Biuret values are extremely high (Table 1) and in the same range as those measured previously when the peptide fragments were taken into account (1).

The absorbance spectra of the Biuret chromogen in a urine sample and an albumin solution are shown in Fig. 1A. The profiles are directly comparable, and there were no anomalous peaks at 550 nm, the wavelength used in the assay.

The Biuret assay showed quantitative recovery (99.3% ± 0.7%; n = 6) of albumin added to human diabetic urine (for controls, the recovery was 98.7% ± 0.5%; n = 5), demonstrating that the urine matrix does not affect the chromogen and thus validating the use of the Biuret assay to measure total protein concentration of urine samples.

To test whether the matrix of the urine (or urinary pigment) contributes to the Biuret chromogen, diabetic urine samples were filtered through an Amicon/Millipore membrane with a molecular mass cutoff of 500 (cat. no. 13022). This filtration indicated that most of the proteinaceous material (85.8% ± 9.4%; n = 5) was retained. Filtration of urine through an Amicon/Millipore membrane with a molecular mass cutoff of 10 000 (cat. no. 13622) gave a recovery of 96.5% ± 0.9% (n = 5) of the Biuret-reactive material in the filtrate. These experiments demonstrate that the protein fragments in diabetic human urine for the samples studied have molecular masses mainly between 500 and 10 000 Da. Similar results were obtained for control urines, where the membrane with a molecular mass cutoff of 500 retained 81.8% ± 6.4% (n = 6) of the material, whereas filtration through the membrane with a cutoff of 10 000 recovered 97.7% ± 0.8% (n = 6) in the filtrate.

The HPLC profile of a control urine sample is shown in Fig. 1B (peaks measured by absorbance at 214 nm were also apparent when measured at 278 nm; not shown). Urine retained by the filter after filtration through a membrane with a molecular mass cutoff of 500 gave a similar profile (not shown). The whole profile was altered substantially by proteolytic digestion of the urine sample by trypsin (Fig. 1C) or Glu C, another endoproteinase (not shown). These studies demonstrate that the HPLC peaks in human urine are proteinaceous materials.

Overall, these studies demonstrate that large quantities of low-molecular mass protein-derived material exists in urine, which had not been recognized previously.

We would like to acknowledge the kind assistance of Shane Reeve and Dr. Ian Smith of the Baker Institute, Melbourne, Australia, in performing the proteolytic digestions.

References

Olanzapine is an atypical antipsychotic drug now considered as a first-line agent to treat schizophrenia and psychotic mood disorders (1). Plasma concentrations indicative of a clinical response are known to be >9 µg/L (2). Knowledge of plasma concentrations, to check compliance or drug-drug interactions, is also necessary in treatment of schizophrenia (3). Moreover, olanzapine toxicity may appear at blood concentrations that are considerably lower than those observed in antidepressant-related deaths (4).

Numerous publications have described the usefulness of a HPLC silica column and aqueous methanol eluents for the analysis of many basic drugs in plasma (5, 6). The use of this type of column provides more reproducible results, making it possible to obtain a sensitive HPLC-ultraviolet (UV) procedure that is easier to perform and less expensive than electrochemical (7, 8) or mass spectrometric (9) detection. We have adapted this HPLC system for routine therapeutic drug monitoring of psychotropic drugs (10–12). To update this system, it is important to include new antipsychotic drugs, such as olanzapine.

The Dionex HPLC system used consisted of a Dionex injector (Model ASI-100), an isocratic pump (Model P-580A), and a photodiode array detector (Model UVD-170S). Chromatograms were processed using the Chromeleon™ chromatographic data collection and analysis system.

Blood samples were collected in tubes containing lithium heparin as an anticoagulant. After centrifugation for 10 min at 3000g, the plasma was immediately separated, supplemented with 250 g/L ascorbic acid (10 µL/mL of plasma), and stored at −20 °C until analysis.

Olanzapine was extracted from 1 mL of serum after the addition of 0.2 mL of bicarbonate buffer (pH 10.5), 20 µL of internal standard working solution (demethylated metabolite of trimipramine; 2 µg/L), and 5 mL of a mixture of hexane–isoamyl alcohol (98:2 by volume). The mixture was shaken for 15 min and centrifuged at 3000g for 5 min. The aqueous layer was discarded, and the organic layer...
was transferred to another glass tube and back-extracted after acidification with 1 mL of 0.2 mol/L sulfuric acid. After shaking and centrifugation, the aqueous layer was alkalized and reextracted with the same mixture. The organic phase was evaporated to dryness under a stream of nitrogen, the residue was dissolved in 100 μL of mobile phase, and 50 μL was injected into the chromatographic system.

All analyses were performed on an Ultremex silica column [250 × 4.6 mm (i.d.); Phenomenex]. The mobile phase consisted of a methanol–deionized water mixture (70:30 by volume) containing 0.110 mL/L butylamine. The mobile phase was filtered through a 0.22 μm filter and degassed before use. The chromatography was carried out at ambient temperature at a flow rate of 1 mL/min. Peaks were monitored at 273 nm.

For calibration, a six-point calibration curve was constructed before each series of assays with calibrators prepared by adding different volumes of olanzapine working solution into drug-free serum to obtain a concentration range of 1.25–80 μg/L. Olanzapine plasma concentrations were quantified using linear regression of response (drug/internal standard peak height ratios) vs concentration. Serum samples, prepared in advance by adding 5 and 40 μg/L olanzapine to a pooled serum and then aliquoting in Eppendorf tubes and storing at −20 °C, were used as quality-control samples.

The retention times of olanzapine and the internal standard were 3.9 and 11.6 min, respectively (Fig. 1). The limit of quantification, defined as the lowest concentration that could be calculated with a CV <10%, was 1 μg/L (n = 10). This detection limit was similar to those reported for an electrochemical detector, i.e., 1 μg/L (8) and 1.2 μg/L (3), but was lower than the limit of quantification for UV detection (6), i.e., 1.56 μg/L. However, some authors have obtained a lower quantification limit (0.25 μg/L) with electrochemical (7) and mass spectrometric detection (9).

The linearity of the extraction procedure was verified over the calibration range by measuring drug-free plasma supplemented with known concentrations of olanzapine. The slope, y-intercept, and correlation coefficient for different calibration curves were 0.012 ± 0.004, 0.069 ± 0.0269 μg/L, and 0.9962 ± 0.0037, respectively. Thus, calibration curves were linear over the range 1.25–80 μg/L.

The absolute recovery of olanzapine was obtained by comparing the peak height of extracted and nonextracted supplemented solutions. The mean extraction recovery was 67.7% ± 10.5% (n = 5). These values were comparable to those obtained by the same extraction method with tricyclic antidepressants (10, 11) or clozapine (12). The three extraction steps, although time-consuming, provided high-purity extracts and increased column longevity.

Precision was estimated from intra- and interday assay variations. The intraday assay variation was determined by analyzing six aliquots of supplemented samples containing 5 and 40 μg/L olanzapine with a calibration curve on the same day. The interday variation was determined by analyzing supplemented serum (5 and 40 μg/L) on 10 different days with an independent calibration curve on each day (Table 1). No chromatographic interference was observed between olanzapine and commonly used psychotropic drugs (amitriptyline, clomipramine, fluoxetine, clozapine, flunitrazepam, levopromazine). The analysis method was applied to monitor plasma samples collected from a small number of patients with chronic schizophrenia. A wide concentration range was observed (3.6–89.4 μg/L) for administered doses between 10 and 20 mg. For a complete analysis, it is important to add more individual data and elements of clinical response in a larger number of patients (13).

As illustrated in our study, the HPLC-diode array detection method with an unmodified silica column and hydrophilic eluents is a powerful and sensitive tool for the efficient separation and identification of psychotropic drugs in plasma. The HPLC analysis requires only 15 min for each sample. With the concomitant use of UV spectral analysis, this system is well suited for routine drug monitoring of multiple, concomitantly used medications, such as sedatives and antidepressants.

<table>
<thead>
<tr>
<th>Added concentration, μg/L</th>
<th>Measured concentration, μg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday (n¹ = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>5.3</td>
<td>9.4</td>
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<tr>
<td>40.0</td>
<td>37.9</td>
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<td>Interday (n¹ = 10)</td>
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<td></td>
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<tr>
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<td>12</td>
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<tr>
<td>40.0</td>
<td>40.7</td>
<td>8.9</td>
</tr>
</tbody>
</table>

¹ n, number of determinations.
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References


Diurnal Variations in Serum and Urine Markers of Type I and Type III Collagen Turnover in Children, Ole D. Wolthers,1* Carsten Heuck,2 and Lene Heickendorff,3 (1 Children’s Clinic Randers, DK-8900 Randers, Denmark; 2 Department of Paediatrics and Institute of Experimental Clinical Research, Medical Research Laboratories, and 3 Department of Clinical Biochemistry, Aarhus University Hospital, DK-8000, Aarhus, Denmark; * address correspondence to this author at: Children’s Clinic Randers, Dytmaersken 9, DK-8900 Randers, Denmark; fax 45-86-43-33-95, e-mail o.d.wolthers@dadlnet.dk)

New serum and urine markers of type I and type III collagen turnover have recently been introduced in children. These markers include the formation markers of type I collagen turnover, serum N-terminal (PINP) and C-terminal (PICP) propeptides of type I procollagen, and serum N-terminal propeptide of type III procollagen (PIIINP), as well as the resorption markers serum cross-linked C-terminal telopeptide of type I collagen (ICTP) and urine cross-linked N-telopeptides of type I collagen (Ntx) and deoxypyridinoline (DPD) (1–5). The aim of the present study was to assess diurnal variations in serum PINP, PICP, ICTP, PIIINP, and urine DPD and Ntx in children.

Two boys and five girls 10.4–14.4 years (mean, 12.2 years) were studied. One boy and three girls were in puberty. Height SD scores varied from −2.5 to 2.2 (mean, 0.6) and weight SD scores from −1.4 to 1.0 (mean, −0.4). The study was approved by the local ethics committee, and informed consent was obtained from all children and their parents.

The first sample was urine collected from 2400 to 0800 on the morning of the day of investigation. Thereafter, urine was collected in 4-h intervals until 2400 and in another 8-h interval from 2400 to 0800. Blood samples were taken at 0900 and every 2 h thereafter until 0700 the following morning. The samples were centrifuged at 3000g for 10 min within 1 h after they were collected. After centrifugation, the samples were stored at −80 °C and batch-assayed at the completion of the study.

Each child received breakfast at 0815, lunch at 1300, an ice cream at 1630, and dinner at 1900. Sleep was permitted from 2400 to 0730.

Serum concentrations of PICP, PINP, ICTP, and PIIINP were determined by specific RIAs based on human antigen (Orion Diagnostica) (1–3). Intra- and interassay variations were 3.5–3.9% and 4.1–7.2%, respectively. Urine DPD was measured by a solid-phase chemiluminescent enzyme immunoassay on an automated instrument (Immulite Pyrilinks-D; Diagnostic Products Corporation) (4). Urine Ntx was measured by the Osteomark immunoassay (Oste) (5). Intra- and interassay variations were 8% and 9% for the DPD assay, respectively, and 8% and 12% for the Ntx assay, respectively.

Data are described as percentages of the overall day mean ± SE of the mean in the 24-h profile. To evaluate the 24-h profiles one-way ANOVA for repeated measurements was performed followed by the Student-Newman-Keuls method for all pairwise multiple comparisons. The 5% level of significance was used.

PICP and ICTP were relatively low during the day (Fig. 1) with increased PICP concentrations from 0100 to 0500 (P = 0.006; F = 5.0) and ICTP concentrations from 0100 to 0700 (P = 0.002; F = 6.2). Peak concentrations of PICP (mean ± SE) occurred at 0500 [342.0 μg/L (91.6 μg/L)] and trough concentrations at 1100 [286.0 μg/L (46.4 μg/L); P = 0.01]. Peak concentrations of ICTP were detected at 0700 [12.1 μg/L (1.4 μg/L)] and trough concentrations at 2100 [10.3 μg/L (1.3 μg/L); P = 0.02]. No significant variations in PINP (F = 2.1; P = 0.17) or PIIINP (F = 2.1; P = 0.15) were detected.

A significant diurnal variation in urine DPD (F = 15.1; P < 0.001) and Ntx (F = 8.2; P < 0.001) was found. Peak concentrations of DPD occurred in urine collected at 0800–1200 [19.7 nmol/mmol (1.6 nmol/mmol)] and trough concentrations in urine collected at 2000–2400 [12.4 nmol/mmol (1.0 nmol/mmol); P < 0.01]. DPD in the urine collected at 0800–1200 and two samples collected at 2400–0800 did not vary, whereas DPD concentrations in each of these periods were higher than in the samples collected at 1200–1600, 1600–2000, and 2000–2400 (P...