Reversed-Phase C\textsubscript{18} Cartridge for Extraction of Estrogens from Urine and Plasma

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We describe a simple method for extraction of estrogens that is efficient over the whole polarity range of estrogen metabolites in urine and plasma. A small reversed-phase C\textsubscript{18} Cartridge (Sep-Pak\textsuperscript{\textregistered} C\textsubscript{18}, Waters Associates, Inc.) is used for this purpose. After application of the biological fluid, followed by an aqueous wash, estrogens are eluted in 3 mL of methanol. For urine the pH should be kept at 3 before the methanol elution. When estrogens are extracted from plasma, the steroid-protein interaction is counteracted by the presence of triethylammonium sulfate and D-norgestrel at pH 5. The recoveries of added \textsuperscript{3}H-labeled estrogens are essentially quantitative. The capacity of the column is high and estrogens can be extracted from large volumes of pregnancy urine without loss in the washing water. Because of the high flow rate (2 mL/min) and the small volume of methanol in which the estrogens are eluted, the whole procedure is convenient and rapid.

Liquid-liquid extraction has been used for decades for the isolation of estrogens from biological fluids. The main disadvantages of these extraction systems are the formation of emulsions and the low recovery of highly polar estrogen conjugates (1).

A variety of adsorbents have been used for extraction of estrogens (1), and one of the best extraction procedures involved Amberlite XAD-2\textsuperscript{8} (2). Because of changes in the polymer, this procedure was later modified by the introduction of an additional wash with an aqueous solution of 0.5 mol/L triethylamine sulfate, to ensure quantitative recovery of polar steroid conjugates (3). The same column was used for the extraction of plasma by keeping the sample and the resin at 64 °C to minimize protein binding (4). The relative disadvantages of extraction with Amberlite XAD-2 are its low capacity (0.5 g of resin per milliliter of urine) and the low flow rate used (about 0.2 mL/cm\textsuperscript{2} per minute).

A liquid-gel method on Lipidex 1000 was recently described for the extraction of unconjugated steroids of low and medium polarity from aqueous solutions (5). Its advantages are high capacity (1–2 g of gel for 40 mL of urine) and high flow rate (5 mL/min). The evident disadvantage is the loss of the most polar steroids. With the same column, however, glycine- and taurine-conjugated bile acids could be extracted as ion pairs with decytrimethylammonium bromide (6). Ion pairs with quaternary ammonium ions had already been used for the extraction of bile acid conjugates in liquid-liquid systems (7, 8).

In the course of studies aimed at developing a gas-chromatographic/mass-spectroscopic method for quantitative determination of the complete estrogen profile of biological fluids, a rapid and efficient extraction method became necessary. This extraction had to be efficient over a wide range of polarities from the least polar unconjugated estrogens to the most polar double and mixed estrogen conjugates. We describe a new extraction technique based on use of a reversed-phase octadecylsilane column (Sep-Pak\textsuperscript{\textregistered} C\textsubscript{18}) and ion-pair formation with a hydrophobic counter-ion (heteron).

Materials and Methods

Chemicals. The methanol and sulfuric acid (analytical grade), triethylamine (synthetic grade), acetic acid (Suprapur\textsuperscript{\textregistered}) and pyridine (Uvasol\textsuperscript{\textregistered}, redistilled before use) were purchased from E. Merck AG, Darmstadt, F.R.G. Potassium acetate was from Noovy-Baker N.V., Deventer, The Netherlands. Hexamethyldisilazane was from Pierce Chemical Co., Rockford, IL 61105, and was of specially purified grade. Tri-methylchlorosilane was from Fluka AG, Buchs SG, Switzerland, and was redistilled before use. Helix pomatia juice was obtained from Réactifs IBF, France. Decytrimethylammonium bromide (DTMABr)\textsuperscript{1} was from Eastman Kodak Co., Rochester, NY 14650. The scintillation liquid used was ACS (Aqueous Counting Scintillant; Amersham Corp., Arlington Heights, IL 60005). D-(++)-Norgestrel was from Leiras Oy, Turku, Finland.

The following potassium acetate buffers were used: 1.5 mol/L, pH 3; 1.5 mol/L, pH 5; 0.15 mol/L, pH 3; and 0.15 mol/L, pH 5.

The 0.5 mol/L solution of triethylammonium sulfate (TEAS) was prepared by adjusting the pH of 7.0 mL of triethylamine to pH 5 (measured with a glass electrode against an aqueous reference buffer) with 1 mol/L sulfuric acid and diluting to 100 mL with water.

DTMABr was used as 0.9 mol/L aqueous solutions. D-Nortestral was used as a 4 mmol/L solution in methanol.

Labeled estrogens. [2,4,6,7-\textsuperscript{3}H]Estrone (E\textsubscript{1}) (about 97 kCi/mol), [2,4,6,7-\textsuperscript{3}H]estradiol (E\textsubscript{2}) (about 108 kCi/mol), and [6,7-\textsuperscript{3}H]estrone sulfate (about 50 kCi/mol) were from New England Nuclear, Boston, MA 02118. [6,9-\textsuperscript{3}H]Estriol-16α-glucuronide (E\textsubscript{3}-16G) (20 kCi/mol) was from the Radioc hemical Centre, Amersham, U.K. Estriol-3-sulfate-16β-glucuronide (E\textsubscript{3S}-3S,16G) was synthesized from [6,9-\textsuperscript{3}H]E\textsubscript{3}-16G according to Levitz et al. (9).

The radioactivity of added estrogens ranged from 16 to 70 nCi.

Measurement of radioactivity. Tritium activity was measured with a liquid scintillation counter (Rackbeta 1215, LKB-Wallac, Turku, Finland) calibrated to correct for quenching. Results were calculated from disintegrations per minute.

Samples. We used 24-h urines from women in late pregnancy, from nonpregnant women, and from men. The plasma pool was collected from men and nonpregnant women.

Apparatus. Octadecylsilane bonded-phase packings (Sep-Pak C\textsubscript{18} cartridges, 9 mm × 10 mm, i.d.) were purchased from Waters Associates, Inc., Milford, MA 01757. Before use we washed the columns with 10 mL of water, then 5 mL of methanol, then 10 mL of water.

\textsuperscript{1} Nonstandard abbreviations used: TEAS, triethylammonium sulfate; DTMABr, decytrimethylammonium bromide; E\textsubscript{1}, estrone; E\textsubscript{1}-16G, estriol-16α-glucuronide; E\textsubscript{3S}-3S, estrone-3-sulfate; E\textsubscript{3S}, estradiol-17β; GC, gas chromatography; GC/MS, gas chromatography/mass spectroscopy.

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We maintained a flow rate of about 2 mL/min with the aid of injection syringes or compressed air; the latter procedure permitted the simultaneous elution of several columns.

Procedures

Urine: The urine samples were equilibrated with labeled estrogens for 30 min at room temperature before further processing. Three different extraction procedures were used.

1. Apply 10 mL of urine to the column, then wash it with 10 mL of water. Elute the estrogens with 3 mL of methanol.
2. Apply to the column 9 mL of urine buffered with 1 mL of 1.5 mol/L acetate buffer, pH 3, then wash it with 10 mL of 0.15 mol/L acetate buffer, pH 3.
3. Apply to the column 90 mL of urine buffered with 10 mL of the same acetate buffer, then wash and elute the sample as in procedure 2.

Plasma: The plasma samples were equilibrated with labeled estrogens for 30 min at room temperature before further processing. Five different procedures were used.

1. Dilute 5 mL of plasma with 4 mL of water and 1 mL of 1.5 mol/L acetate buffer, pH 3, and apply this to the column; then wash the column with 10 mL of 0.15 mol/L acetate buffer, pH 3. Elute estrogens with 3 mL of methanol.
2. Dilute 5 mL of plasma with 2 mL of 1.5 mol/L acetate buffer, pH 5, and 1.2, 2.4, or 10 mL of 0.5 mol/L TEAS (final TEAS concentrations 30, 60, and 250 mmol/L, respectively); add water to make 20 mL final volume. After letting it stand 20 min at room temperature, apply the sample to the column, then wash with 10 mL of 0.15 mol/L acetate buffer, pH 5. Elute estrogens with 3 mL of methanol.
3. Dilute 5 mL of plasma with 2 mL of 1.5 mol/L acetate buffer, pH 5, and 0.67, 1.33, or 5.6 mL of 0.9 mol/L DTMABr (final DTMABr concentrations 30, 60, and 250 mmol/L, respectively), add water to make 20 mL. Further treatment is as in plasma procedure 2.
4. Mix 5 mL of plasma and 100 nmol of D-norgestrel and allow to stand for 30 min at room temperature. Then dilute with 3 mL of water, 2 mL of 1.5 mol/L acetate buffer, pH 5, and 10 mL of 0.5 mol/L TEAS. Further treatment is as in plasma procedure 2.

Re-Usability of the Sep-Pak C18 Cartridge

The re-usability of the Sep-Pak C18 cartridge was studied by repeated extractions on the same column. Urines were extracted with method 2. After each extraction the cartridge was regenerated with 10 mL of distilled water, 5 mL of methanol, and again 10 mL of distilled water (procedures A-E). However, different regeneration procedures were also tested (procedure F). The whole experimental protocol consisted of the following procedures.

A. The methanol fraction of a blank extraction with distilled water was analyzed by gas chromatography and mass spectrometry (GC and GC/MS) and used as reference for comparison with urine extractions.

B. A 9-mL portion of late-pregnancy urine was extracted on a Sep-Pak C18 cartridge. The methanol fraction was then enzymically hydrolyzed with *Helix pomatia* digestive juice and re-extracted on the same cartridge. Finally a blank was extracted and the methanol fraction was analyzed by GC and GC/MS.

C. A 9-mL portion of non-pregnancy female urine was processed as described in B.

D. Five 9-mL portions of late-pregnancy urine—to which radiolabeled E2-16G standard had been added—were extracted successively on the same Sep-Pak C18 cartridge. Aliquots of the methanol fractions were taken for radioactivity measurement.

E. The rest of the methanol fractions obtained with the above procedure were hydrolyzed with *Helix pomatia* and the hydrolysates were successively extracted on the same Sep-Pak C18 cartridge. Radioactivity was measured in an aliquot of the methanol fraction of each extraction. A blank extraction was finally performed, the methanol fraction of which was analyzed by GC and GC/MS.

F. Three 9-mL portions of late-pregnancy urine were processed as in procedure B. The difference was that after each extraction the column was regenerated by increasing the methanol wash (between the two 10-mL water washes) to 10 mL, 20 mL, and 50 mL, respectively.

Hydrolysis: The methanol fraction of the Sep-Pak C18 extraction was evaporated to dryness and redissolved in 5 mL of acetate buffer, 0.15 mol/L pH 4.1. Fifty microliters of *Helix pomatia* were added to give a concentration of 1.007 U/mL and the sample was incubated overnight at 40 °C (10, 11).

Derivatization: The trimethylsilyl derivatives of estrogens were formed by adding to the dry residue 250 μL of pyridine/hexamethyldisilazane/trimethylchlorosilane (9/3/1 by vol) and incubating for 90 min at 60 °C (10, 12).

GC/MS analysis. We used a Perkin-Elmer Sigma 1 analyzer housed with 25-m SE-50 and OV-210 WCOT capillary column, a flame-ionization detector, and H2 carrier gas.

*GC/MS* analysis. We used a Hewlett-Packard 5990A GC/MS system equipped with a 25-m WCOT OV-101 capillary column. The selected-ion monitoring technique was used to monitor the characteristic fragment ions of estradiol, which is the most abundant urinary estrogen.

Results and Discussion

Octadecylsilane-bonded columns have already been used with great success in reversed-phase liquid chromatography (13). The compatibility of the C18 column with aqueous mobile phase (biological fluid), the rapidity of the analyses, and the possibility of trace enrichment make it suitable for study as an alternative extraction method for estrogens.

Extraction of Estrogens from Urine

Labeled E1, E2-16G, and E2-3,5,16G were selected to represent the whole range of estrogens with different polarities excreted in urine. Urines with high (late-pregnancy) and low (male) estrogen content were used. The results are summarized in Table 1. When a water wash was used without pH adjustment, E1 and E2-16G were recovered quantitatively in the methanol fraction, but almost 30% of the E2-3,5,16G was lost in the water wash. However, when the pH was kept at 3 with the aid of acetate buffer, E2-3,5,16G was not lost in the buffer wash, but was recovered quantitatively in the methanol fraction. The low pH partly suppressed the ionization of this highly polar conjugate and, by rendering it more neutral, enhanced its adsorption on the stationary phase.

To check the capacity of the C18 cartridge, we loaded it with 90 mL of late-pregnancy urine. The recovery of labeled E2-16G

| Table 1. Extraction of Labeled Estrogens Added to 9 mL of Late-Pregnancy and Male Urine |
|-----------------------------------------------|-----------------|-----------------|
| Late-pregnancy urine (n = 5)                  | Male urine (n = 5) |
| [3H] E1                                       | 97.8 (2.92)     | 98.4 (2.30)     |
| [3H] E2-16G                                   | 97.4 (3.41)     | 99.1 (1.84)     |
| [3H] E2-3,5,16G                               | 95.8 (1.90)     | 95.9 (2.86)     |

Urine buffered with 1 mL of 1.5 mol/L acetate buffer, pH 3, was applied to the reversed-phase C18 column, which was then washed with 10 mL of 0.15 mol/L acetate buffer, pH 3. Estrogens were eluted with 3 mL of methanol. Flow rate was 2 mL/min.
in the methanol fraction was about 90%. It therefore seems probable that larger volumes of non-pregnancy urine can be applied to the column without loss of steroids. The high concentration coefficient obtained with this column is favorable when the aim is to quantify the complete profile of estrogens in non-pregnancy urine by GC or GC/MS. The amount of urine that can be handled is sufficient to meet the sensitivity requirements of the quantification methods.

The methanol fraction eluted about 3% of the total urinary solids, mainly colored urinary compounds. The urine effluent was colorless, and contained about 80% of the solid materials, mainly salts. The remaining 15% was eluted in the buffer wash.

**Extraction of Estrogens from Plasma**

In experiments with plasma, because of the protein–steroid interaction, we included two more labeled estrogens: E₁-S, an estrogen known to be tightly bound to proteins such as albumin, and E₂, an estrogen specifically bound to the sex-hormone-binding globulin.

The results of the first experiments, made in conditions similar to those used for urine, are shown in Table 2. The recoveries of E₁, E₁-S, and E₂-16G, though relatively satisfactory, were not quantitative, about 10% being lost in the plasma effluent. About 90% of E₂-3S,16G was lost in the plasma effluent. The use of pH 3 obviously did not completely suppress the ionization of this very acidic conjugate and thus did not inhibit ion-pair formation with the amino groups of proteins. We therefore investigated the possibility of using a hydrophobic hetaeron at a pH near the isoelectric point of albumin to compete with it for ion-pair formation with estrogens. Moreover, ion-pair formation with hydrophobic hetaeron would enhance the retention of estrogens on the stationary phase.

Two different hetaeras were tested: DTMABr, a quaternary ammonium salt previously used for the extraction of bile acids on Lipidex 1000, and TEAS, previously used to elute polar steroid conjugates from Amberlite XAD-2. Because both parabolic and hyperbolic dependence of the capacity factor on hetaeron concentration have been observed (6,14), three different concentrations of the hetaeras were tested; the pH was 5, to be near the isoelectric point of albumin. The results are summarized in Table 3. With DTMABr the capacity factor showed a parabolic dependence on the concentration of the hetaeron, a result in agreement with previous observations in the extraction of bile acids (6). With TEAS, in contrast, the capacity factor showed hyperbolic dependence, the three concentrations of the hetaeron being in the plateau region of the curve, where the effect of the hetaeron on retention was greatest. The differential effect of the concentration and nature of the different hetaeras on the capacity factor has been explained extensively by Horvath et al. (14). From the results it was obvious that TEAS was more suitable for use. The advantage of working in the plateau region of the curve is that there the capacity factor is least sensitive to small fluctuations in hetaeron concentration. Consequently, we used 0.25 mol/L TEAS, although smaller concentrations would have been equally effective, the recovery of E₂-16G being about 95% with all three concentrations used (Table 3).

When labeled E₂ was equilibrated with plasma and thereafter extracted with TEAS, almost 50% was lost in the plasma effluent (Table 4), primarily because of specific binding to sex-hormone-binding globulin. Because 2-methoxyestradiol has been shown to be bound to this protein with an even higher affinity than testosterone and E₂ (relative affinities 2, 1, and 0.39, respectively) (15), we tested two different extraction procedures for plasma to ensure the quantitative recovery of this group of estrogens in the methanol fraction. In one, plasma was incubated for 30 min at 64 °C, in the other 100 nmol of D-norgestrel was added to the sample to compete for the binding sites (16). The results are shown in Table 4. Incubation at 64 °C did not significantly increase the recovery of E₂, probably because the presence of bound steroid protects the binding site (17). In contrast, addition of D-norgestrel dramatically increased the recovery of E₂, to about 95%. Because D-norgestrel is separated from estrogens in anion-exchange chromatography (10,18) and so does not interfere with the quantitative determination of estrogens in GC or GC/MS, we decided to use it in the final plasma extraction method.

Table 5 shows the recoveries of all representative labeled estrogens extracted by the procedure finally adopted for

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**Table 2. Extraction of Labeled Estrogens Added to 5 mL of Plasma**

<table>
<thead>
<tr>
<th>Mean recovery (and SD), % *</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H] E₁</td>
<td>92.1 (1.89)</td>
</tr>
<tr>
<td>[³H] E₁-S</td>
<td>87.9 (2.39)</td>
</tr>
<tr>
<td>[³H] E₂-16G</td>
<td>87.4 (1.71)</td>
</tr>
<tr>
<td>[³H] E₂-3S,16G</td>
<td>8.2 (9.5)</td>
</tr>
</tbody>
</table>

Plasma, diluted with 4 mL of water and 1 mL of 1.5 mol/L acetate buffer, pH 3, was applied to the column, which was then washed with 10 mL of 0.15 mol/L acetate buffer, pH 3. Estrogens were eluted with 3 mL of methanol. Flow rate was 2 mL/min. * n = 5 for each estrogen.

**Table 3. Effect of Concentration and Nature of the Hetaeron on Extraction of Labeled E₂-3S,16G Added to Plasma**

<table>
<thead>
<tr>
<th>Conc, nmol/L</th>
<th>DTMABr</th>
<th>TEAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>W</td>
</tr>
<tr>
<td>30</td>
<td>8.1</td>
<td>3.7</td>
</tr>
<tr>
<td>60</td>
<td>7.1</td>
<td>1.4</td>
</tr>
<tr>
<td>250</td>
<td>78.7</td>
<td>6.7</td>
</tr>
</tbody>
</table>

P = plasma effluent; W = wash with 0.15 mol/L acetate buffer, pH 5 (10 mL); Me = methanol (3 mL).

**Table 4. Extraction of Labeled E₂ Added to Plasma**

<table>
<thead>
<tr>
<th>Recovery, %</th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma effluent</td>
<td>51.9</td>
<td>52.3</td>
<td>48.5</td>
</tr>
<tr>
<td>Buffer wash</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Methanol</td>
<td>42.9</td>
<td>47.3</td>
<td>51.2</td>
</tr>
</tbody>
</table>

Method 1: plasma extraction procedure. Method 2: same as 1, but heated before addition of TEAS. Method 3: same as 1, but plasma incubated with D-norgestrel. See text for specific details.

**Table 5. Effect of Norgestrel and TEAS on Extraction of Labeled Estrogens Added to Plasma**

<table>
<thead>
<tr>
<th>Mean recovery (and SD), % *</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H] E₁</td>
<td>95.8 (1.97)</td>
</tr>
<tr>
<td>[³H] E₂</td>
<td>94.4 (1.99)</td>
</tr>
<tr>
<td>[³H] E₁-S</td>
<td>93.7 (2.55)</td>
</tr>
<tr>
<td>[³H] E₂-16G</td>
<td>98.1 (1.92)</td>
</tr>
<tr>
<td>[³H] E₂-3S,16G</td>
<td>95.9 (3.64)</td>
</tr>
</tbody>
</table>

* n = 5 for each estrogen.

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plasma. They were all essentially quantitative, regardless of polarity or the degree of protein binding of the different estrogens.

Re-Usability without plasma.

These sensitive chromatograms were producible.

The extraction of a blank on the Sep-Pak C18 cartridge without any previous extraction of urine on the same cartridge (procedure A) gave a very clean gas chromatogram. The gas chromatograms obtained when analyzing the methanol fractions of the blank extractions of procedures B, C, E, and F—after previous extraction of urine on the Sep-Pak C18 cartridges—did not show any additional peaks. The GC/MS analysis of the same fractions with the highly selective and sensitive selected-ion monitoring technique detected about 3 ng of estril in procedures B and E, respectively. Because these procedures involved analysis of late-pregnancy urine, containing about 25 mg of estril per 24-h urine, the cross-contamination can be considered minimal. However, in procedure F, where 10, 20, and 50 mL of methanol was used for washing of the column, 2700 pg, 50 pg, and no detectable quantities, respectively, of estril were obtained. In procedure C, where non-pregnancy urine was used, no estril fragment ions were detected with GC/MS.

The recovery of the radioactively labeled E2-16G standard obtained from five successive urine extractions on the same Sep-Pak C18 cartridge (procedure D) was identical to the results obtained when five urines were extracted separately on five different Sep-Pak C18 cartridges (Table 1). However, when the hydrolysates of five urines were extracted on the same Sep-Pak C18 cartridge (procedure E), the recovery of the hydrolyzed labeled E2-16G standard was 3 to 5% lower in every sequential extraction. In addition, higher pressure was necessary to obtain the same flow rate because of accumulation of solid material on the filter of the cartridge, derived from denaturation of proteins of the crude Helix pomatia digestive juice. This accumulation obviously produced absorption sites or traps resulting in reduction of the recovery of the subsequent extraction.

We conclude that Sep-Pak C18 cartridge can be used for extractions for at least five times after proper regeneration. However, if the biological fluid contains particles that block the filter so that increased pressure is needed to obtain the optimum flow rate, the cartridge is not suitable for further extraction—the recovery will probably be lower and not reproducible. The capillary GC did not show any cross-contamination of the subsequent extractions on the same column. However, GC/MS analysis with the selected-ion monitoring technique revealed a little cross-contamination when urines with very high estrogen content (late-pregnancy urines) were used. This cross-contamination was eliminated by increasing the volume of the methanol wash to 50 mL during regeneration. However, it would be wise not to extract biological fluids of high and low estrogen content on the same cartridge. When non-pregnancy female urine was used, no cross-contamination was evident, even with the highly sensitive selected-ion monitoring technique.

In summary, extraction of estrogens from urine and plasma by the method described has considerable advantages over previous methods. The time of extraction is shorter, owing to the high flow rate (2 mL/min). The volume of methanol with which estrogens are eluted from the column is as small as 3 mL, which considerably reduces the time required for evaporation of the eluate. Extraction is equally efficient over the whole polarity range of estrogen metabolites. The capacity of the reversed-phase C18 cartridge is remarkably high for all the estrogen metabolites, permitting analysis of large sample volumes. The great problem of steroid–protein interaction in plasma is avoided, which makes it possible to dispense with procedures such as heating or denaturation of protein.

We thank Ulla-Maj Pomoell, M.Sc., for her valuable advice during this study. The work was supported by a grant from the Ford Foundation, New York, and contract CB 74104 from the National Cancer Institute through the Breast Cancer Task Force Committee.

Note added: After this work had been submitted for publication, a study dealing with the extraction of urinary steroids on Sep-Pak C18 appeared in the literature (Shackleton, C. H. L., and Whitney, O. J., Use of Sep-Pak cartridges for urinary steroid extraction. Evaluation of the method for use prior to gas-chromatographic analysis. Clin. Chim. Acta 107: 231–243, 1980). However, plasma was not tried in the study and the work was confined mainly to free and monoconjugated steroids. Mixed and double-conjugated steroids were not studied. Their results for urine samples agree well with the results presented here.

References