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Effect of temperature on free β-human chorionic gonadotropin and pregnancy-associated plasma protein-A concentration

J. CRUZ*†, G. CRUZ*, R. MINEKAWA*, N. MAIZ*‡ and K. H. NICOLAIDES*†

**Harris Birthright Research Centre for Fetal Medicine, King's College Hospital and †Department of Fetal Medicine, University College Hospital, London, UK and ‡Fetal Medicine Unit, Centro Sanitario Virgen del Pilar, San Sebastian, Spain*

KEYWORDS: first-trimester combined screening; free β-hCG; nuchal translucency; PAPP-A; trisomy 21

ABSTRACT

Objective *To examine the effect of the duration of storage of serum and whole blood at different controlled temperatures on the concentrations of both serum free-β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A) in first-trimester screening for aneuploidies.*

Methods *The concentrations of free β-hCG and PAPP-A were measured in samples collected from 10 pregnant women and stored as whole blood or serum for 1–8 days at 4, 20 or 40°C. The concentrations measured were adjusted to take day-to-day variations into account and were expressed as a percentage of the values on day 0. In a second study involving 10 pregnant women, free β-hCG was measured at 10 min and at 2, 4, 8 and 12 h after collection and storage at 30 or 40°C, either as separated serum or as whole blood.*

Results *The change in the levels of PAPP-A in the separated serum at all three temperatures and in whole blood at 4°C was always less than 10% throughout the 8 days of storage. In whole blood stored at 20 and 40°C, the percentage variation was less than 10% only if the storage period was shorter than 4 days. The concentration of free β-hCG was not altered by storage of either whole blood or separated serum at 4°C throughout the 8 days of storage. At 20°C, reliable results were obtained only if the maximum storage time was 2 days for separated serum and 1 day for whole blood. At 30°C, reliable results were obtained only if the samples were analyzed within 2 h of collection, and at 40°C the concentrations increased by more than 50% within 2 h and by about 500% after 1 day of storage.*

Conclusion *In first-trimester screening for aneuploidies, analysis of blood samples should be undertaken within a few minutes of collection, otherwise the samples should be refrigerated at 4°C throughout the interval between collection and analysis. Copyright © 2010 ISUOG. Published by John Wiley & Sons, Ltd.*

INTRODUCTION

Effective screening for trisomy 21 and other chromosomal defects is carried out at 11–13 weeks of gestation by analysis of a combination of maternal age, maternal serum free-β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A), and the sonographic measurement of fetal nuchal translucency (NT) thickness[1,2]. In some specialist centers the ultrasound scan and biochemical tests are performed in the same patient visit to a one-stop clinic for assessment of risk (OSCAR). Such OSCAR services have become possible by the development of automated biochemical machines that provide reliable results within 40 min of blood collection. However, in the vast majority of patients undergoing first-trimester combined screening, the analysis of blood is performed in laboratories remote from the clinics in which the blood is collected.

Several studies have reported that a high storage temperature and a long interval between collection and analysis of the sample produce an increase in the concentration of free β-hCG because it is liberated by the dissociation or degradation of intact hCG[3–10]. In whole blood kept at room temperature, the mean serum concentration of free-β-hCG was reported to increase by 10–15% after 24 h, by about 25% after 3 days and by 45% after 4 days[3–5]. Another study reported that free β-hCG levels

Correspondence to: Prof. K. H. Nicolaides, Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, Denmark Hill, London SE5 9RS, UK (e-mail: fmf@fetalmedicine.com)

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are stable in whole blood stored at room temperature for 34 h[6]. A study involving incubation of whole blood at 3, 20 and 30°C for 72 h demonstrated that the median serum concentration of free β-hCG did not change if the temperature was 3°C, but increased by 10% after 24 h and by 16% after 72 h if the temperature was 20°C and by 47% and 119%, respectively, after incubation at 30°C[7].

The level of free β-hCG also increases with temperature and incubation time, even in separated serum. Zimmermann *et al.* reported that in separated serum kept at room temperature the mean concentration of free β-hCG increased by 3% after 24 h and by 14% after 48 h[8]. Kardana and Cole showed that the level of free β-hCG did not change significantly during a 4-week period of incubation at 4°C, but with incubation at 21°C there was a 37% increase within 4 days[9]. Similarly, Cowans *et al.* reported that free β-hCG is stable in serum for 94 days at refrigerator temperature, for 3 days at room temperature and for 12 h at 30°C[10].

The aims of this study were, first, to examine the effect of the duration of storage of serum and whole blood at different controlled temperatures on the concentrations of both serum free-β-hCG and PAPP-A in individual samples and, second, to define a policy in the handling of samples for biochemical testing that will produce reliable results.

METHODS

We describe two prospective studies in pregnant women attending for their routine first hospital visit at King's College Hospital. In this visit, which is held at $11+0$ to $13+6$ weeks of gestation, all women have an ultrasound scan to, first, confirm gestational age from the measurement of the fetal crown–rump length (CRL), second, diagnose any major fetal abnormalities and, third, measure fetal NT thickness as part of screening for chromosomal defects[1,2]. In addition, the maternal serum levels of PAPP-A and free β-hCG are measured using an automated machine (Delfia Express system; Perkin Elmer, Waltham, MA, USA) and the results are combined with the fetal NT to calculate the patient-specific risk for trisomy 21. In addition to blood samples obtained to measure the concentrations of free β-hCG and PAPP-A, blood is collected for research. Written informed consent was obtained from the women agreeing to participate in the study, which was approved by King's College Hospital Ethics Committee.

Study 1

A total of 35 mL of blood was collected from each of 10 pregnant women into seven siliconized glass tubes with no additive (BD vacutainer®; Becton, Dickinson, UK Ltd, Oxfordshire, UK) (i.e. 5 mL of blood in each tube). Four of the tubes were centrifuged at 3000 rpm for 10 min and the serum was separated and transferred into four new siliconized glass tubes with no additive. The other three samples were kept as whole blood in the original tubes. The sample in one of these tubes was used to measure the concentrations of PAPP-A and free β-hCG within 20 min of blood sampling (day 0). The samples in the remaining six tubes were used for the experiment, which involved measuring the concentrations of PAPP-A and free β-hCG every 24 h from days 1 to 8 (Figure 1). One set of tubes (i.e. one tube containing whole blood and one tube containing serum) was placed in a fridge at 4°C (blood 4°C and serum 4°C), another set of tubes was placed in a water bath at 20°C (blood 20°C and serum 20°C) and another set of tubes was placed in a water bath at 40°C (blood 40°C and serum 40°C). Every day, an aliquot of 0.2 mL was aspirated from each of the six tubes, placed in a falcon tube and this was placed in the automated machine to measure the concentrations of PAPP-A and free β-hCG. Therefore, for each patient 49 measurements were obtained.

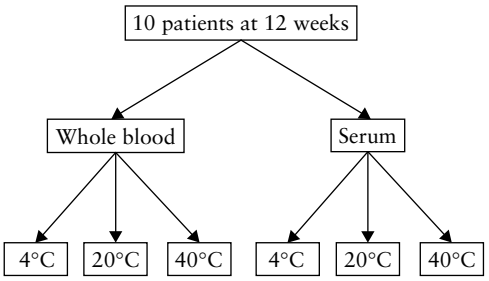

Figure 1 Flow diagram showing the samples collected and experimental conditions in Study 1: whole blood and separated serum were obtained for each patient and stored for 8 days at 4, 20 and 40°C.

The assays and handling of reagents were carried out as recommended by the manufacturers. The whole study was conducted using a single kit lot, and the exact PAPP-A and free β-hCG concentrations for standards/calibrators were given in the lot-specific certificates. A full calibration curve was run in duplicate for the kit lot before the start of the experiment. Subsequently, every day we used three quality controls in duplicate to validate the calibration before analysis of the study samples. Throughout the study period the measured concentrations of the quality control samples was within the acceptable recommended limits and there was no need to recalibrate the machine. The acceptable variation was defined as plus or minus 10% from the given value of the quality control sample values.

Study 2

Study 2 was carried out after the results of Study 1 demonstrated that in the case of β-hCG the percentage difference from day 0 was more than 10% within 1 day of storage at 40°C and within 1–2 days for samples kept at 20°C (see the Results).

Blood was collected from 10 pregnant women, and a similar methodology was used as in Study 1. The serum concentration of free β-hCG was measured at 10 min and 2, 4, 8 and 12 h after collection and storage at 30 or 40°C, either as separated serum or as whole blood. Therefore,

for each patient we had a total of 17 measurements (serum at 10 min; serum after storage at 30°C for 2, 4, 8 and 12 h; serum after storage at 40°C for 2, 4, 8 and 12 h; blood after storage at 30°C for 2, 4, 8 and 12 h; and blood after storage at 40°C for 2, 4, 8 and 12 h).

Statistical analysis

In the analysis of the data from Study 1 the following three steps were taken. First, each of our 49 measurements of PAPP-A and free β-hCG from each of the 10 patients was adjusted to take day-to-day variations into account by multiplication with the ratio of the average of the expected to observed measurements of the three quality control samples (which were provided by the manufacturer and measured on the same day). Second, in each patient the difference in the adjusted measurements of PAPP-A and free β-hCG on days 1–8 from those on day 0 were expressed as a percentage of the values on day 0. Third, the mean and 95% CI of the percentage difference from day 0 for each of the 8 days and three temperatures of storage in the serum and whole blood samples was calculated.

In the analysis of the data from Study 2, the same approach described above was used, except that step 1 was omitted (it was unnecessary because all the samples were analyzed on the same day).

The mean percentage difference of PAPP-A and free β-hCG on the different days (Study 1) and different hours (Study 2) of measurement was compared with the measurement on day 0 by the Mann–Whitney *U*-test with post hoc Bonferroni correction, dividing the critical value for significance (0.05) by the number of tests we conducted, which was eight for Study 1 and four for Study 2.

RESULTS

Study 1

The mean and 95% CI of the percentage difference in the concentrations of serum PAPP-A and free β-hCG from the measurement on day 0 for each of the 8 days and three temperatures of storage in the whole blood and separated serum samples are shown in Figure 2 and Tables 1 and 2.

In the case of PAPP-A in the separated serum, the percentage difference from day 0 was always less than 10% throughout the 8 days of storage at all three temperatures. Similarly, there was a low percentage difference in the whole blood stored at 4°C throughout the study period. However, in the whole blood stored at 20 and 40°C, the percentage variation was less than 10% only if the storage period was less than 4 days.

In the case of free β-hCG, the percentage difference from day 0 was less than 10% throughout the 8 days of storage in both the separated serum and whole blood kept at 4°C. In the samples kept at 20°C, reliable measurements were obtained only if the maximum storage was 2 days in the case of separated serum and 1 day for whole blood. In both the separated serum and whole blood kept at 40°C, the concentrations increased by about 500% after 1 day of storage and by about 1000% at day 3.

Study 2

The percentage differences in concentration of free β-hCG in serum at 2, 4, 8 and 12 h relative to the measurement at 10 min after collection, in whole blood and separated serum samples stored at 30°C and 40°C, are shown in Figure 3 and Table 3. The mean percentage difference was

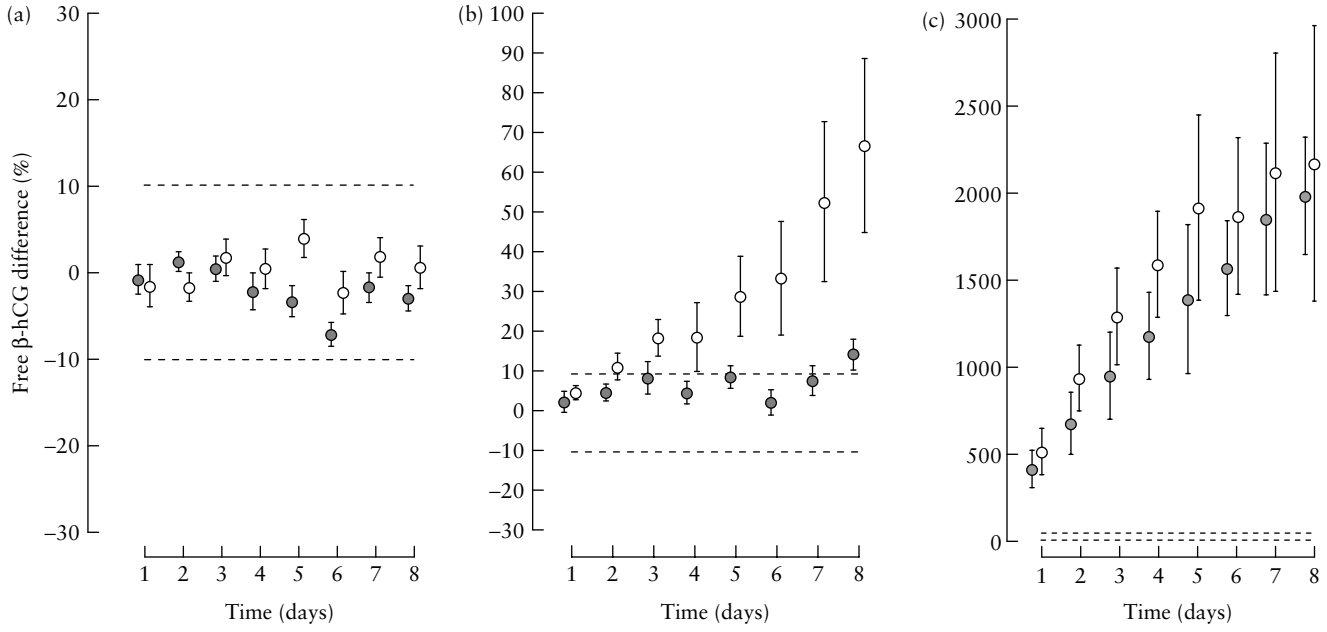

Figure 2 Mean and 95% CIs of percentage difference between serum free β-human chorionic gonadotropin (β-hCG) concentration measured on day 0 and that measured on each of the 8 days and three temperatures (4°C (a), 20°C (b) and 40°C (c)) of storage in whole blood (○) and separated serum (●) in Study 1. Horizontal dashed lines at −10% and 10% represent the acceptable limits of variation in analyte concentrations.

Table 1 Percentage difference between serum pregnancy-associated plasma protein-A (PAPP-A) concentration measured on day 0 and on each of the 8 test days at three storage temperatures in whole blood and separated serum samples

	PAPP-A percentage difference (mean (95% CI))		
	4°C	*20°C*	*40°C*
Separated serum			
1 day	0.7 (−0.5 to 1.8)	0.9 (−0.6 to 2.5)	0.5 (−1.4 to 2.4)
2 days	0.5 (−1.7 to 2.7)	3.2 (1.3 to 5.0)*	5.9 (4.4 to 7.4)*
3 days	−0.5 (−1.7 to 0.6)	2.7 (0.9 to 4.4)	1.8 (0.1 to 3.5)
4 days	2.9 (1.1 to 4.7)*	3.8 (1.7 to 6.0)*	2.4 (0.7 to 4.1)*
5 days	−0.7 (−2.1 to 0.6)	2.5 (1.2 to 3.7)*	3.2 (1.3 to 5.1)*
6 days	−1.4 (−3.7 to 0.9)	−2.8 (−4.6 to −1.0)*	−2.2 (−3.9 to −0.5)
7 days	−1.5 (−4.1 to 1.1)	0.6 (−1.5 to 2.7)	0.9 (−1.0 to 2.8)
8 days	−0.7 (−2.6 to 1.2)	−0.9 (−2.8 to 1.0)	−2.1 (−4.2 to 0.0)
Whole blood			
1 day	0.9 (−0.8 to 2.5)	4.2 (2.4 to 6.0)*	4.1 (1.7 to 6.5)*
2 days	1.0 (0.2 to 1.8)	5.5 (4.5 to 6.6)*	−2.1 (−4.4 to 0.3)
3 days	1.8 (0.7 to 2.8)*	7.4 (5.7 to 9.1)*	−4.2 (−6.5 to −1.9)*
4 days	4.4 (2.6 to 6.3)*	9.7 (7.3 to 12.2)*	−1.7 (−4.5 to 1.2)
5 days	4.1 (3.3 to 4.9)*	10.3 (7.9 to 12.7)*	−2.4 (−4.7 to 0.0)*
6 days	3.8 (1.5 to 6.1)*	3.2 (−0.3 to 6.7)	−7.4 (−11.8 to −2.9)*
7 days	2.4 (0.2 to 4.7)	4.7 (0.8 to 8.6)	−11.7 (−15.9 to −7.5)*
8 days	1.2 (−0.4 to 2.9)	3.1 (−0.4 to 6.5)	−15.4 (−24.4 to −6.3)*

*Statistically significant difference at the level of $P < 0.00625$; Mann–Whitney *U*-test with post-hoc Bonferroni correction.

Table 2 Percentage difference between serum free-β-human chorionic gonadotropin (β-hCG) concentration measured on day 0 and on each of the 8 days at three storage temperatures in whole blood and separated serum samples

	β-hCG percentage difference (mean (95% CI))		
	4°C	*20°C*	*40°C*
Separated serum			
1 day	−0.9 (−2.6 to 0.9)	2.8 (0.3 to 5.3)	415.5 (308.8 to 522.3)*
2 days	1.2 (0.1 to 2.3)*	5.2 (2.9 to 7.4)*	677.9 (500.3 to 855.5)*
3 days	0.4 (−1.1 to 1.8)	8.8 (4.9 to 12.7)*	951.4 (701.5 to 1201.3)*
4 days	−2.2 (−4.2 to −0.2)	5.1 (2.3 to 7.9)*	1180.1 (930.6 to 1429.5)*
5 days	−3.4 (−5.1 to −1.6)	8.9 (6.3 to 11.7)*	1390.8 (963.7 to 1818.0)*
6 days	−7.2 (−8.6 to −5.8)*	2.7 (−0.3 to 5.7)	1568.7 (1296.0 to 1841.3)*
7 days	−1.8 (−3.5 to −0.1)	8.1 (4.5 to 11.7)*	1851.3 (1415.6 to 2287.0)*
8 days	−3.0 (−4.4 to −1.6)	14.7 (10.8 to 18.6)*	1984.3 (1647.5 to 2321.0)*
Whole blood			
1 day	−1.6 (−4.1 to 0.9)	5.2 (3.5 to 6.8)*	515.8 (381.6 to 649.9)*
2 days	−1.7 (−3.3 to −0.2)	11.5 (8.2 to 14.8)*	938.3 (748.9 to 1127.6)*
3 days	1.7 (−0.4 to 3.7)	18.8 (14.1 to 23.4)*	1291.4 (1014.3 to 1568.4)*
4 days	0.4 (−1.8 to 2.6)	19.0 (10.6 to 27.5)*	1591.0 (1287.0 to 1895.0)*
5 days	3.8 (1.6 to 6.1)*	29.3 (19.3 to 39.2)*	1917.0 (1385.4 to 2449.4)*
6 days	−2.4 (−4.8 to 0.1)	33.7 (19.6 to 47.8)*	1868.1 (1418.3 to 2318.0)*
7 days	1.7 (−0.6 to 4.0)	52.8 (32.9 to 72.8)*	2119.8 (1436.3 to 2803.2)*
8 days	0.5 (−1.9 to 3.0)	66.8 (45.2 to 88.5)*	2170.8 (1379.9 to 2961.6)*

*Statistically significant difference at the level of $P < 0.00625$; Mann–Whitney *U*-test with post-hoc Bonferroni correction.

less than 10% only for the samples analyzed within 2 h of collection after storage at 30°C. In both the separated serum and whole blood kept at 40°C, the concentrations increased by more than 50% within 2 h of storage.

DISCUSSION

The findings of this study demonstrate that the maternal serum concentration of free β-hCG and PAPP-A is not altered by storage, at 4°C, of either whole blood or separated serum, even after at least 1 week. By contrast, storage at 20–40°C was associated with a substantial elevation in the concentration of free β-hCG in serum, which increased with both the duration and temperature of storage. At 20°C, reliable results were obtained only if the maximum storage period was 2 days for separated serum and 1 day for whole blood. At 30°C reliable results were obtained only if the samples were analyzed within 2 h of collection, and at 40°C the measurements increased by more than 50% within 2 h.

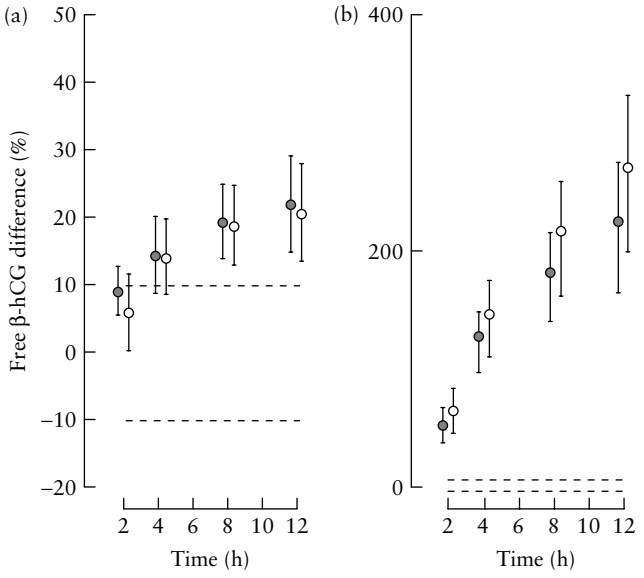

Figure 3 Mean and 95% CIs of percentage difference relative to serum free β-human chorionic gonadotropin (β-hCG) concentration measured at 2, 4, 8 and 12 h relative to that measured 10 min after collection, in whole blood (○) and separated serum (●), after storage at 30°C (a) and 40°C (b), in Study 2. Horizontal dashed lines at −10% and 10% represent the acceptable limits of variation in analyte concentrations.

Table 3 Percentage difference between serum free β-human chorionic gonadotropin (β-hCG) concentration measured at 2, 4, 8 and 12 h and that measured 10 min after collection, in whole blood and separated serum samples stored at 30°C and 40°C

	β-hCG percentage difference (mean (95% CI))	
	30°C	*40°C*
Whole blood		
2 h	5.7 (0.5 to 10.9)	64.4 (45.9 to 82.9)*
4 h	14.3 (8.7 to 19.9)*	143.2 (110.8 to 175.6)*
8 h	18.9 (13.0 to 24.8)*	212.2 (163.5 to 260.9)*
12 h	20.8 (13.6 to 27.9)*	266.0 (199.7 to 332.3)*
Separated serum		
2 h	9.3 (5.7 to 12.9)*	53.2 (39.0 to 67.3)*
4 h	14.6 (9.0 to 20.2)*	123.2 (96.5 to 149.9)*
8 h	19.5 (14.0 to 25.1)*	178.4 (139.9 to 217.0)*
12 h	22.1 (14.9 to 29.2)*	220.2 (164.7 to 275.6)*

*Statistically significant difference at the level of $P < 0.0125$; Mann–Whitney *U*-test with post-hoc Bonferroni correction.

In the case of PAPP-A, reliable measurements were obtained in separated serum, even after storage at 20–40°C for 8 days, whereas with whole blood the results were reliable if the period of storage was less than 4 days, presumably because of the extensive hemolysis observed in such samples. A recent study reported that PAPP-A levels are stable in serum for 142 days at refrigerator temperature, for 37 days at room temperature and for 20 days at 30°C[10]. The study also examined whole blood for 3 days and showed that the level of PAPP-A was stable during this period of storage at all three temperatures.

The findings on free β-hCG are in general agreement with those of previous studies that reported an increase in levels of β-hCG with time in samples kept at 'room' temperature. The reported average daily increases in free β-hCG were 3.8–14% for whole blood and 7% for separated serum[3,6,8,11]. As shown by our studies, in addition to the effect of the interval between sample collection and analysis there is a substantial influence of the exact temperature at storage and there are major differences in these effects on individual samples. Consequently, it would not be valid to correct for such storage time- and temperature-related effects by simple mathematical adjustments based on the average time-related effects of 'room' temperature. In the context of sample collection and transportation in different countries and seasons, the concept of 'room' temperature is meaningless and a policy of storing samples at such a temperature, even if they are separated, is unsatisfactory and likely to produce erroneous results.

The results have major implications on the implementation of first-trimester combined screening for trisomy 21 and other major aneuploidies. In the case of OSCAR services, analysis of blood samples is undertaken within a few minutes of collection and therefore reliable results are obtained irrespective of the room temperature. However, in most screening units the blood is collected either before, or at the time of the ultrasound scan and are sent to a different location for biochemical testing. Our findings indicate that such practice can produce reliable results only if the whole blood or separated serum samples are refrigerated at 4°C throughout the interval between collection and analysis. Recommendations cannot be made on the basis of the concept that there is such a thing as a 'room temperature', which is universally applicable to all countries, in all seasons, at all times and within each room of all hospitals, as well as in all transport vehicles. The application of alternative policies, such as the use of dried whole-blood spots on filter paper because in this medium free-β-hCG is stable for several days[6], requires rigorous investigation, as applied in the present study.

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