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Human Chorionic Gonadotropin Detection by Means of Enzyme Immunoassay: A Useful Method in Forensic Pregnancy Diagnosis in Bloodstains

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ABSTRACT: This paper reports on human chorionic gonadotropin (HCG) detection in bloodstains using a commercial kit based on enzyme immunoassay. The specificity and sensitivity of the method is tested, as well as HCG stability over time in the samples. Detection of this hormone in bloodstains is of special interest for pregnancy diagnosis in forensic science applications.

The experimental series comprises 60 bloodstains prepared in our laboratory and obtained from blood samples taken from 30 pregnant women and 30 healthy, nonpregnant individuals. The bloodstains were studied at different stages of aging over a 6-month period, each sample being stored at 2 different temperatures throughout the process.

The experimental evidence proves HCG to be a useful and stable diagnostic indicator of pregnancy in bloodstains. In addition, the technique used is fast, as well as highly sensitive and specific.

KEYWORDS: pathology and biology, human chorionic gonadotropin, pregnancy, immunoassay, bloodstains, enzyme immunoassay

Human chorionic gonadotropin (HCG) is a glycoprotein, its constituents being two polypeptidic chains linked by noncovalent bonds (α = 18 kD and β = 32 kD), as well as carbohydrates [*1*] (sialic acid NA-NA), glucose, galactose (GAL), mannose (MAN), *N*-acetylglucosamine (GLUNAC), and galactosamine), bonded to these chains in different combinations [*2*]. Biochemically, HCG is similar to other glycoproteinic hormones, such as the luteinizing hormone (LH), the follicle-stimulating hormone (FSH), and the thyroid-stimulating hormone (TSH) [*3*]. The α subunits containing these hormones are highly similar among themselves, whereas the β subunits vary widely in their amino acid chains [*4*], as compared to the other hormones mentioned above. This fact allows for convenient HCG identification using specific antibodies vis à vis a β subunit [*5*].

HCG secretion occurs initially through the trophobastic cells of the developing blastocyte and later through the placental syncytiotrophoblasts. HCG plays a significant role in corpus luteum maintenance which keeps up progesterone and estradiol production during pregnancy. It also intervenes as a stimulating agent in Leydig cell formation as well as in testosterone production in male fetuses and supports fetal adrenal growth in the early stages of pregnancy.

Aschheim [*6*] (1934) used bioassays in mice for pregnancy tests which were based on

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HCG detection. In 1948 Berg [*7*], starting from Friedman's experiments in 1932 [*8*], obtained satisfactory results with HCG detection in bloodstains applying a low sensitivity bioassay. In 1964, Abelly and Colb [*9*] used a hemagglutination-inhibition technique for HCG detection, obtaining results with bloodstains of up to 13 days when stored at 4°C, yet only up to 8 days when kept at room temperature. In 1967, Tesar [*10*] achieved HCG detection in up to 20-day-old bloodstains from pregnant women using a commercial kit based on hemagglutination-inhibition techniques. In 1980, Low-Beer and Lappas [*11*] described a cross-electroimmunodiffusion technique for HCG using bloodstains containing approximately 100 to 200 μL of blood and obtained positive results with bloodstains from samples which had been taken on Day 30 of pregnancy.

This research reports on the application of a fast, specific, and highly sensitive enzyme immunoassay kit for clinical use, which in our laboratory is used for qualitative HCG detection in bloodstains. It proved to be an effective tool in forensic pregnancy diagnosis. The technique applied in our research to date has not been described in the literature as an efficient method for pregnancy testing in bloodstains.

Material and Methods

Preparation of Bloodstains and Extracts

Using whole blood, 60 samples were taken from donors whose HCG levels were approximately known a priori: 30 pregnant women (all of them within Month 1 to 6 of pregnancy), 15 healthy adult men, and 15 healthy menopausic women. HCG concentration was determined in each of the 60 samples of the β-HCG 15/15 kit (Abbott Laboratories Diagnosis Division). The bloodstains were prepared at a ratio of 50 μL of whole blood per 100 mm^2 of cotton cloth. The 60 bloodstains thus obtained were each dried and divided into 2 series of 60 specimens for storage at 2 different temperatures, 56°C and room temperature, over a maximum period of 6 months. HCG activity was assessed qualitatively or quantitatively or both in our laboratory at different stages of the aging process, that is, after 1 week, 3 months, and 6 months. HCG was determined from bloodstain extracts. For this purpose, each sample was macerated and soaked in 300 μL of distilled water per 100 mm^2 of stained cloth for 2 h at room temperature. The extracts were obtained centrifuging the cloths for 10 min at 200*g*.

Enzyme Immunoassay

Qualitative Enzyme Immunoassay for HCG Determination in Bloodstains—The 60 bloodstain samples were tested on a commercial testpack HCG serum kit (Abbott Laboratories). The kit is a sandwich enzyme immunoassay. Figure 1 illustrates the principle underlying this method.

From the 60 bloodstains, 300-μL extracts were incubated with a goat anti-human α-HCG alkaline phosphatase conjugate for 30 to 60 s at room temperature. The product was then transferred to a membrane containing mouse monoclonal anti-human β-HCG immobilized on the membrane support and was subjected to a second incubation for 2 min at room temperature. Then it was washed once with a sodium chloride solution. Upon addition of three drops of chromogenic reagent (registered trademark, Abbott Laboratories), which is the substrate for the conjugated enzyme, color development set in after 2-min reaction time.

Figure 2 shows the results obtained. A (+) indicates high HCG content in the extract, whereas (−) means absence of detectable HCG.

Quantitative Enzyme Immunoassay for HCG Determination in Bloodstains—Quantitative analysis was applied to 30 bloodstains (only the stains with positive results in the

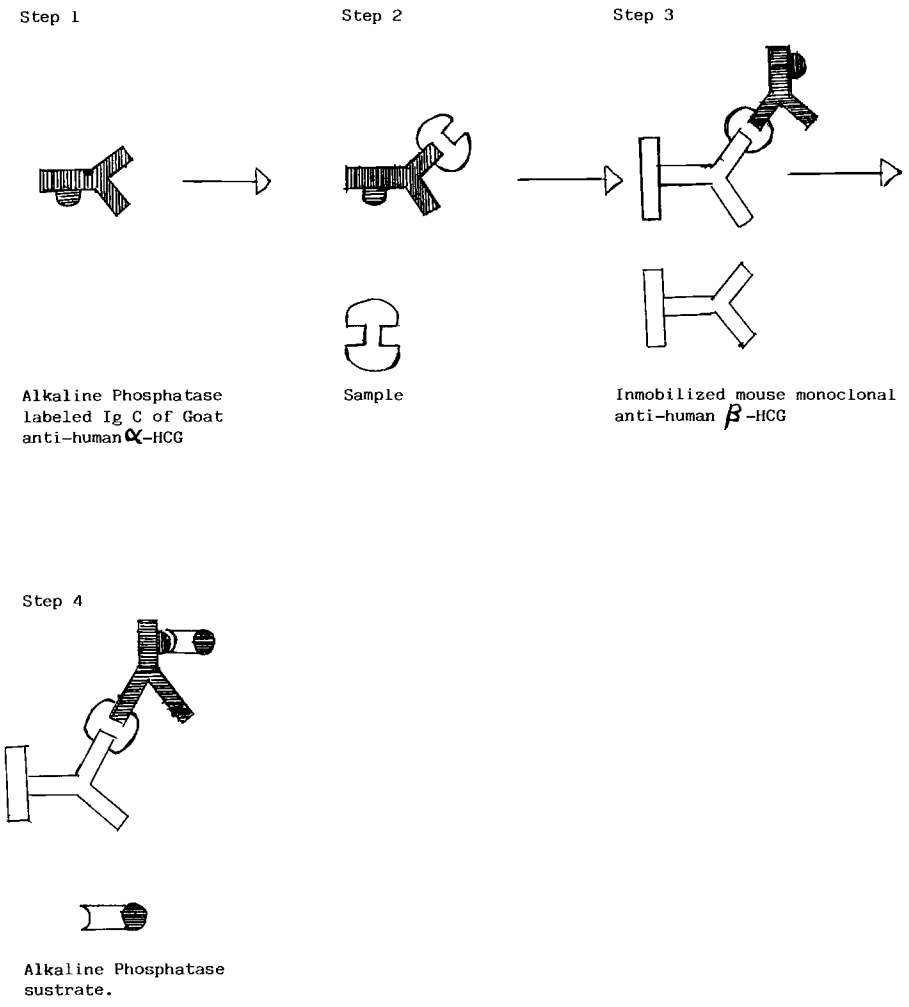

FIG. 1—*Principle of sandwich enzyme immunoassay method.*

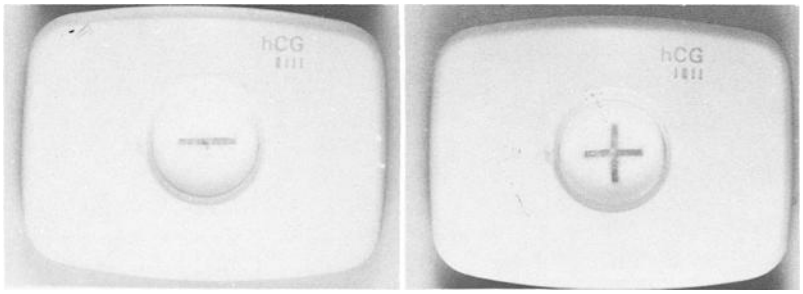

FIG. 2—*Results of sandwich enzyme immunoassay method. A (+) indicates high HCG content in the extract, whereas (−) means absence of detectable HCG.*

qualitative determination with the HCG testpack were considered for evaluation) and 60 plasmas, obtained from the respective 60 whole blood samples from which the bloodstains had been prepared. For quantification, a commercial β-HCG 15/15 kit from Abbott Laboratories was used, that is, a solid phase sandwich immunoassay. Quantitative plasma determination was conducted strictly following the instructions in the kit manual. For quantitative HCG determination in the 30 extracts from pregnancy bloodstains, 100 μL of extract from the 30 bloodstains, standards, and controls had been incubated with goat anti-human β-HCG immobilized on polystyrene beads and a goat anti-human β-HCG horseradish peroxidase conjugate. Unbound materials were removed by washing the beads. Subsequently, the beads were incubated with *o*-phenylenediamine (OPD) substrate solution containing hydrogen peroxide. As color developed from this solution, color intensity was measured in a spectrophotometer set at 492 nm.

Results and Discussion

Table 1 shows the results from HCG quantification by means of enzyme immunoassay applied to 60 plasmas and bloodstain extracts of 30 pregnant women. These latter were assessed at 2 different stages of aging, that is, at 1 week and 6 months. In addition, Table 1 indicates the range of HCG activity of the 60 plasma samples and the 30 bloodstains from the pregnant group.

The HCG plasma levels for the 30 pregnant subjects ranged between 1640 and 134 140 mIU/mL HCG [*12*]. The values obtained for the 15 healthy adult men varied from 0 to 10 mIU/mL of HCG, and those for the 15 healthy menopausic women from 0 to 13 mIU/mL of HCG. HCG determination in bloodstain extracts of the pregnant group preserved at room temperature for 1 week ranged between 801 to 57 900 mIU/mL of HCG, whereas the respective 6-month-old samples yielded values of 78 to 6148 mIU/mL of HCG. The data obtained for the lot stored for 1 week at ambient temperature suggest that the treatment applied to these samples provides optimum conditions for maximizing HCG extraction from this kind of support. On the other hand, HCG stability in pregnancy bloodstains stored at room temperature for 6 months proved to be poor, as a result of the considerable loss in hormone activity, as came to light in comparative analysis of our quantitative data.

Tables 2, 3, 4, and 5 compile the results from qualitative HCG determination by means of enzyme immunoassay applied to 60 plasma samples and 30 extracts from the bloodstains of the pregnancy group. Each of these samples was treated at the 2 experimental temperatures and assessed at 3 different stages of the aging process, that is, at 1 week, 3 months, and 6 months.

The results of qualitative HCG assessment of the 60 plasma samples, as shown in Table 2, were positive for the whole pregnancy group and negative for all controls, that is, the 15 samples from adult men and 15 samples from menopausic women.

HCG marker stability in bloodstains from pregnant women was tested by means of qualitative immunoassay in different storing times, up to a maximum of six months, and at different temperatures, with the aim of determining the influence of these factors on hormone stability. Table 3 summarizes the qualitative results. The bloodstain extracts stored for 1 week and 3 months at either temperature, ambient or 56°C, showed positive results of comparable intensity in the 30 samples in each of the 4 lots of stains under study. The last 2 aged for 6 months showed positive results for the 30 samples preserved at ambient temperature, 26 of which (86.6%) showed HCG activity levels which resulted positive in the qualitative test, however, at the sensitivity limit (25-mIU/mL HCG, according to testpack manual).

The ratio between mIU/mL and ng/mL is expressed as: 1-mIU/mL HCG = 0.08-ng/mL HCG [*13*]. In the 30-sample lot aged for 6 months at 56°C, positive results were

TABLE 1—*Results of the quantification of HCG in plasma of 30 pregnant women, 15 menopausic women, 15 men, and extracts of stains from 30 pregnant women.*

	Range of Activity in mIU/mL										
	0	1000	2000	3000	6000	12 000	20 000	30 000	50 000	100 000	150 00
30 Plasmas—pregnancy		2		4			1	19	2	2	
15 Plasmas—menopausic women	15										
15 Plasmas—men	15										
30 Extracts—bloodstains one week old	2	2	2			13	8	2	1		
30 Extracts—bloodstains six months old	27	2			1						

TABLE 2—*Reaction of 60 blood samples in qualitative HCG assessment by enzyme-immunoassay.*

	Results Negative	Results Positive
30 Plasmas—pregnancy	. . .	30
15 Plasmas—adult men	15	. . .
15 Plasmas—menopausic women	15	. . .

TABLE 3—*Reaction of extracts of aged bloodstains from 30 pregnant women.*

Aging	Room Temperature	56°C
One week	30	30
Three months	30	30
Six months	30[a]	29[b]

[a]Twenty-six extracts at sensitivity limit.
[b]Twenty-seven extracts at sensitivity limit.

TABLE 4—*Reaction of extracts of aged bloodstains from 30 pregnant women.*

Dilutions of Extracts = 1/100	Room Temperature	56°C
One week	24	24
Three months	24	24
Six months	1[a]	. . .

[a]One extract at sensitivity limit.

TABLE 5—*Reaction of extracts of aged bloodstains from 30 pregnant women.*

Dilutions of Extracts = 1/200	Room Temperature	56°C
One week	4	4
Three months	3	3
Six months	. . .	. . .

obtained for 29 samples (96.6%), 27 of which (90.0%) achieved positive results in the qualitative test, however, all of them at the sensitivity limit. The negative stain in the qualitative test corresponded to the lowest plasma level of this group, that is, 1640-mIU/mL HCG.

In addition, the absolute sensitivity limit of the test was determined with the bloodstains from pregnant women, starting from the data provided in the kit manual (sensitivity limit 25-mIU/mL HCG or 2-ng/mL HCG) and comparing this data with the data from the quantitative tests performed in our laboratory with the blood samples from which the bloodstains had been prepared.

The extracts obtained from the 30 pregnancy bloodstains were assessed at the afore-

mentioned stages of the aging process, after 1 week, 3 months, and 6 months storage, and each of these at the 2 experimental temperatures. Before HCG detection, all extracts were diluted to 1/100 and 1/200 with bidistilled and deionized water.

As shown in Tables 4 and 5, the samples diluted to 1/100 and stored for a week and 3 months at ambient temperature and 56°C, respectively, yielded 24 positives out of 30 specimens in each lot (80.0%). The negative results corresponded to dilutions of bloodstain extracts whose HCG activity was below 15 000 mIU/mL. Inversely, the only positive result (3.3%) among the extracts at 1/100 dilution from bloodstains stored for 6 months at ambient temperature pertained to a blood sample with an HCG activity of 134 140 mIU/mL, which, in addition, approaches the upper detection threshold of the test. All the samples of the group aged for 6 months at 56°C and 1/100 dilution yielded negatives in qualitative HCG determination.

At 1/200 dilution, laboratory tests showed the following results: four samples (13.3%) were positive in both the ambient temperature and 56°C lots with a storing period of one week. Three positives for the lots aged for three months at both temperatures, and no positives for the six months lost at either temperature. In the one-week lots, the positives had been obtained for samples with a plasma HCG activity above 58 700 mIU/mL, and in the three-month lots only for samples above 65 000-mIU/mL HCG.

In the light of our data it is legitimate to consider the qualitative enzyme immunoassay sufficiently sensitive for the detection of HCG activity in bloodstains from pregnant women, apart from the fact that it has proved to possess a high degree of specificity [*14*].

The sensitivity threshold of the HCG serum testpack is defined as 2-ng/mL HCG in the Abbott manual. Our laboratory tests have, however, demonstrated that the actual sensitivity limit approaches 0.8-ng/mL HCG. Hence, for HCG activity detection with this test, extremely small amounts of whole blood are required. Considering that the normal plasma levels of HCG in pregnant women fluctuate around 80 ng/mL after 40 days of pregnancy [*15*], only 20 μL of whole blood would be needed to produce a functional bloodstain in this test.

HCG activity in the stains aged for up to three months does not appear to be affected, and thus hormone degradation does not occur during this period. In contrast, after six months of aging, considerable deterioration in hormone activity is observed. The effect of the storage temperature, within the experimental range, on HCG stability in cotton cloth bloodstains is practically negligible.

Conclusions

Qualitative HCG determination by means of enzyme immunoassay has proved to be an acceptable method for the detection of this hormone in blood stains. It constitutes a fast, sensitive, and highly specific tool, especially indicated for pregnancy diagnosis in bloodstains and hence of specific interest in forensic science applications.

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