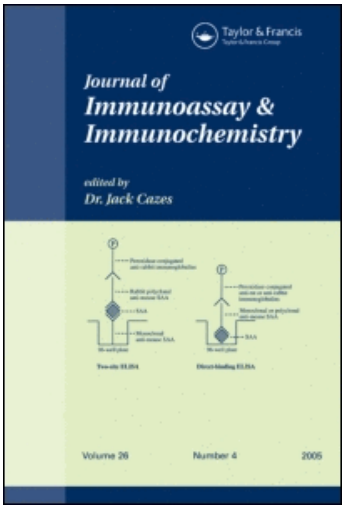

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Development of an Indirect Enzyme Immunoassay using Monoclonal Antibodies for the Measurement of 17α-Hydroxyprogesterone in Human Serum

Heilly Chong[a]; Swee Hung Cheah[a]; M. Ragavan[a]; V. T. Johgalingam[a]
[a] Monoclonal Laboratory, Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

To cite this Article Chong, Heilly , Cheah, Swee Hung , Ragavan, M. and Johgalingam, V. T.(2009) 'Development of an Indirect Enzyme Immunoassay using Monoclonal Antibodies for the Measurement of 17α-Hydroxyprogesterone in Human Serum', Journal of Immunoassay and Immunochemistry, 30: 2, 166 — 179
To link to this Article: DOI: 10.1080/15321810902782863
URL: http://dx.doi.org/10.1080/15321810902782863

Journal of Immunoassay and Immunochemistry®, 30: 166–179, 2009

ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810902782863

Taylor & Francis
Taylor & Francis Group

Development of an Indirect Enzyme Immunoassay using Monoclonal Antibodies for the Measurement of 17α-Hydroxyprogesterone in Human Serum

Heilly Chong, Swee Hung Cheah, M. Ragavan, and V. T. Johgalingam
Monoclonal Laboratory, Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Abstract: An indirect enzyme immunoassay for the measurement of total 17α-hydroxyprogesterone (17OHP) in serum using monoclonal antibodies generated in our laboratory was developed. Here, (a) instead of extraction with solvents, serum was heated to free protein-bound 17OHP and assay was performed at pH 9.6, (b) to ensure uniform assay conditions for both standards and samples, buffer for standards contained charcoal-stripped pre-heated pooled cord serum. Assays were done in 96-well EIA microplates pre-coated with 17α-hydroxyprogesterone-3-(o-carboxymethyl)oxime: bovine serum albumin. Secondary antibody was horseradish peroxidase-linked sheep anti-mouse IgG polyclonal antibody. The method was accurate and suitable for screening for congenital adrenal hyperplasia.

Keywords: 17α-Hydroxyprogesterone, Indirect enzyme immunoassay, Monoclonal antibody

INTRODUCTION

The measurement of 17α-hydroxyprogesterone (17OHP) in serum or plasma is clinically useful for the diagnosis and management of congenital adrenal hyperplasia (CAH). CAH is caused by the deficiency of

Address correspondence to Dr. Swee Hung Cheah, Department of Physiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. E-mail: cheahsh@ummc.edu.my

21-hydroxylase, a cytochrome P450 enzyme, which mediates the conversion of 17OHP and progesterone to 11-deoxycortisol and deoxycorticosterone, respectively.[1,2] Deficiency of 21-hydroxylase activity results in elevated levels of steroids proximal to the enzyme block, notably 17OHP. Classical 21-hydroxylase deficiency occurs with a frequency of about 1:10,000 to 1:15,000 births in various populations.[1] The conventional method used for measuring 17OHP is based on competitive-binding radioimmunoassay (RIA)[3,4] using polyclonal antibodies and often involves solvent extraction of samples. However, in the past decade, laboratories are leaning more towards using enzyme immunoassays (EIA) for easier mass screening of steroids in patients' sera or plasma. EIA possesses several advantages over RIA. These include the avoidance of problems associated with the use of radioisotopes, the shorter incubation period, and less work involved in the overall procedure. Additionally, immunoassays are now increasingly based on monoclonal antibodies (Mabs) instead of antisera (polyclonal antibodies). This is due to the fact that Mabs possess uniform characteristics, are well-defined proteins, and are readily available in unlimited supplies.

The measurement of 17OHP by immunoassay may involve extraction with organic solvent, which is tedious and time consuming. Immunoassay has also been done directly on the sample without extraction; however, interference of the serum and plasma constituents with immunoassays has been reported.[5,6] Steroid hormones bind to serum and plasma proteins[5,6] and, thus, the quantities measured may vary with the degree of binding of steroid. Blood transported on filter paper has also been "extracted" using PBS; however, it also suffers from the possible different degrees of detachment of 17OHP both from blood proteins and also the matrix of the filter paper.

In this paper, we report on the development and performance of an indirect competitive EIA using Mabs generated in our laboratory. Our assay varies from conventional assays in 2 important factors: (i) total 17OHP is measured without prior extraction with organic solvents: bound 17OHP is freed from any proteins by a simple heating step and by performing the assay at alkaline pH; (ii) incubation matrix: the standard curve is generated from standard 17OHP solutions that contain blood proteins that had been stripped of steroids in equivalent quantities as the samples. The standard curves and samples are run under exactly the same conditions; this gives parallelism between the standard curve and dilutions of samples, thus making the assay valid. In this assay "total" steroid instead of "free" steroid is measured, thus eliminating the uncertainties in actual 17OHP levels due to binding to proteins in serum or plasma. This procedure is based on a concept first described by Dhar and Ali[7] for the measurement of testosterone in human serum.

The immunoassay system is based on competitive binding between steroids from the samples (and standards) with the 17OHP immobilized within the wells for the limited antibody binding sites in the Mab added. The quantity of the binding of Mab to immobilized steroids is inversely proportional to the quantity of free 17OHP in the samples. The bound Mab is detected by a secondary antibody specific for mouse IgG to which horseradish peroxidase (HRP) has been conjugated. Following color development, the optical density would be inversely proportional with increasing amounts of 17OHP that have been introduced into the well. The quantity of 17OHP present in a sample can then be determined from the standard curve generated.

EXPERIMENTAL

Chemicals and Reagents

Flat bottom 96-well EIA plates were purchased from Nunc, Denmark. The automatic microtiter plate reader was from Bio-Tek Instruments, Inc, USA. 17α-hydroxyprogesterone-3-carboxymethyl-oxime-bovine serum albumin (17OHP:BSA) was from Sigma, USA, 17α-hydroxyprogesterone from ICN, USA, ABTS (2,2′-Azinobis[3-ethylbenzthiazoline-sulfonic acid]) tablets and ABTS buffer from Roche-Boehringer, Germany. Secondary antibody (sheep anti-mouse IgG) conjugated to HRP was from Chemicon, USA. All other analytical grade chemicals were purchased from BDH, UK, unless specified.

Buffers

Coating buffer was $Na_2CO_3/NaHCO_3$ (0.05 M, pH 9.6). ''Alkaline buffer'' contained $Na_2CO_3/NaHCO_3$ (0.25 M, pH 9.6). Washing buffer was NaCl (0.9% with 0.1% Tween-20). Blocking buffer was PBS (pH 7.0) containing 1% bovine serum albumin (BSA) and 0.1% Tween-20.

Working Solutions for Standards

The standard solutions were made with buffer solution that contained steroid-free blood proteins in equivalent concentration as the sample during the assay.

This ''working solution'' for standards was made from pooled cord blood serum that had been heat-treated-and then charcoal-stripped to remove the endogenous steroids. Serum was obtained from cord blood

collected from the Clinical Diagnostics Laboratory, University of Malaya Medical Center. Briefly, the working solution was prepared by diluting pooled cord serum (1:2) with alkaline buffer, and heating the mixture in a water bath at 70–75°C for 30 min. The endogenous steroids were removed from the mixture by stirring with Norit-A charcoal (4 g/L) at room temperature for 1 hr. The mixture was left to stand overnight at 4°C. Next, the charcoal was removed from the mixture by centrifugation (10,000 rpm for 5 min at 4°C) and any charcoal residues were removed by filtration using a 0.2 μm cellulose acetate syringe filter. This working solution could be stored in aliquots at −20°C for several months.

Chromogen

The substrate solution for HRP was prepared by dissolving an ABTS tablet (50 mg/tablet) in 50 ml of ABTS buffer (Roche, Germany). This mixture was stored at 4°C and used within two weeks of preparation.

17OHP Standards

17OHP for standards was prepared from a stock solution of 17OHP dissolved in methanol (1 mg/mL) and stored at 4°C. Twelve working standards (5–100 ng/mL) were prepared by diluting appropriate amounts in working solution.

Mab Preparation

Mab was generated within our laboratory using 17OHP:BSA as immunogen.[8] Several stable hybridomas producing highly specific antibodies of high affinity were obtained.[8] Before use, hybridomas which were kept in liquid nitrogen were thawed and expanded to obtain the requisite number of cells for use.

Laboratory scale production of Mab against 17OHP was done through the mouse ascites fluid method. The use of animals was passed by the University of Malaya Faculty of Medicine Animal Use Ethics Committee. Briefly, Balb/C mice were primed with pristane (Sigma, USA) at a dose of 0.5 mL/mouse) and one week later, one mL of hybridoma cells in PBS were injected i.p. into each mouse (3–5 million cells per mouse). The Mab (IgG fractions) from the ascites fluid was separated by ammonium sulfate precipitation (50% saturated) and centrifugation. The pellet was re-dissolved in PBS, dialyzed against PBS and purified by affinity chromatography using a protein G column (Roche,

Germany). The purified IgG was stored in aliquots at −20°C. Working Mab solutions were made by appropriate dilutions of aliquots with PBS (pH 7) and stored at 4°C. The Mab used for developing the immunoassay was P192 (derived from hybridoma coded 192) and has an affinity constant of 7.77×10^{-7} M^{-1}.[8] In general, the Mab was diluted to a concentration that gave about 60–70% of maximum binding in the 17-OHP-BSA wells in the absence of added free 17OHP.

For the secondary antibody, sheep anti-mouse IgG conjugated to HRP (Chemicon, Cat no: AQ32BP, USA) was used at a dilution of 1 in 6,000.

Coating of 96-Well EIA Plates

Wells of the EIA microtiter plate were coated by incubating 100 μL of 17OHP:BSA dissolved in coating buffer (0.25 μg/mL in terms of BSA concentration) overnight at 4°C. Each molecule of BSA carried between 20 and 30 molecules of 17OHP, according to the supplier. The following day, the incubation solution was decanted and plates were washed 2 times with washing buffer. Wells were refilled with blocking buffer, and incubated for 1.5 hr at 37°C. Finally, the blocking buffer was removed and wells were rinsed three times with washing buffer. Coated plates were stable for several months if stored at 4°C.

Preparation of Serum Samples

Serum samples from patients and subjects (200 μL) were diluted two-fold with alkaline buffer in Eppendorf tubes. The tubes were then heated at 70–75°C in a water bath for 30 min. The samples were cooled to room temperature, vortexed, and were ready to be used in the assay.

17OHP Enzyme Immunoassay

Fifty μL of prepared serum samples (heat-treated) or standards (prepared in working solution) were transferred to appropriately marked wells of a 17OHP:BSA coated plate, followed by 50 μL of Mab solution (diluted in 1:5,000 in PBS). The plate was shaken for 15–20 sec using a plate shaker, and then transferred into a humidified container and incubated at 37°C for 1.5 hr. The wells were then washed three times with 200 μL portions of washing buffer. One hundred μL of second antibody (anti-mouse IgG conjugated to HRP) were then added into each well and the plate shaken for 10 sec. The plate was re-incubated in the dark for another 1 hr at

room temperature. Finally, the wells were washed as before (three times) with washing buffer. Next, 100 μL of chromogen solution (ABTS) were added into each well and the plate was shaken again for 10 sec. Optimum color development was achieved after 40–60 min incubation with ABTS buffer. The absorbance was measured using a microplate reader at 405 nm.

Specificity and Accuracy Studies

The specificity of the Mab P192 was assessed by the EIA method. Mab was incubated in the presence of several steroids that are structurally related to 17OHP, and the cross-reactivity was calculated using the procedure described by Abraham.[9]

The accuracy of the assay was assessed by a linearity study. Two serum samples were diluted 2- to 4-fold with the "0 ng/mL standard" and assayed and the measured and expected levels of 17OHP were compared.

Intra-assay and inter-assay coefficients of variation (CV) were determined by repeated measurement on a limited number of serum samples obtained from blood of adult male volunteers.

RESULTS

Analytical Variables

Figure 1 depicts an optimized 17OHP standard curve covering a range of 5–300 ng/mL. This standard curve range is adequate to measure, accurately, the range of total 17OHP in human serum. The sensitivity of the 17OHP assay is taken as the minimum point that was 2 times the SD from 0 ng/mL. This is typically about 0.2–1 ng/mL.

Working solution containing heat-charcoal-treated newborn serum derived from cord blood was used in preparing the standard solutions. As shown in Figure 2, heat-treatment of the serum in the working solution increased the sensitivity and working range of the standard curve compared with when non-heated serum was used. The working solution with heat-treated serum gave a curve that was more sensitive in the range of between 2.5–30 ng/mL.

Figure 3 is a profile of two binding curves of standard calibrators (5–500 ng/mL) performed using alkaline buffers for standards that were prepared differently. In the H−C curve, the buffer for standards was heated first, then stripped with charcoal. In the C−H curve, the buffer was stripped with charcoal first, then heat-treated. The results

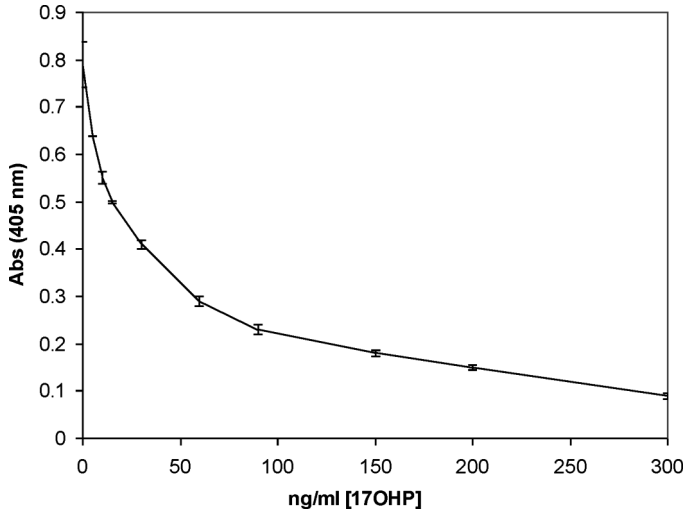

Figure 1. Standard curve for 17OHP assay in serum. Each point represents the mean and standard deviation of 3 measurements in duplicate. Plates were coated with 17OHP:BSA at 0.25 μg/ml (in terms of BSA concentration).

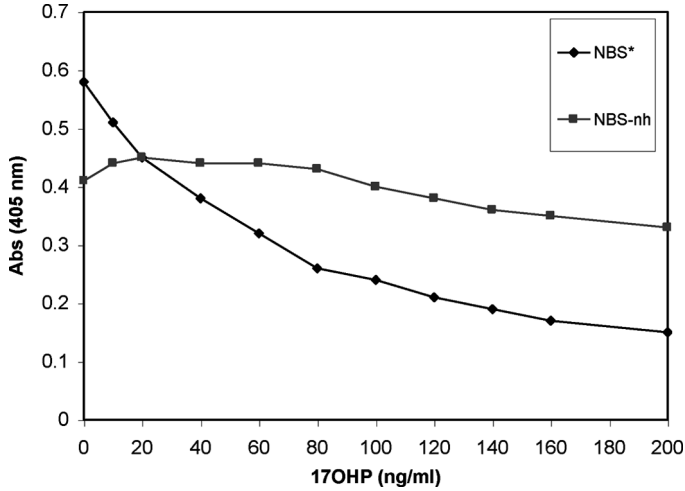

Figure 2. Standard curves obtained with diluents containing heat-treated newborn pooled serum (diamond shape point, NBS*) and unheated newborn pooled serum (square shape point, NBS-nh). Both serum samples were stripped off of endogenous steroid by using Norit-A charcoal (Sigma, USA).

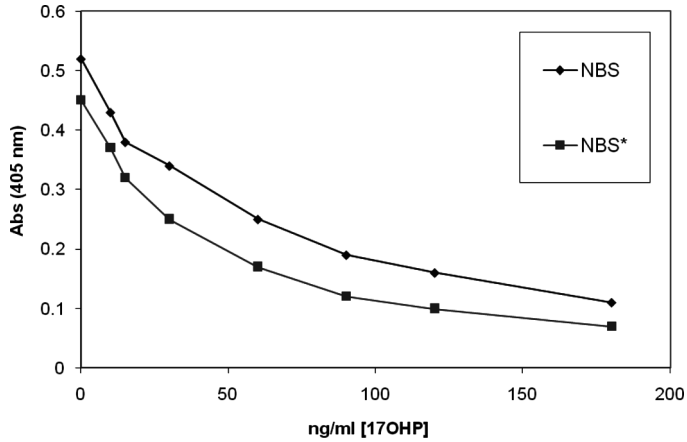

Figure 3. Comparison of binding curves of standard 17OHP solutions prepared in buffers containing newborn serum that had been heated then stripped by charcoal (NBS*) or stripped by charcoal first then heated (NBS). Each point represents the mean of 3 measurements in duplicates.

showed that the curves were approximately parallel but the C–H curve consistently gave apparent slightly higher values than the H–C curve.

Figure 4 shows standard curves in which assay buffers contained either newborn serum (NHC) or adult serum (AHC). Both had been

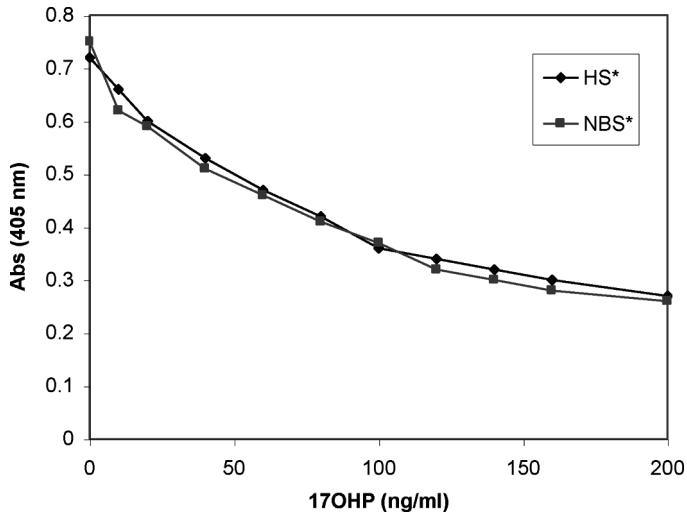

Figure 4. Comparison of standard curves for 17OHP made from diluents containing human pooled serum (HS*), and in newborn pooled serum (NBS*). Both sera had been similarly heated then stripped by charcoal.

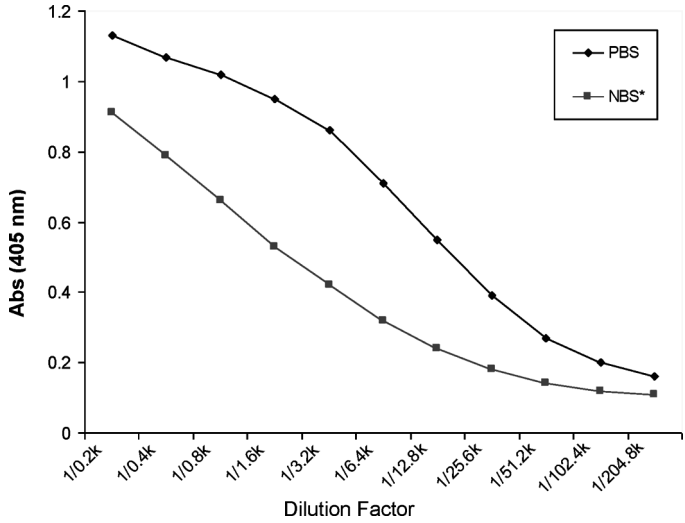

Figure 5. Comparison of binding curves of 17OHP calibrators that had been prepared in different buffers: PBS and working solution containing heat-treated charcoal-stripped newborn serum (NBS*).

heat-treated and then stripped with charcoal. The results show that the curves almost overlapped with each other and, in general, were not significantly different.

Figure 5 shows the comparison of standard curves of 17OHP calibrators that had been prepared in different working buffers: PBS and working solution containing heat-treated charcoal-stripped newborn

Table 1. Cross-reactivity study of P192 monoclonal antibody with various steroids

Steroid	Cross-reactivity (%)*
17alpha-hydroxyprogesterone	100
Progesterone	<1
Dehydroepiandrosterone-sulphate	<1
17alpha-hydroxypregnenolone	<1
5-pregnen-3beta-ol-20-one	<1
Androsterone	<1
4-androstene-3,17-dione	<1
Beta-estradiol	<1
Dehydroisoandrosterone	<1

*Percentage indicates the cross-reactivity for each steroid at 50% displacement, calculated using criteria described by Abraham, 1969.

Table 2. Linearity studies. Two serum samples containing 24.5 and 65 ng/ml of endogenous 17OHP were diluted serially with zero standards and assayed by indirect EIA procedure. Recorded values depict the mean of two measurements done in duplicate

Sample	Dilution	Measured (ng/ml)	Expected (ng/ml)	Recovery (%)
Serum A	Neat	24.5	–	–
	1:2	13.5	12.5	110
	1:4	6.2	6.12	101
Serum B	Neat	65	–	–
	1:2	35	32.5	107
	1:4	17.7	16.25	107

serum (NBS*). Results clearly showed that there was no parallelism between the two curves, indicating that the presence of serum proteins had an effect on the standard curve.

Specificity

Table 1 summarizes the cross-reactivity study of P192 with other steroids. There was little cross-reactivity (<1%) with other steroids tested.

Accuracy

Comparison of the measured and expected concentrations is shown in Table 2. The results show that, within the limits of the assay conditions, the measured and expected results were in close agreement.

Table 3. Intra-assay CV. For the intra-assay variation study, five serum samples from healthy adult males were assayed in replicate by indirect EIA. Recorded values depict the mean of n measurements in duplicate

Serum ID	n	Mean (ng/ml)	SD	CV (%) Intra-assay
Mc1	4	7.25	0.5	6.9
Mc2	3	26	2.07	7.9
Mc3	4	9.1	0.2	2.3
Mc4	4	8.7	1	11.5
M6	3	19.2	2.33	12.1

Table 4. Interassay CV. For the inter-assay variation study, five serum samples from healthy adult males were assayed in replicate using indirect EIA. Each sample was measured two times in duplicate on n different days

Serum ID	n	Mean (ng/ml)	SD	CV (%) Inter-assay
Mc1	3	7.19	1.37	19
Mc2	3	25.25	2.88	11.4
Mc3	3	9.27	1.10	11.9
Mc4	3	10.99	3	27.5
M6	3	19.06	2.22	11.6

Validation of the Method

Total 17OHP concentrations in 80 normal adult male samples (volunteers) were measured by the optimized EIA. The results for intra- and inter-assay CV are summarized in Tables 3 and 4, respectively. The intra-assay CV varied from 2.3 to 12.1%, while the inter-assay CV was between 11.4 to 27.5%.

DISCUSSION

In the present report, we have described the development of a competitive EIA for the measurement of total 17OHP in serum. This assay is relatively simple to perform, as it needs no extraction by organic solvent, is more rapid than RIA, and does not produce large amounts of hazardous radioactive wastes. The assay is developed from Mabs that were generated in the lab[8] and is of sufficient sensitivity, rapidity, and ease of use for the screening of large numbers of newborns blood for elevated serum 17OHP, which is a sign of CAH.

The final assay conditions were optimized following extensive studies using different buffer reagents, different dilutions of Mab for coating wells, conditions such as time and temperature of incubation, and with trials using serum from adult and newborns in the buffers for standard curves. We, finally, found that the most stable and consistently optimum condition was to use heat-treated and then charcoal-stripped (in that order) serum in the generation of the standard curve. Sample serum was similarly diluted in alkaline buffer, heat-treated, and the assay was done in alkaline conditions. This released the steroid from the binding proteins in the serum and allowed the assay of total 17OHP.

The buffer for standard was carefully selected to give the closest conditions in which serum or plasma samples were measured. In our assay, both standards and samples contained similar biological matrices,

especially in terms of protein content. Standard solutions prepared in PBS (without the heat-treated serum and alkaline conditions), as done in most commercially available assay kits, would not be expected to give standard curves that are parallel with serum samples serially diluted with PBS (refer to Figure 5). This non-parallelism is likely to be caused by differences in biological matrix content between standard (which do not contain serum proteins) and serum samples. This would give rise to erroneous readings of 17OHP in the samples. Thus, the use of buffer for standards that contained heat-treated charcoal-stripped pooled serum from cord blood would negate this source of error and make the results more accurate and valid.

The measurement of 17OHP in a limited number of normal adult sera showed that the results obtained were between 7–25 ng/mL. To our knowledge, there has been no published data on total 17OHP in adult or newborn serum. The reference value for free 17OHP in normal adults is between 0.4 to 3.3 ng/mL.[1,10–14] It is known that, normally, a substantial quantity of steroids in blood circulate bound to serum proteins or exist as conjugates (about 91–98% of total), and only 2 to 9% exist in the free form.[15–18] Thus, if we assumed that, of the total 17OHP in the sample sera, about 10% at the maximum is in free from, then the free 17OHP in these sera would lie between 0.2–6.5 ng/mL, which is in approximate agreement with the reference range. Thus, the total 17OHP values would be approximately within the accepted normal range of male adults.

The Mab used is highly specific for 17OHP and shows little cross reactivity with other steroids, including progesterone. Therefore, the assay should reliably measure 17OHP throughout the menstrual cycle of females.

The recovery studies show that the measured and expected values of 17OHP were in agreement. The intra- and inter-assay CV were within the accepted range.

We are, at present, using this assay to measure 17OHP in a large number of adult serum samples from the blood bank and from normal babies in order to start building up a baseline database of total serum 17OHP for the Malaysian population. As more samples are measured, it is expected that the intra-assay and inter-assay CV values will be narrowed.

CONCLUSIONS

In summary, we have successfully used a Mab that was generated within the country in order to develop an EIA for 17OHP. This assay is stable enough and easy to use, which will be useful for the screening of newborn blood at relatively low cost.

ACKNOWLEDGMENTS

We wish to thank Dr. J. V. Gavilondo and Dr. M. F. de Cossio from the Center of Genetic Engineering and Biotechnology, Havana, Cuba, for their excellent technical assistants and technology transfer whose travel was supported by a grant from The International Atomic Energy Agency, Vienna. This work is supported by grants from the Malaysia Government Ministry of Science, Technology and Innovation (MOSTI) and the University of Malaya. Dr. Chong was a recipient of a National Science Foundation training grant from MOSTI during her PhD training. This work was conducted in partial fulfillment for Dr. Chong's PhD degree at the University of Malaya.

REFERENCES

1. Pang, S.; Wallace, M.A.; Hofman, L.; Thuline, H.C.; Dorce, C.; Lyon, I.C.T.; Doin, R.H.; Kling, S.; Fujide, K.; Suwa, S. Worldwide experience in hyperplasia due to 21-hydroxylase deficiency. Pediatrics **1988**, *81*, 866–874.
2. Perrin, C.W.; Speiser, P.W. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocrine Rev. **2000**, *21*, 245–291.
3. Hofman, L.F.; Klaniecki, J.E.; Smith, E.K. Direct solid-phase radioimmunoassay for screening 17α-hydroxyprogesterone in whole-blood samples from newborns. Clin. Chem. **1985**, *31*, 1127–1130.
4. Milewicz, A.; Vecsei, P.; Korth-Schutz, S.; Haack, D.; Rosler, A.; Litchwald, K.; Lweicka, S.; Mitterslstaedt, G.V. Development of plasma 21-hydroxycortisol radioimmunoassay and application to the diagnosis of patients with 21-hydroxylase deficiency. J. Steroid Biochem. **1984**, *21*, 185–191.
5. Selby, C. Interference in immunoassay. Ann. Clin. Biochem. **1999**, *36*, 704–721.
6. Tate, J.; Ward, G. Interferences in immunoassay. Clin. Biochem. Rev. **2004**, 105–120.
7. Dhar, K.T.; Ali, E. Direct microtiter plate of enzyme immunoassay of testosterone in unextracted serum. J. Immunol. Meth. **1992**, *147*, 167–172.
8. Chong, H.; Cheah, S.H.; Ragavan, M.; Johgalingam, V.T. Production and characterization of monoclonal antibodies against 17α-hydroxyprogesterone. Hybridoma **2006**, *25*, 34–40.
9. Abraham, G.E. Solid phase radioimmunoassay for estradiol-17β. J. Clin. Endocrinol. Metab. **1969**, *29*, 866–870.
10. Berry, J.; Betts, P.; Wood, P.J. The interpretation of bloodspot 17α-hydroxyprogesterone levels in term and pre-term neonates. Ann. Clin. Biochem. **1986**, *23*, 546–551.
11. Azziz, R.; Zacur, H.A. 21-hydroxylase deficiency in female hyperandrogenism: screening and diagnosis. J. Clin. Endocrinol. Metab. **1989**, *69*, 577–584.
12. Cutfield, W.; Webster, D. Newborn screening for congenital adrenal hyperplasia in New Zealand. J. Pediatr. **1995**, *126*, 118–121.

13. Carlson, A.D.; Obeid, J.S.; Kanellopoulou, N.; Wilson, R.C.; New, M.I. Congenital adrenal hyperplasia: Update on prenatal diagnosis and treatment. J. Steroid Biochem. Mol. Biol. **1999**, *69*, 19–29.
14. Cartigny-Maciejewski, M.; Guilley, N.; Vanderbecken, S.; Gonde, S.; Stuckens, C.; Ponte, C.; Weill, J.; Farriaux, J.P.; Paux, E. Neonatal screening of congenital adrenal hyperplasia due to 21-hydroxylase deficiency: Lille experience 1980–1996. Arch. Pediatr. **1999**, *6*, 151–158 [French].
15. Joseph, D.R. Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. Vitam. Horm. **1994**, *49*, 197–280.
16. Hammond, G.L.; Bocchinfuso, W.P. Sex hormone-binding globulin/androgen-binding protein: Steroid-binding and dimerization domains. J. Steroid Biochem. Mol. Biol. **1995**, *53*, 543–552.
17. Pekary, A.E.; Hershman, J.M. Hormone Assays. In: *Endocrinology and Metabolism*; Felig, P.; Frohman, L.A.; Eds.; McGraw-Hill Professional: New York, 2001; Ch. 5, 91–108
18. Huang, W.; Kalhorn, T.F.; Baillie, M.; Shen, D.D. Thummel, K.E. Determination of free and total cortisol in plasma and urine by liquid chromatography-tandem mass spectrometry. Ther. Drug Monit. **2007**, *29*, 215–224.

Received September 14, 2008
Accepted October 27, 2008
Manuscript 3321