	Ramon	4	8	7,000	1/16 ≡ 12 i.u. per 0.1 ml.		Yes	60
6	Pregnyl	Ramon	1	6	7,000	Nil			Rabbit died on day 52
7A	Pregnyl	Ramon		6	6,000	Nil	Yes		
7B	Pregnyl	Freund		2	3,000	1/16 ≡ 3 i.u. per 0.1 ml.		No	58
9	Leo	Freund		2	3,000	1/16 ≡ 3 i.u. per 0.1 ml.		No	38
10	Leo	Freund		2	3,000	1/4 ≡ 3 i.u. per 0.1 ml.		Yes	38
11	Pregnyl	Freund		2	3,000	Nil			
12	Leo	Freund		2	3,000	Nil			
13	Pregnyl	Aluminium phosphate		2	3,000	Nil			
14	Leo	Aluminium phosphate		2	3,000	Nil			
15	Pregnyl	Freund		4	6,000	1/8 ≡ 1.5 i.u. per 0.1 ml.		Yes	45
16	Leo	Freund		2	3,000	1/16 ≡ 3 i.u. per 0.1 ml.	Yes	Not determined	17
17	Pregnyl	Aluminium phosphate		6	9,000	Nil			
18	Leo	Aluminium phosphate		4	6,000	Nil			
19	Leo	Freund		2	4,500	1/8 ≡ 1.5 i.u. per 0.1 ml.		Yes	24
20	Pregnyl	Freund		3	4,500	1/16 ≡ 1.5 i.u. per 0.1 ml.		Yes	38
21	Leo	Freund		4	6,000	1/16 ≡ 1.5 i.u. per 0.1 ml.	Yes	Not determined	59
22	Pregnyl	Freund		6	9,000	1/8 ≡ 1.5 i.u. per 0.1 ml.		Yes	108
23	Pregnyl	Freund		3	4,500	1/16 ≡ 1.5 i.u. per 0.1 ml.		Yes	59
26	Leo	Freund		2	9,000	1/4 ≡ 1.5 i.u. per 0.1 ml.		Yes	38

¹ International units as stated by the manufacturers.

0.1 ml. 3M.H.D. complement plus 0.1 ml. diluent. Known positive and negative control urine extracts are put up at the same time as the test series. All tubes are incubated at 37°C. for one hour. At the end of this time 0.2 ml. volumes of a 2% suspension of sensitized sheep red cells are added to each tube and after further incubation at 37°C. for 30 minutes readings are made. A positive reading is given by those tubes showing no lysis of the red cells and a clear supernatant. A supernatant showing haemolysis indicates a negative result. Any urine extracts showing anticomplementary activity should be discarded and a fresh early morning specimen of urine requested.

In the quantitative determination of human chorionic gonadotrophin the procedure adopted is exactly as described above with the difference that doubling dilutions of the urine extract in barbitone complement-fixation test diluent are required. The end-point is taken as the highest dilution tube showing a clear supernatant. Readings may

be simplified by centrifugation of the tubes at the end of the test.

ANTICOMPLEMENTARY PROPERTIES OF CERTAIN URINE EXTRACTS In our early experiments it was soon discovered that a large number of extracts of neat urine prepared by the Scott technique were anticomplementary. This difficulty appeared at first to have been resolved by diluting the urine extracts 1 in 2 with barbitone complement-fixation test diluent. Since the publication of our preliminary communication (Lynch and Schwabacher, 1962), we have encountered a very small but appreciable number of urine extracts which showed anticomplementary activity even at a 1 in 2 dilution (Table II). Such extracts are quite unsuitable for examination and should be discarded.

TABLE II

ANTICOMPLEMENTARY PROPERTIES OF URINE EXTRACTS

No. of Undiluted Urine Extracts Tested	No. of Undiluted Urine Extracts Anticomplementary	No. of Urine Extracts Diluted 1 in 2 with Buffer Diluent	No. of Urine Extracts Diluted 1 in 2 Anticomplementary
67	23 (33%)	287	13 (4.5%)

Attempts were also made to abolish anticomplementary effects by treating affected urine extracts with the solvent Arcton 113. This proved unsatisfactory because it was established that human chorionic gonadotrophin passes into the solvent phase. The anticomplementary effects shown by these urine extracts may well be accounted for when it is appreciated that there is often a considerable delay from the time of collection of an early morning specimen of urine to its eventual extraction. Transit in the post is most favourable to bacterial multiplication in urine specimens. It is therefore important to test the urine extracts for human chorionic gonadotrophin activity as soon as possible after extraction. If this is impracticable the urine extracts should be stored for not more than 48 hours at 0 to 2°C. before testing. In our preliminary communication we reported that there seemed to be no appreciable loss of human chorionic gonadotrophin activity in extracts of neat urine kept at this temperature for as long as seven days. Although this proviso still holds, anticomplementary affects frequently prove troublesome in stored extracts.

IMMUNODIFFUSION OF HUMAN CHORIONIC GONADOTROPHIN ANTISERUM

The method of Elek (1948) and Ouchterlony (1949) and the micro-method of May and Rawlins (1962) using ion agar were used in a number of experiments. Specific anti-human chorionic gonadotrophin sera failed consistently to yield precipitin lines against known Hogben-positive urine extracts or against various concentrations of standard human chorionic gonadotrophin in normal saline. Certain anti-human chorionic gonadotrophin sera which at first appeared satisfactory in the complement-fixation test were later discovered to fix complement when tested against known Hogben-negative male and female urine extracts. These non-specific antisera yielded precipitin lines against Hogben-positive urine extracts but failed to yield precipitin lines against various concentrations of the standard human chorionic gonadotrophin preparation. Such false positive reactions may be due to union of a mucoprotein in the urine extracts with a non-specific antibody in the anti-human chorionic gonadotrophin serum. Reference to Table I shows that antisera

from rabbits 7B and 9 had non-specific properties. An attempt to remove these non-specific reactions by the method of Brody and Carlström (1962) was successful. Titrations of non-specific antisera saturated with an equal volume of serum taken from a non-pregnant, non-menopausal subject failed to give false positive reactions with Hogben-negative male and female urine extracts.

DISCUSSION

The complement-fixation test may be used as a qualitative determination of urinary human chorionic gonadotrophin in pregnancy and as a quantitative determination of urinary human chorionic gonadotrophin in clinical conditions associated with a high urinary output of human chorionic gonadotrophin *e.g.*, hydatidiform mole, chorion epithelioma, and in Rhesus-negative mothers bearing severely affected Rhesus-positive infants (Scott, 1958).

It is interesting to compare the accuracy of the complement-fixation test with the Hogben test in pregnancy. Reference to the table (Table III) shows close agreement of results in the small series of parallel studies undertaken. The main difficulty in the clinical application of the complement-fixation test lies in the production of human chorionic gonadotrophin antiserum of suitable potency and of suitable specificity. In connexion with the immunizing procedure it should be remembered that injections at long intervals with human chorionic gonadotrophin plus Freund's adjuvant give better results than repeated injections at short intervals. The course of injections should be relatively short so as to minimize the development of secondary antibodies and sheep red cell haemolysins.

In the clinical application of the complement-fixation test the obvious disadvantage of the method is the large percentage of urine extracts (4.5% in the present series) showing pronounced anticomplementary activity. Such anticomplementary activity is largely due to delay in collection and extraction of the urine samples which tends to favour bacterial multiplication. If, therefore, the method is to be adopted by routine laboratories the collection and extraction of urine specimens would preferably be done on the same day.

TABLE III

COMPARISON OF COMPLEMENT-FIXATION TEST WITH HOGBEN TEST

Total No. of Urine Extracts Examined	Hogben Test		Complement-fixation Test			False Positive Result in Com- plement-fixation Test	False Negative Result in Com- plement-fixation Test
	Positive	Negative	Positive	Negative	Anti- complementary		
140	64	76	61	72	7	—	2

The advantages of the complement-fixation test over the Hogben test are as follows:—1 The test is easy to perform and the results can be obtained on the day of the test. If there is no urgency for results, the test can be run in parallel and on the same day as the Wassermann reactions. More than 100 tests can be completed with ease in a morning. 2 No animals are required with a resulting saving in technical staff and materials. 3 The test can be adapted to yield quantitative results with a much more satisfactory and reliable end-point than can be obtained by present-day bio-assay methods. 4 Facilities are available in all laboratories for the preparation of anti-human chorionic gonadotrophin sera.

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Institute for Medical Research, London, for supplying us with the international standard preparation of human chorionic gonadotrophin.

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