Improved Methods for Isolating Cortisol Metabolites from Neonatal Urine

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Urine was collected from newborns, children, and adults who had received a tracer dose of [3H]cortisol. The free and different types of conjugated cortisol metabolites were separated by ion-exchange chromatography. A modified Amberlite XAD-2 procedure was used, which allowed quantitative extraction of the extremely polar cortisol metabolites from urine of newborns. Large differences were observed between the ion-exchange chromatographic patterns for neonates and adults. Urine from neonates was shown to contain quantitatively important amounts of an as-yet-unidentified conjugate that was absent from urine of adults. The effects of three different hydrolytic methods on the neonatal steroid conjugates were studied and it was found that the unknown conjugate in urine from the newborn could be hydrolyzed by solvolysis only.

Additional Keyphrases: steroids · steroid metabolism · pediatric chemistry · relative effectiveness of hydrolytic techniques

Several authors have reported on the isolation, identification, and quantification of cortisol metabolite fractions in the urine of the human newborn (1-4). In these studies, radioactive cortisol was given intravenously to newborns and the radioactive metabolites in the urine were subjected to isolation and identification procedures. It was found that the urine of the newborn contains relatively large amounts of unconjugated cortisol metabolites, but only relatively small amounts of glucuronides as compared to the amounts found in the urine of adults. Minor amounts of sulfates hydroxylizable by the sulfatases present in the digestive juice of Helix pomatia were also present in neonatal urine (5). One of the major problems encountered in all these studies was the inability of the then-available methods to isolate the radioactive metabolites from neonatal urine to any quantitative extent: 45-70% of the radioactive content of neonatal urine could not be extracted at all, even after several hydrolytic procedures, in contrast to adult urine, from which nearly all radioactivity could be extracted.

More recently, better techniques for the isolation of steroids from biological fluids have been developed (5, 6). However, the performance of these techniques in the isolation of cortisol metabolites from neonatal urine has not been evaluated by radiotracer methods. Such methods offer the advantage that the efficiency of the isolation procedures can be determined very easily.

The aim of the work described here was to design a procedure for quantitatively isolating cortisol metabolites from neonatal urine. To avoid the use of solvent-solvent partitioning methods in the separation of unconjugated steroids and several types of conjugated steroids, we separated these different groups of cortisol metabolites by ion-exchange chromatography. The procedure was tested with use of various hydrolytic methods.

Materials and Methods

All analytical-grade solvents, reagents, and ready-made thin-layer chromatography plates were purchased from Merck, Darmstadt, Germany. Radioactive steroids were obtained from New England Nuclear, Dreieichenhain, Germany, and were purified by thin-layer chromatography to give at least 95% purity. Nonradioactive reference steroids were from Steraloids, Pawling, N.J., and Servachrom (Amberlite XAD-2), particle sizes 100-200 μm and 300-1000 μm, from Serva, Heidelberg, Germany. Ketodase was obtained from Warner-Chilcott, Morris Plains, N.J., and Glusulase from Endo Laboratories, Garden City, N.Y. DEAE-Sephadex A-25 and LH-20 lipophilic Sephadex were from Pharmacia, Uppsala, Sweden.

[4-14C]Cortisol-21-sulfate, sodium salt, was prepared from [4-14C]cortisol according to the method of Gribesch and Garn (7). The product was separated on a unreacted starting material by Sephadex LH-20 column chromatography in the system chloroform/methanol (1/1), 10 mmol/liter NaCl, as described by Sjövall and Vikho (8). The product melted at 179-180 °C (ref. 7: 185 °C) and the infrared spectrum confirmed the structure, exhibiting strong absorption bands at 1250 and 1060 cm⁻¹ and medium bands at 1130-1160 cm⁻¹.

Subjects. After fully explaining the study to both parents, written parental consent was obtained to administer intravenously 0.15 μCi (3.3 × 10⁶ dpm) of [1,2,6,7-3H]cortisol (specific activity, 80-100 Ci/mmol), dissolved in 50 μl of ethanol and added to 5 ml of an aqueous glucose solution (50 g/liter), to four newborn infants suffering from various diseases (see Table 2). Urine was collected during two 24-h periods. The urine not needed for the determination of the cortisol production rate was used in the experiments described below.

Also, we used the remainders of 48-h urine collections from two adults and three children who received 0.2-1.0 μCi of tritiated cortisol to estimate their cortisol production rate. Radioactivity was determined by counting duplicate 1-ml samples, to which 10 ml of scintillation cocktail was added (9), in a Nuclear Chicago Mark III liquid scintillation counter. The results were corrected for quenching by the external standard method.

Amberlite ion-exchange procedures. Several different Amberlite XAD-2 methods were investigated. In all instances the columns were prepared as described by Shackleton et al. (6).

Method 1a: 5 ml of urine were applied to a 1.2 × 13.5 cm column packed with Amberlite XAD-2 of particle size 300-
1000 µm. The column was washed with 6 × 25 ml of water and eluted with 2 × 25 ml of ethanol.

Method 1b: As under 1a, except that Amberlite XAD-2 of particle size 100–200 µm was used.

Method 2: Urine, 5 ml, to which 250 g of sodium chloride was added per liter, was applied to a 1.2 × 13.5 cm column packed with Amberlite XAD-2 of particle size 300–1000 µm. The column was washed with 6 × 25 ml of a 250 g/liter sodium chloride solution in water and eluted with 2 × 25 ml of ethanol.

Method 3: Various amounts of urine (maximum, 40 ml) or aqueous urinary fractions (maximum, 70 ml) obtained by DEAE-Sephadex chromatography were applied to 1.2 × 13.5 or 1.4 × 25 cm columns packed with Amberlite XAD-2 of particle size 100–200 µm. After application of the sample, the column was immediately eluted with 100 ml of ethanol.

In all instances the ethanol fraction was collected from the precise moment that this solvent reached the lower end of the column. Duplicate 1-ml portions were taken from the sample, the aqueous eluate, and the ethanol eluate, and assayed for radioactivity. The ethanol eluate was then filtered and evaporated under reduced pressure and the residue was used in further experiments.

DEAE-Sephadex ion-exchange chromatography. DEAE-Sephadex A-25, a weakly basic ion-exchanger, was swollen in water for at least three days at room temperature. A Pharmacia K-9 column, 60 × 1 cm, was packed with the DEAE-Sephadex gel, and 50 ml of water was put through it. Then 10–50 ml of urine or of aqueous urine fraction was applied and the column was eluted with a linear NaCl gradient in water, starting at zero concentration and reaching a concentration of 0.3 mol/liter after 400 ml of eluent had passed the column. The flow rate was 1.05 ml/min and 10.5-ml fractions were collected, of which 1-ml portions were removed and their radioactivities counted.

Essentially, this procedure was described before by Hobkirck and Davidson (10) for the separation of free and conjugated 17-ketosteroids.

Hydrolytic methods. Several hydrolytic methods were applied consecutively to the different fractions obtained by DEAE-Sephadex chromatography from one neonatal and one adult urine, as will be discussed below. These hydrolytic procedures were as follows.

Ketodase Hydrolysis. The dry urine extracts (obtained by the Amberlite XAD-2 method) were dissolved in 20 ml of acetate buffer (pH 5.0, 0.2 mol/liter) and 2 ml of Ketodase (0.52 U of β-glucuronidase, EC 3.2.1.31) and a few drops of chloroform (to prevent bacterial growth) were added. The solution was incubated for 65 h at 37 °C.

Glusulase Hydrolysis. The dry urine extracts were each dissolved in 20 ml of acetate buffer pH 4.7 (0.2 mol/liter) and 0.2 ml of Glusulase (0.79 U of β-glucuronidase and 84 U of aryl-sulfatase, EC 3.1.6.1), and a few drops of chloroform were added. This solution was incubated for 65 h at 37 °C.

Solvolyis. The dry urine extracts were dissolved in 2 ml of methanol and 38 ml of peroxide-free tetrahydrofuran and 35 µl of a 70% (by weight) solution of perchloric acid in water was added. The reaction mixture was allowed to stand at 37 °C for 15 h. The reaction was stopped by adding 0.2 ml of pyridine and the solvents were evaporated under reduced pressure. The dry residue was dissolved in 10 ml of water.

Experiments and Results
Isolation with Amberlite XAD-2

When neonatal urine was subjected to Amberlite XAD-2 isolation according to method 1a, substantial amounts of radioactivity eluted from the column during the washing with water (see Figure 1). This was not the case when an adult urine sample was processed. This phenomenon may be explained by the much higher polarity of neonatal urinary cortisol metabolites as compared to those in adult urine (1–4). When Amberlite XAD-2 of particle size 100–200 µm was used (method 1b), similar results were obtained, but the elution of radioactivity during the washing of the column tended to be somewhat delayed (see Figure 1).

Adding sodium chloride to both the neonatal urine sample and the water used for washing the column (method 2) increased the amount of radioactivity eluted in the ethanol fraction to >95% of the amount applied to the column (see Table 1). However, the presence of large amounts of sodium chloride in the ethanol eluate hampered further processing and this procedure was abandoned.

From Figure 1 it can be deduced that the analytical recovery of neonatal cortisol metabolites in the ethanol eluate could be quite satisfactory if Amberlite XAD-2 of particle size 100–200 µm was used, the amount of urine applied to the column was not excessive, and the column was not washed with water after applying the urinary sample. Table 1 shows that under these conditions good results were obtained for both original urine samples and urine fractions obtained by DEAE-Sephadex chromatography. In the course of the succeeding experiments Amberlite XAD-2 method 3 was used throughout and the amounts of radioactivity recovered in the ethanol fractions were never <94% of the amounts applied to the columns.

DEAE-Sephadex Chromatography

When urine samples from adults were chromatographed on DEAE-Sephadex according to the method of Hobkirck and Davidson (10), 80–90% of the radioactive cortisol metabolites eluted as anticipated (11) in the glucuronide fraction (see Figure 2). Small amounts of radioactivity were found in the unconjugated and sulfate fractions. However, when neonatal urine samples were subjected to the same procedure, completely different elution patterns were observed (Figure 2). Relatively large amounts of radioactivity were found in the
Table 1. Performance of Different Amberlite XAD-2 Methods in the Isolation of Radioactive Cortisol Metabolites from Neonatal Urine

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>Amounts of radioactivity in the different eluates[^b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonatal urine, 5 ml</td>
<td>1a</td>
<td>Aqueous: 32.5, Ethanol: 65.1, Overall recovery: 97.6</td>
</tr>
<tr>
<td>Adult urine, 5 ml</td>
<td>1a</td>
<td>Aqueous: 101.6, Overall recovery: 101.6</td>
</tr>
<tr>
<td>Neonatal urine, 5 ml</td>
<td>1b</td>
<td>Aqueous: 31.0, Ethanol: 88.0, Overall recovery: 99.0</td>
</tr>
<tr>
<td>Neonatal urine, 5 ml</td>
<td>2</td>
<td>Aqueous: 1.8, Ethanol: 95.4, Overall recovery: 97.2</td>
</tr>
<tr>
<td>Neonatal urine, 40 ml</td>
<td>3</td>
<td>Aqueous: 2.9, Ethanol: 93.1, Overall recovery: 96.0</td>
</tr>
<tr>
<td>Unconjugated fraction, 40 ml[^c]</td>
<td>3</td>
<td>Aqueous: 3.6, Ethanol: 94.7, Overall recovery: 98.3</td>
</tr>
<tr>
<td>Glucuronide fraction, 70 ml[^c]</td>
<td>3</td>
<td>Aqueous: 3.6, Ethanol: 94.7, Overall recovery: 98.3</td>
</tr>
<tr>
<td>Presulfate fraction, 70 ml[^c]</td>
<td>3</td>
<td>Aqueous: 3.6, Ethanol: 94.7, Overall recovery: 98.3</td>
</tr>
</tbody>
</table>

[^a]: See text.
[^b]: Presented as a percentage of the amount applied to the column.
[^c]: These fractions were obtained by DEAE-Sephadex chromatography of 50 ml of neonatal urine No. 1 and they were processed on 1.5 x 25 cm Amberlite XAD-2 columns.

unconjugated fraction, while the relative amounts eluting in the glucuronide fraction were small as compared to the amounts found in the same fraction of adult urinary cortisol metabolites. The relative amounts of radioactivity found in the sulfate fraction were also larger than those found in the same fraction of adult cortisol metabolites.

Undoubtedly, the most striking difference between adult and neonatal elution patterns was the presence of a large peak of radioactivity eluting between the glucuronide- and sulfate-conjugate fractions of neonatal urine. This peak was virtually absent in the adult elution patterns. The fraction associated with this peak will be referred to as the “presulfate” fraction. Table 2 shows the relative amounts of radioactivity found in each of the fractions mentioned before from the urine samples of four newborn infants, three children, and two adults. In each case the total amount of radioactivity eluted was at least 96% of the amount applied to the DEAE-Sephadex column.

Hydrolytic Experiments

An aliquot from neonatal urine no. 1 was subjected to Glusulase hydrolysis and chromatographed on DEAE-Sephadex subsequently. Figure 2 shows that the peaks due to both the glucuronide- and sulfate-conjugated fractions had disappeared from the elution patterns, but that the presulfate peak was still present. Fifty milliliters of the same urine was chromatographed on DEAE-Sephadex and both the glucuronide and presulfate fractions were subjected to several consecutive hydrolytic procedures, as outlined in Figure 3. DEAE-Sephadex chromatography and Amberlite XAD-2 isolation were used to separate and concentrate the hydrolysis products.
Table 2. Radioactivity Found in the Different Fractions Obtained by DEAE-Sephadex Chromatography of Urine Samples from Patients Who Received Tritiated Cortisol

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Disorder</th>
<th>Uncon.</th>
<th>Glucuron.</th>
<th>Presulfate</th>
<th>Sulfate</th>
<th>Nonspecific</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult no. 1</td>
<td>31 y</td>
<td>♂</td>
<td>obesity</td>
<td>4.9</td>
<td>87.3</td>
<td>4.1</td>
<td>2.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Adult no. 2</td>
<td>55 y</td>
<td>♂</td>
<td>obesity</td>
<td>6.2</td>
<td>90.0</td>
<td>1.7</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Child no. 1</td>
<td>5½ y</td>
<td>♂</td>
<td>C.A.H. c</td>
<td>14.0</td>
<td>72.4</td>
<td>5.7</td>
<td>5.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Child no. 2</td>
<td>1¾ y</td>
<td>♂</td>
<td>C.A.H.</td>
<td>19.9</td>
<td>67.8</td>
<td>3.6</td>
<td>7.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Child no. 3</td>
<td>4 mon.</td>
<td>♂</td>
<td>hypogl. d</td>
<td>14.8</td>
<td>76.5</td>
<td>3.3</td>
<td>4.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Neonate no. 1</td>
<td>2 days</td>
<td>♂</td>
<td>C.A.H. e</td>
<td>29.6</td>
<td>33.3</td>
<td>27.3</td>
<td>8.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Neonate no. 2</td>
<td>2 days</td>
<td>♂</td>
<td>hypogl.</td>
<td>44.5</td>
<td>26.1</td>
<td>17.7</td>
<td>10.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Neonate no. 3</td>
<td>2 days</td>
<td>♂</td>
<td>hypogl.</td>
<td>38.9</td>
<td>29.4</td>
<td>23.7</td>
<td>6.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Neonate no. 4</td>
<td>2 days</td>
<td>♂</td>
<td>C.A.H.</td>
<td>38.4</td>
<td>28.0</td>
<td>20.4</td>
<td>12.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Expressed as a percentage of the total eluted radioactivity.
* Radioactivity not eluted in one of the fractions described.
* Congenital adrenal hyperplasia, non-salt-losing form.
* Hypoglycemia.
* Congenital adrenal hyperplasia, salt-losing form.

products after each step. Figure 3 presents the relative amounts of radioactivity found in each of the fractions obtained by these hydrolytic procedures. The glucuronide fraction mainly consisted of material hydrolyzable by β-glucuronidase. Only a very small part of the radioactive cortisol metabolites present in the presulfate fraction could be hydrolyzed by the mixed-enzyme preparation, Glusulase. The bulk of the material present in this fraction could be hydrolyzed by solvolysis only. Both the glucuronide and presulfate fractions contained small amounts of cortisol metabolites that could not be hydrolyzed by any of the procedures tested.

When the glucuronide fraction of adult urine no. 1 was subjected to Ketodase hydrolysis, the hydrolysis products were recovered almost quantitatively in the unconjugated fraction after DEAE-Sephadex chromatography.

**Discussion**

Inadequate extraction methods have greatly hampered previous investigations into neonatal cortisol metabolism (1–4). Using the modified Amberlite XAD-2 procedure presented here, it is shown that cortisol metabolites can be extracted almost quantitatively from neonatal urine or aqueous fractions of urine.

The DEAE-Sephadex method was introduced to avoid use of solvent–solvent extraction methods to separate free and several types of conjugated steroids. The relative amounts as they were found by this method in the unconjugated fractions of neonatal cortisol metabolites (30–45% of the total urinary radioactivity) were considerably higher than the proportions extracted by Danilescu-Goldenberg et al. (4) and Aarskog et al. (2) from neonatal urine by ethyl acetate extraction (9–17% and 14–27%, respectively). This is attributable to the inability of conventional extraction-based methods or solvent–solvent partition to extract the highly polar neonatal cortisol metabolites effectively.

The amounts of radioactivity found in the neonatal glucuronide fractions by the DEAE-Sephadex method agreed fairly well with the data presented by others (2–4), who used solvent extraction to isolate the cortisol metabolites liberated from the glucuronides by enzymatic hydrolysis. The same authors reported that these extracts predominantly contained corticosteroids of moderate polarity, such as tetrahydrocortisone, which are easily extracted by conventional methods. This explains the good agreement between our data and those reported by these authors for the glucuronide fraction.

From the literature it was already known that neonatal urine contains relatively increased amounts of steroid sulfates (2–4) as compared to the amounts found in adult urine. Our results agree with these data.

The most interesting fraction of the neonatal cortisol metabolites is the presulfate fraction; this fraction was virtually absent from the urine of older children and adults. This fraction may contain a type of sulfate that cannot be hydrolyzed by enzymatic methods, but only by solvolysis, such as 3α- or 6-sulfates. However, it is not clear why such sulfates would elute so much earlier than (e.g.) cortisol-21-sulfate in DEAE-Sephadex chromatography. Therefore, it seems likely that this fraction contains a new, as-yet-unidentified conjugate. Solvolysis seems to be the only way to hydrolyze this conjugate, but because corticosteroids are readily rearranged under solvolytic conditions (12) further study on the applicability of this method seems necessary.

The neonatal urines contained small amounts of radioactivity that behaved like conjugates on DEAE-Sephadex but resisted all hydrolytic methods. Possibly this material might consist of C-21 carboxylic acid steroids, which were identified in adult urine by Bradlow et al. (13). Unfortunately, suitable reference compounds are not available to test this hypothesis.

We conclude that the combination of DEAE-Sephadex chromatography and the modified Amberlite XAD-2 procedure constitutes a rational approach to the difficult problem of analysis for neonatal cortisol metabolites. Furthermore, we have shown that mere enzymatic hydrolysis leaves a considerable fraction of the neonatal corticosteroid conjugates unhydrolyzed. Further study to identify the type of conjugate present in the presulfate fraction is essential for a complete understanding of neonatal cortisol metabolism.

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**References**


