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Original

Hematocrit: an uncontrollable variable in newborn screening?

El hematocrito: ¿una variable incontrolable en el cribado neonatal?

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ABSTRACT

Introduction: dried blood spots (dbs) are valuable samples for the newborn screening laboratory. Cut-off values for neonatal screening determinations are based on an average hematocrit value (55 %). Newborns that exceed the cut-off value of the determinations are cited again for a confirmatory sample collection.

Objective: to develop a laboratory technique that allows knowing the value of the hematocrit in the dbs samples of neonatal screening and to study the impact of the hematocrit variable on the results of neonatal screening.

Material and methods: 1124 samples from two neonatal screening laboratories were analyzed. The hematocrit in the DBS samples was estimated using the sodium lauryl sulfate reagent (1.7 g/L). Thyrotropin (TSH), immunoreactive trypsin (IRT), and 17-hydroxyprogesterone (17-OHP) were determined. The results were evaluated with and without hematocrit correction taking into account the estimated value.

This work was presented at the Congress of the Latin American Society of Inborn Errors of Metabolism and Neonatal Screening 2019, May 12 to 15 in Buenos Aires (Argentina), winner as Best Oral Communication of Neonatal Screening.

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Results: 183 samples (without correction) exceeded the cut off value proposed by each laboratory: 49 for TSH, 44 for IRT, and 90 for 17-OHP. Using the hematocrit estimation, 18 (36.7 %) newborns exceeded the TSH cut-off value, 25 (56.7 %) in the IRT measurement and 39 (43.3 %) in 17-OHP determination. If the correction of the packed red cell had been used in the DBS samples, only 44.8% of the 183 newborns would have had to be cited again.

Conclusion: the estimation of the hematocrit would allow the correction of the volume in the DBS samples. We suggest the use of hematocrit estimation for samples that exceed the cut-off value. By lowering the percentage of requests for new samples we would avoid the anguish that it causes in the family.

Palabras clave:
Hematocrito. Cribado neonatal. Valor de corte. Tirotropina. Tripsina inmunorreactiva. 17-hidroxiprogesterona.

RESUMEN

Introducción: las gotas de sangre en papel de filtro (GSPF) son muestras valiosas para el laboratorio de cribado neonatal. Los valores de corte para las determinaciones del cribado neonatal están basados en un valor de hematocrito promedio (55 %). Los recién nacidos que superan el valor de corte de las determinaciones son citados de nuevo para una toma de muestra confirmatoria.

Objetivo: desarrollar una técnica de laboratorio que permita conocer el valor del hematocrito en la GSPF del cribado neonatal. Estudiar el impacto de la variable del hematocrito en los resultados del cribado neonatal.

Material y métodos: se analizaron 1124 muestras de dos laboratorios de cribado neonatal. El hematocrito en las GSPF se estimó utilizando el reactivo lauril sulfato de sodio (1,7 g/L). Se realizaron determinaciones de tirotropina (TSH), tripsina inmunorreactiva (IRT) y 17-hidroxiprogesterona (17-OHP). Los resultados fueron evaluados con y sin corrección del hematocrito teniendo en cuenta el valor estimado.

Resultados: 183 muestras (sin corrección) superaron el valor límite propuesto por cada laboratorio: 49 para TSH, 44 para IRT y 90 para 17-OHP. Utilizando la estimación del hematocrito, 18 (36,7 %) recién nacidos superaron el valor de corte de la TSH, 25 (56,7 %) en la medición de IRT y 39 (43,3 %) con 17-OHP. Si se hubiera utilizado la corrección del paquete globular en las GSPF, solo hubieran tenido que ser citados de nuevo el 44,8 % de los 183 recién nacidos.

Conclusión: la estimación del hematocrito permitiría la corrección del volumen en la muestra GSPF, que es muy sencilla de calcular. Sugerimos el uso de la estimación del hematocrito para muestras que excedan el valor de corte. Al bajar el porcentaje de solicitudes de nuevas muestras evitaríamos la angustia que causa a la familia.

INTRODUCTION

Newborn screening (NBS) for congenital diseases began in the 1960s when microbiologist Robert Guthrie and biochemist Louis Woolf developed a simple and sensitive test for the detection of phenylketonuria. In 1968, Wilson and Jungner published criteria for evaluating screening programs, stating that they should be "an ongoing process and not a one-time test" (1).

NBS allows for the diagnosis of diseases that in their natural course would be life-threatening and/or compromise the intellectual development of affected infants, with a high social and psychological cost for the patients, their families, and the society. These diseases account for a major economic burden due to the increased use of social and healthcare resources (2,3). Although incidence of each of these disorders is low, their impact on public health is substantial. NBS

programs are preventive and essential for healthcare systems; however, the implementation strategies vary in different countries (4). Adequate detection with timely intervention reduces disease-associated morbidity, mortality, and disability. NBS is low cost and greatly reduces the economic burden related to these conditions for both families and healthcare systems (2).

Detection times are important for confirmatory testing and early treatment initiation in identified cases, before symptoms of the disease appear or irreparable damage is done (2-4).

In Argentina, testing for phenylketonuria, congenital hypothyroidism, cystic fibrosis, galactosemia, congenital adrenal hyperplasia, biotinidase deficiency, retinopathy of prematurity, Chagas disease, and syphilis, followed by treatment in positive cases as well as monitoring of these tests is mandatory in all public, state-run, social security, and private institutions in the country where deliveries take place and/or newborns are seen (National Law 26279/2007) (5,6).

The dried blood spot (DBS) sample used for NS has many advantages: minimal volume, favorable stability of numerous analytes, reduced costs associated with shipping and storage, and is less invasive than venous sample collection (7,8. Nevertheless, the concentration of red blood cells per unit volume of blood influences DBS analysis (7,9). One of the main sources of error is the packed red blood cells-to-plasma ratio. This variable could be determined by estimating the hematocrit level (through the measurement of hemoglobin). When blood is applied to the filter paper, it will diffuse at different rates depending on the sampling technique and variables related to the newborn and the filter paper, affecting the size of the blood spot. By using a fixed punch size for sample analysis, the packed red blood cells could affect the results of the different analytes used for newborn screening. The hematocrit behaves as a variable in the analysis of the results (10). The normal hematocrit range for a healthy newborn is from 42 % to 64 %. Abnormal levels may be characteristic of polycythemia or anemias, which cause an extremely high and low hematocrit, respectively (11,12). In some cases, volume correction could mean the difference between a positive and negative result (7,11). Since the cut-off points are established independently of the hematocrit value, not knowing the hematocrit level may lead to a false positive results. Therefore, determining the hematocrit level may improve the positive predictive value of this test (11).

The aims of our study were to develop a laboratory technique that allows us to determine the hematocrit value in the DBS of the NBS, to assess the effect of the variable hematocrit on the results of thyrotropin (TSH), immunoreactive trypsinogen (IRT) and 17-Hydroxyprogesterone (17OHP), and to analyze its impact on the results.

MATERIAL AND METHODS

Study design and samples used

A retrospective, cross-sectional study was conducted. The samples were used once the mandatory NBS was completed, between May 3, 2018 and January 29, 2019. A total of 1124 samples from preterm and term newborns collected by two NBS laboratories were analyzed. Gestational age of hospitalized and non-hospitalized infants ranged from 24 to 41 weeks. Sampling for term newborns was performed between the 2nd and 5th day of life with at least 24 hours of protein feeding (breast milk or formula milk), before the mother and newborn were discharged. In the case of premature infants, the sampling was repeated at 15 days or when they reached the age of 37 weeks. The samples were collected in the hospital or maternity ward and once dry, they were stored at 4 °C until the time of testing. Samples with blood spots with diameters less than 10 mm, poor quality specimens, or incomplete card data were excluded.

The sample had to be accompanied by a complete information card to be properly identified. The data from the newborn were necessary for the correct interpretation of the results, while those from the mother and the responsible physician allowed rapid communication of the results (2).

In case of an inadequate sample, doubtful results, or results above the established cut-off points, a new sample was requested and the analysis was repeated, which was considered urgent in all cases (2).

On the other hand, a complete blood count was performed in 114 children, by medical request, on the same day as the DBS collection. Hematocrit values from the complete blood counts were used to perform a linear regression analysis to validate the method of hematocrit estimation in DBS.

Ten DBS samples from adult patients (hematocrit range, 29.2 % to 42.0 %) were included in the study and were analyzed on 5 consecutive days to evaluate repeatability.

Hematocrit estimation

A simple technique, available at any NBS laboratory, was used. The procedure was based on measuring hemoglobin (Hb) from a DBS sample and subsequent estimation of the percent hematocrit.

A 6-point standard curve was developed with known hemoglobin concentrations using samples of patients who underwent the same procedure as the study tests. Hb was measured in triplicate in calibrators using a Sysmex XE-2100 autoanalyzer (Kobe, Japan). The calibrators were dispensed on S&S#903 Whatman cards (13) with venous whole blood collected in EDTA-K3 tubes (Beckton Dickinson plastic vacutainer tube lilac capped with 5.4 mg EDTA K3 without separator,

Oxford, UK) and then allowed to dry at room temperature. The curve was used in each series to estimate the hematocrit value of samples and controls. Hb and hematocrit values from the analyzer were considered as the true value and used as a reference for the curve and controls. The curve consisted of a blank (card without blood) and the following hemoglobin points: 4.3 g/dL; 10.1 g/dL; 15.4 g/dL; 21.4 g/dL, and 26.9 g/dL (hematocrit: 12.8 %; 29.7 %; 45.4 %; 64.2 %, and 80.7 %, respectively). The same procedure was performed with 3 control levels - low, medium, and high - with hematocrit levels of 17.4 %, 40.7 %, and 60.7 % (hemoglobin 5.7 g/dL; 13.5 g/dL; 20 g/dL), respectively, also from patient samples.

A 3.2 mm punch (disk) of each of the samples, standards and controls, was placed in a 96-well V-bottom reaction plate. Both standard and control samples were added in duplicate. Subsequently, 200 µL of 1.7 g/L sodium lauryl sulfate (SLS) was added to each well and incubated at room temperature for 1 hour. The supernatant was transferred to a reading plate and incubated for 10 minutes, avoiding bubbles that could affect the reading. Absorbance was measured using a Biotek Elx800 plate reader (Vermont, United States) at a wavelength of 550 nm and plotted on the curve to determine the hemoglobin concentration. This colorimetric method (spectrophotometry) described by Richardson et al. (12) forms an SLS-Hb complex with an optical absorbance between 500 and 560 nm. The absorbance was linear in the expected concentration range in the patient samples, obeying the Beer-Lambert law (11,12). The readings were stable on the plate at room temperature and protected from light for at least 12 hours.

From the calculated hemoglobin data, the hematocrit concentration was obtained using a factor of 3.0 (hematocrit/hemoglobin ratio from the measurement of the hemogram used to make the curve) based on which the value of the packed red blood cells of each DBS could be determined. The factor ratio will depend on the analyte used and should be calculated by creating a new curve (Fig. 1).

Measurement of thyrotroponin, immunoreactive trypsinogen, and 17-hydroxyprogesterone

NBS measurements were performed according to the technique used at each laboratory. In the first laboratory, after extraction was performed using Diluent Universal Roche (Mannheim, Germany), TSH levels were determined by electrochemiluminescence using a COBAS 6000 Roche (Rotkreuz, Switzerland). IRT levels were measured with reagents (MP Biomedicals, California, United States) and read in a Biotek Elx800 spectrophotometer (Vermont, United States). 17OHP was determined using a coated tube radioimmunoassay (MP Biomedicals) with a Wallac AN-LKB Dream Gamma-10 reader (Mount Waverley, Australia). In the second NBS laboratory, the DELFIA Neonatal 17 alpha-OH-Progesterone kit - Perkin Elmer (Turku, Finland) was used for 17OHP, the DELFIA Neonatal IRT kit - Perkin Elmer for IRT, and the DELFIA Neonatal hTSH kit -

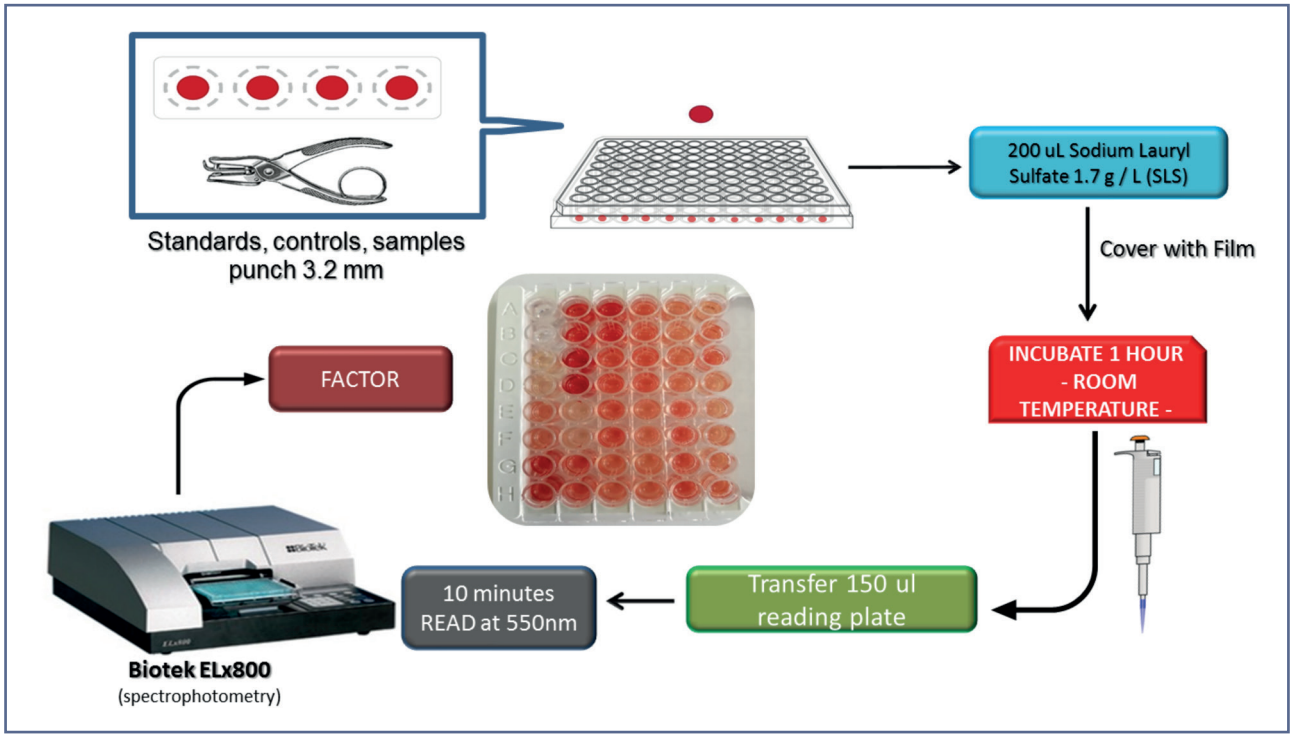

Figure 1 – *Schematic diagram of the hematocrit estimation technique.*

Perkin Elmer for TSH, while a Victor 2D 1420 Multilaber Counter - Perkin Elmer reader was used for all analytes.

Statistical analysis

Data analysis was performed using the IBM SPSS v22 statistical program (New York, United States). The statistical methods used were the Kolmogorov-Smirnov test, linear regression with Pearson correlation, Spearman correlation, Deming regression, and Bland-Altman analysis. To assess repeatability, the coefficient of variation (CV) and standard deviation (SD) were determined. Results were evaluated with and without hematocrit correction. The independent samples t-test were performed. The t-test was used to compare means and bilateral significance. In addition, the relationship between the value obtained and the cut-off value was analyzed for each measurement.

RESULTS

General characteristics of the newborns

Of the total number of newborns (1,124), 79.5% (893) were term, between 37 and 41 weeks of gestation and 20.5% were either preterm (36-28 weeks) or immature (< 27 weeks).

Mean birth weight was 3083 g (range, 620-4830 g). Overall, 81.5% were adequate weight (> 2500 g), 17.3% low weight (< 2,500-1,000 g), and 1.2% extremely low weight (< 1,000 g).

Correlation between both variables - birth weight and weeks of gestation - showed that 866 (77%) newborns were born full term with an adequate birth weight.

Validation of the hematocrit measurement method

Linear regression analysis was performed on the hematocrit results in DBS compared to the hematocrit from the hemogram, both obtained on the same day (n = 114); the independent variable (x) was hematocrit concentration determined in the whole blood sample (hemogram) and the dependent variable (y) was the result obtained in DBS. Mean hematocrit was 39.6 % (median 38.4 %; range, 19.8 % to 61.0 %) in the DBS and 40.7 % (median 39.3 %; range, 22.1 % to 61.0 %) in the hemogram. The Kolmogorov-Smirnov test showed that both had a normal distribution. Pearson's correlation coefficient was 0.957 showing a linear association between both methods ($p < 0.001$). The Deming regression coefficient was 0.989, the slope 1.006 (95 % CI, 0.957 to 1.056), and the ordinate at the origin 0.803 (95 % CI, -1.292 to 2.898) demonstrating the interchangeability of the two methods. Mean CV of the results was 4.4 % with a SD of 1.7 %. The Bland-Altman plot, allowing for analysis of the difference between a new method and an established one, identified a bias of -1.06, showing that the new method was reliable and reproducible compared to the reference method (95 % CI, 4.46 to -6.57) (Fig. 2).

In the study of the 10 DBS samples to evaluate repeatability, a mean CV of 3.4% and a SD of 1.2% were obtained (Table I).

Effect of correction on newborn screening measurements

Considering the hematocrit estimation in the DBS samples, a consensus hematocrit value of 55% was used. Based on this value, a correction factor was applied to the NBS results. The new result was then compared

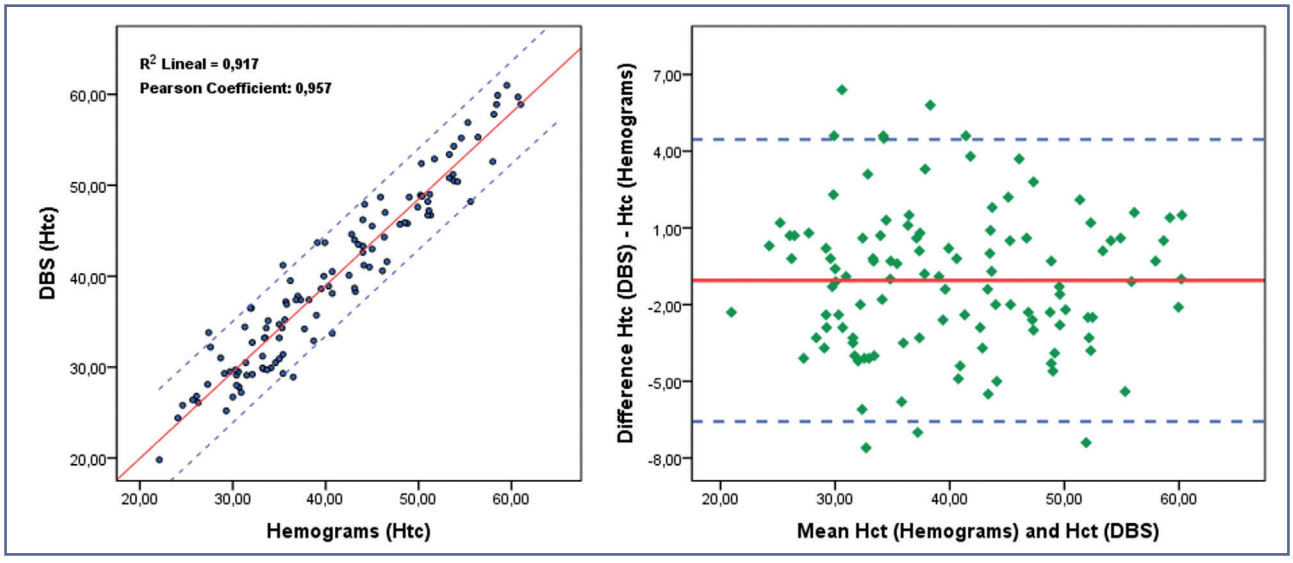

Figure 2 – *Comparison of hematocrit measurement in hemogram and blood spots on filter paper using linear regression and Bland-Altman analysis. DBS: Dry blood spots on filter paper; Hct: hematocrit.*

Table I.
Repeatability analysis of the method for the determination of hematocrit in adult patient samples

Patient	Hemogram (% hematocrit)	DBS hematocrit (%) and days of repetitions					CV (%)	SD (%)	Mean
		Day 1	Day 2	Day 3	Day 4	Day 5			
1	40,9	38,9	42,2	40,4	41,7	40,5	3,17	1,29	40,7
2	39,6	40,6	39,9	39,3	39,6	41,8	2,51	1,01	40,3
3	31,9	28,6	31,4	29,7	29,9	28,1	4,28	1,26	29,6
4	39,6	39,3	36,9	39,4	40,3	40,7	3,77	1,48	39,3
5	42,0	39,1	41,8	39,7	38,9	43,5	4,90	1,99	40,6
6	30,2	28,3	29,4	28,6	28,9	29,8	2,12	0,62	29,0
7	29,2	28,7	28,8	26,9	29,2	28,4	3,12	0,89	28,4
8	32,3	32,3	30,9	30,8	29,8	30,7	2,91	0,90	30,9
9	34,9	35,0	33,6	34,1	32,0	33,5	3,29	1,11	33,7
10	41,1	40,7	39,9	38,6	42,9	39,8	3,95	1,60	40,4

DBS: dry blood spots on filter paper; CV: coefficient of variation; SD: standard deviation.

to the cut-off value (TSH: COBAS Roche: 12 µUI/ml – Perkin Elmer: 8 µUI/ml; 17OHP: MP Biomedicals: 22 ng/ml – Perkin Elmer: 23 nmoles/l; IRT: MP Biomedicals: 130 ng/ml – Perkin Elmer: 60 ng/ml) and its impact as well as the action to be taken by laboratory were evaluated (Table II).

Of the 1124 samples analyzed without hematocrit correction, 183 patients exceeded the cut-off value in the original analysis of any of the 3 analytes tested: 49 patients for TSH, 90 for 17OHP, and 44 for IRT. The results of the confirmatory tests were abnormal in three patients (2 TSH and 1 IRT).

Thyrotropin

The ratio between the value obtained and cut-off value (O/C) allows us to compare the different methods and cut-off values, and determine the positivity ratio. In 49 samples, TSH was above the cut-off value with an O/C ratio of 1.14. When the samples were reevaluated applying the hematocrit correction factor, 63.3 % of these cases were negative.

The median hematocrit value was 48.2 % (O/C ratio 1.08) in positive and 45.0 % (O/C ratio: 0.89) in negative cases. In two cases, patients with normal values of 7.8 µIU/ml and 7.9 µIU/ml (cut-off value 8 µIU/ml) were reclassified, as they exceeded the cut-off with results of 8.0 µIU/ml and 8.4 µIU/ml when the correction factor was applied (hematocrit 56 % and 59 %, respectively) (Table III).

17-OH-progesterone

Ninety samples with 17OH above the cut-off value and an O/C ratio of 1.19 were detected. When the sam-

Table II.
Application of the hematocric correction factor

Hematocrit in DBS	Factor	TSH (µUI/ml)		IRT (ng/ml)		17 OHP (ng/ml)	
		Without correction	With correction	Without correction	With correction	Without correction	With correction
12,1 %	0,22	28,0	6,2	52,0	11,4	74,8	16,5
29,5 %	0,54	12,0	6,4	50,4	27,0	28,8	15,4
54,1 %	0,98	11,2	11,0	33,0	32,4	9,3	9,1
66,1 %	1,20	4,9	5,9	21,4	25,7	10,3	12,4

DBS: Dry blood spots on filter paper; TSH, thyrotropin; IRT, immunoreactive trypsinogen; 17OHP, 17-hydroxyprogesterone.

ples were reevaluated applying the hematocrit correction factor, 56.7% of the cases were negative.

The median hematocrit value was 49.7 % (O/C ratio 1.16) in positive cases and 41.7 % (O/C ratio: 0.88) in negative cases. Three patients with normal values of 21.5 ng/ml, 21.8 ng/ml, and 21.2 ng/ml (cut-off value: 22 ng/ml) were reclassified and exceeded the cut-off value with results of 22.0 ng/ml, 23.6 ng/ml, and 22.3 ng/ml when the hematocrit correction factor was applied (hematocrit: 56 %, 59 %, and 58 %, respectively) (Table III).

Immunoreactive trypsinogen

Forty-four samples with IRT above the cut-off value with an O/C ratio of 1.46 were detected. When the samples were reevaluated applying the hematocrit correction factor, 43.2 % of the cases showed to be negative.

It should be remembered that this marker is an acute phase reactant and several of the patients were hospitalized for various causes. Median hematocrit value was 45.8% (O/C ratio 1.39) in positive and 35.6 % (O/C ratio: 0.88) in negative cases. No normal results were reclassified (Table III).

Overall newborn screening results

Of the 183 patients with levels above the cut-off value without hematocrit correction, 82 continued to exceed the cut-off value when the packed red blood cell correction was applied. These patients had a mean hematocrit of 50.3 %, while the remaining 101 patients who obtained normal values had a mean hematocrit of 39.8 % (Table IV).

In addition, five patients were reclassified as positive. In all cases the results were doubtful, and very close

Table III.
Number of patients who had to be recalled after application of the correction factor

	Without correction		With correction					
	Patients recalled without correction	O/C without correction	Patients recalled with correction	O/C with correction	Patients NOT recalled with correction	O/C with correction	t	p-value
TSH	49	1,14	18	1,08	31	0,89	2,596	0,012
IRT	44	1,46	25	1,39	19	0,88	3,778	< 0,001
17OH	90	1,19	39	1,16	51	0,88	6,653	< 0,001

O/C: Value obtained/Cut-off value; t: t-test for equality of means; TSH, thyrotropin; IRT, immunoreactive trypsinogen; 17OHP, 17-hydroxyprogesterone.

Table IV.
Number of samples exceeding the cut-off point according to the test used, the analyte, and the application of the hematocrit correction factor

	Brand	Without Hct	With Hct	%
TSH	MP Biomedicals	23	7	30,4
	Perkin Elmer	26	11	42,3
	Subtotal	49	18	36,7
IRT	MP Biomedicals	3	1	33,3
	Perkin Elmer	41	24	58,5
	Subtotal	44	25	56,8
17OH	MP Biomedicals	76	34	44,7
	Perkin Elmer	14	5	35,7
	Subtotal	90	39	43,3
Total		183	82	44,8

Hct, hematocrit; TSH, thyrotropin; IRT, immunoreactive trypsinogen; 17OHP, 17-hydroxyprogesterone.

to the cut-off values, which were exceeded when hematocrit correction was considered. There were 47.5 % (87) requests for a new sample compared to 183 based on the initial data, accounting for a decrease in requests of 52.5% and a total of 96 patients less.

CONCLUSION

Hematocrit estimation allows for correction of the volume of the packed red blood cells in the DBS sample. Knowing the value of this variable the differences in the newborn screening analytes, susceptible to the hematocrit effect, can be normalized (14,15).

Here we describe, analyze, and validate a simple and rapid method to measure hemoglobin and estimate hematocrit in a DBS sample, using equipment that is normally found in the NBS laboratory. Although the method is destructive, it uses a small fragment of the sample, leaving the remainder for other necessary analyses. As more analytes are continuously included in NBS programs, hematocrit should not be routinely measured.

Although false positives are common in NBS due to the attempt to minimize false negative results, the former cause unnecessary distress in the parents of the babies that are recalled (16-18). Moreover, performing new tests in these patients prolongs the time to accurate diagnosis, which is crucial for early treatment initiation to avoid the morbidity and mortality associated with these diseases (19).

The main limitation of our study is the small number of samples processed. Due to the low prevalence of these disorders, further research is needed to confirm the behavior of samples with abnormal or doubtful results when hematocrit is corrected. In addition, a study with larger numbers of samples should be conducted in preterm and low-birth-weight patients, in order to evaluate NBS samples (these are valuable and very scarce samples) in the population that requires hematocrit correction. Another limitation is the possibility of analytical interference in the measurement of the SLS-Hb complex, since we did not know if our patients had lipemia or jaundice, conditions that may have affected the results of this colorimetric technique.

Our results show that hematocrit estimation, which is very easy to calculate, would allow for volume correction in the DBS sample. We suggest the use of hematocrit correction for samples exceeding the cut-off value, those that have doubtful results, and in those of premature and/or low-birth-weight patients.

In conclusion, our study shows requests for a second sample for disorders included in NBS may be reduced by incorporating a variable that is not commonly used in the laboratory. Hematocrit correction using SLS is a simple method with an encouraging technical performance that may be used to reduce the number of false positive results.

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