

Supplementary

Specifications of the Danish neonatal screening for congenital adrenal hyperplasia

Methods

First-tier: Immunoassay

From January 2009 to March 2018 17-hydroxyprogesterone (17OHP) was measured with the AutoDELFIA Neonatal 17 α -OH-progesterone kit (Perkin Elmer, Turku Finland). From March 2018 17OHP it was measured on the Perkin Elmer genetic screening processor (GSP) with the GSP Neonatal 17 α -OH-progesterone kit (Perkin Elmer, Turku Finland). The detection limit was 1.2 nmol/L, quantification interval was set to 1.2-300 nmol/l. Intra assay coefficient of variation (CV) was 4.7% and inter assay CV 6.7%.

Second-tier: Liquid chromatography tandem mass spectrometry (LC-MS/MS)

The second tier analysis was an in-house developed LC-MS/MS method. Calibrators were produced by spiking a steroid depleted whole blood matrix[1] to different concentrations between 3.1-400 nmol/L of 17OH, cortisol (cor) and 4-androstenedione (4AD). For control samples venous blood samples were spiked. Both calibrators and controls were spotted to filter paper and dried overnight. The extraction buffer was a 50:25:25 methanol:acetonitrile:acetone enriched with deuterated internal standards, d4-cor and d8-17OHP (also used for 4AD).

Patient dried blood spot sample (DBSS), calibrators and controls were punched into a 96 well microplate and 100 μ l extraction buffer was added. The plate was sealed and shaken at 300 rpm for 30 min. 80 μ l was transferred to a new microplate, dried under a stream of N₂ and reconstituted by 80 μ l 50:50 methanol:water + 0.1% formic acid. The plate was heat sealed and shaken at 300 rpm for 15 min. several LC-MS/MS methods were used during the study period, all using triple quadrupole mass spectrometers and a 5 minute gradient. Table 1 displays the differences in methods over time. The inter assay CV was 9-15%.

Quality control

The laboratory participates in the Center for Disease Control and Intervention (CDC) Newborn Screening Quality Assurance Program (NSQAP) for both first and second tier analyses and both methods are accredited (ISO17025).

Table 1. Differences in the LC-MS/MS method over time

Time	January 2009 – December 2010	January 2011 – August 2017	September 2017 – present time
Number of 3.2 mm punch per well	2	1	1
Injection volume	20 µl	20 µl	50 µl
Solvents used in gradient	A: 95:5 Water: Methanol + 0.1% Formic Acid, B: 5:95 Water: Methanol + 0.1% Formic Acid	A: 95:5 Water: Methanol + 10mM Amoniumacetate, B: 25:25:25:25 Water: Acetronitril: Methanol: Isopropanol + 0,2% Formic Acid	A: Water + 0.1% Formic Acid, B: Methanol + 0.1% Formic Acid
Column	Thermo Hypersil Gold C18, 3 µm, 2.1 x 50 mm	Waters Aquity UPLC BEH C18, 1.7 µm, 2.1 x 50 mm	Agilent Poroshell 120 C18, 2.7 µm, 2.1 x 50 mm
Ionization technique	Atmospheric pressure chemical ionization	Electrospray	Electrospray
Instrumentation	CTC Pal autosampler, Surveor MS Pump Plus, TSQ Quantum Ultra mass spectrometer	Acquity UPLC, Xevo TQ-S mass spectrometer	Thermo Pal autosampler, Ultimate LPG-3400RS pump, Quantiva mass spectrometer
Instrument Vendor	Thermo Fisher Scientific, Waltham, Massachusetts, USA	Waters, Milford, Massachusetts, USA	Thermo Fisher Scientific, Waltham, Massachusetts, USA

Table 2. Changes of cut-off levels over time in the Danish neonatal screening program for congenital adrenal hyperplasia (2009-2018).

Date	GA (week)	1. tier 17-OHP (nmol/L)	2. tier 17-OHP (nmol/L)	Ratio (17OHP+4AD/Cortisol)	True positive (n)	False Positive (n)	True negative (n)	False negative (n)
February 2009	<34	60	38	3.75	0	2	83554	0
	>=34	30						
June 2010	<34	60	19	3.75	1	0	37532	0
	>=34	30						
January 2011	<34	80	19	3.75	1	0	68283	0
	>=34	20						
March 2012	<34	20	19	3.75	1	1	44145	0
	>=34	20						
December 2012	<34	20	19	3.75	2	1	69333	0
	>=34	15						
March 2014	<34	20	19	1.5	11	0	75552	1
	>=34	15						
July 2015	<38	20	19	1.5	10	16	173357	0
	>=38	15	9	0.5				
May 2018	<38	20	19	1.8	3	3	41679	0
	>=38	15	11	0.8				

Numbers of true positive, false positive, true negative and false negative are shown for each period separately. For numbers of true negative, the live birth rate of the given period is used [2].

GA: Gestational age, 17-OHP: 17-hydroxyprogesterone, 4AD: Adrostendione

References

1. Lacey JM, Minutti CZ, Magera MJ, Tauscher AL, Casetta B, McCann M, et al. Improved specificity of newborn screening for congenital adrenal hyperplasia by second-tier steroid profiling using tandem mass spectrometry. *Clin Chem.* 2004;50(3):621-5.
2. Denmark S. Live births by birth month, day of birth and time 2020 [Available from: <https://www.statistikbanken.dk/statbank5a/SelectVarVal/Define.asp?MainTable=BEV3A&PLanguage=1&PXSID=0&wsid=cftree>].