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Pregnanetriolone in paper-borne urine for neonatal screening for 21-hydroxylase deficiency: The place of urine in neonatal screening

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ABSTRACT

The standard method of primary neonatal screening for congenital adrenal hyperlasia (CAH), determination of 17-hydroxyprogesterone (17OHP) in heelprick blood, is the object of recurrent controversy because of its poor diagnostic and economic efficiency. The superior ability of urinary pregnanetriolone levels to discriminate between infants with and without classical CAH has been known for some time, but has not hitherto been exploited for primary screening. Here we propose an economical neonatal CAH-screening system based on fluorimetric determination of the product of reaction between urinary pregnanetriolone and phosphoric acid.

1. Introduction

Congenital adrenal hyperplasia (CAH) is an inherited metabolic disorder caused by autosomal recessive defects in the genes encoding enzymes involved in the biosynthesis of mineralocorticoids, glucocorticoids or sex steroids in the adrenal glands [1]. The most common such defect, accounting for 90–95% of CAH cases [2], affects *CYP21A2*, the gene encoding 21-hydroxylase, which transforms progesterone into deoxycorticosterone and 17α-hydroxyprogesterone into 11-deoxycortisol. Unless otherwise stated, "CAH" hereinafter refers to CAH caused by 21-hydroxylase deficiency (21OHD). The reported frequency of this condition ranges from 1:28,000 in China to 1:280 among Yupik Eskimos, with an average of around 1:14,200 [3].

Depending on the specific mutation suffered, loss of 21-hydroxylase activity may be complete or only partial, and the degree of 21OHD correlates well, though by no means perfectly, with clinical phenotype [4]: total loss of activity typically results in the most severe form, salt-wasting CAH; mutations averaging 98% loss in simple virilizing CAH; and mutations causing 80–90% loss in mild, "non-classical" (late-onset) CAH. Non-classical CAH, which affects 0.1–0.2% of Caucasians [5–8], gives rise to no symptoms in early childhood, but later results in excess androgen levels and accelerated bone aging, and adolescent and adult females may suffer hirsutism, menstrual irregularity and infertility. In simple virilizing CAH (about a quarter of all "classical" CAH cases) the effects of excess androgen are already pronounced in early childhood; newborn girls may have ambiguous external genitalia. Salt-wasting CAH is a potentially life-threatening condition that, untreated, leads within 1–3 weeks of birth to acute adrenal crises, with poor weight gain, vomiting, diarrhoea, dehydration, failure to thrive, lethargy, hyponatraemia (due to 21OHD-induced aldosterone deficiency), hyperkalaemia, and shock.

Because of its high frequency and life-threatening potential, and because it can be treated effectively by corticoid replacement therapy, CAH is in many countries included among the inherited metabolic disorders screened for at birth. The standard primary screening method measures the 21-hydroxylase substrate 17-hydroxyprogesterone (17OHP) in the same kind of sample as is generally employed for other neonatal screening tests, blood obtained by heel prick and transported to the laboratory in sorbent paper. However, whatever the analytical technique used to determine 17OHP in these blood samples - the most widespread is dissociation-enhanced lanthanide fluoroimmunoassay DELFIA® (PerkinElmer Life Sciences-Wallac Oy, Turku, Finland) – this approach is bedeviled by the fact that 17OHP levels are generally high in unaffected newborns, especially if premature, sick or stressed [9–13]. In healthy newborns 17OHP levels rapidly fall, but their descent is often not completed before heelprick for neonatal screening (nowadays usually performed no later than the third day of life), which results in widely disperse values [23]. Additionally, immunoassays suffer from a degree of cross-reactivity with sulphated steroids produced by the foetal and neonatal adrenal gland [9–14]. As a result of these two influences, there is considerable overlap between the 17OHP levels in heelprick samples of newborns with and without CAH: if a 17OHP screening threshold of adequate sensitivity is set, around 1% of all newborns test positive, and about 99% of these positive results will be false [9], making screening for CAH one of the least cost-effective of neonatal screening procedures [15–17].

For premature newborns the discrimination problem can be alleviated to some extent by using different 17OHP thresholds for different gestational age groups [2,18–20]. More generally, it has been reported that satisfactory sensitivity and positive predictive value can be achieved by subjecting positive-testing samples to a second-tier test in which liquid chromatography followed by tandem mass spectrometry (LC-MS/MS)

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is employed to determine both 17OHP and other steroids (androstenedione, cortisol, 11-deoxycortisol, 21-deoxycortisol), and steroid ratios are used as the test criterion [9,11,21]. However, LC-MS/MS is an expensive procedure with low throughput (as is genotyping, another second-tier option [22]), and its application is costly, given the large number of first-tier false positives.

The basis for a radical possible solution to these problems was laid more than a decade ago when it was found that urinary pregnanetriolone differentiated perfectly between newborns with and without 21OHD [24–26]. The study of Homma et al. [24], was small on the non-21-OHD side (83 non-21OHD newborns with elevated blood 17OHP versus 50 term and 9 pre-term newborns with classical 21OHD), and the analytical method, gas chromatography with mass spectrometry, was not suitable for primary screening; but its findings have since been confirmed in a larger study [13], and there appears to be no reason why the inexpensive fluorimetric analytical procedure by means of which pregnane¬triolone was first identified in patients' urine [27–32] cannot be readily adapted for reliable use in a screening programme. The major obstacle to the adoption of urinary pregnanetriolone as the analyte of choice for classical 21OHD screening seems to be the fact that the collection of urine samples – the basis of the pioneering neonatal screening programmes of Berry [33,34], Woolf [35–38] and others – has by many screening laboratories never been practised, and was discontinued long ago by most of those that did originally practise it.

The neonatal screening laboratory of Galicia (NW Spain) is one of the few that still collect urine samples from all newborns to screen for amino acid disorders, sugar disorders, mucopolysaccharidoses and oligosaccharidosis, and we are currently working to cover lysosomal storage disorders (glycosphingolipidoses, GM2 gangliosidoses, neutral glycosphingolipidoses, glycoproteinoses, mucolipidoses, leukodystrophies and others), which are far easier to detect reliably using urine samples than in blood samples, in which the concentrations of marker substances are several times lower than in urine [38]. In the remainder of this article we describe the practical details of newborn urine sample collection, define a fluorimetry-based method of pregnanetriolone determination that we propose to validate, and outline a broader project for the evaluation of urinary-pregnanetriolone-based 21OHD screening in which we hope to enjoy the collaboration of other centres. We end with some final remarks on the future role of urine analysis in newborn screening.

2. Urine collection

In our screening system, urine samples, like heelprick blood, are taken on the third day of life [39], using a piece of Whatman 903 or Munktell TFN sorbent paper included in the screening kit (Fig. 1). Before heelprick, half the paper is placed over the infant's genitals (which should be clean, with no cream, talc, oil or other product that might contaminate the urine) and is held in place by a diaper or napkin. Upon heelprick, the infant will generally urinate, after which the adsorbent paper can be removed and set apart to dry in a horizontal position on a support with which it makes minimal contact (e.g. a "bed of nails" made of pins stuck in cardboard, as in Fig. 2) [40]. In a maternity ward where samples are taken in a single session from all the infants born three days previously, it is of course necessary to write each infant's identity data on the sorbent paper before use; this should be done with a pencil so as to avoid contamination of the sample by ink. If upon heelprick the infant defecates as well as urinating, the sample should be discarded (even though the filter paper shows no visible faeces stain, it may bear fecal components eluted by the urine); in these cases, the other half of the filter paper is held in place with a diaper or napkin until the infant has urinated again. If the procedure takes place before discharge, the sample enters the laboratory on the day of collection; otherwise, if brought by hand or sent by mail.

3. Determination of pregnanetriolone

Sixteen discs 3 mm in diameter, or four 6 mm in diameter, are punched from the urine-bearing sorbent papers upon their arrival at our laboratory, and are stirred in the wells of 96-well microtitration plates containing 300 μL of water each (2 h, or 10 min if a 96-probe ultrasound device is used). The eluates thus prepared are currently employed to screen for the disorders mentioned in the Introduction by means of a battery of analyses that includes determination of creatinine for normalization [41,42]. Though subject to eventual optimization, the subsequent steps of the pregnanetriolone determination procedure that we propose to validate, adapted from Refs. 45 and 46 (see also Refs. 43 and 44), are as follows.

- Transfer 20 μL of eluate to a well of a black quartz microtitration plate (quartz to withstand heating, black to prevent interference between cells during fluorimetry – see below).

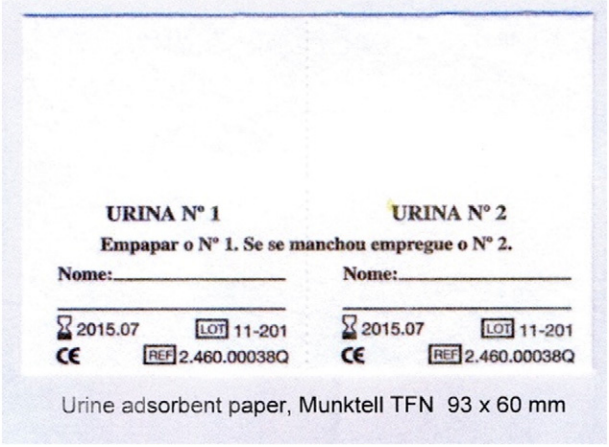

Fig. 1. Sorbent paper.

Fig. 2. Bed of nails.