We optimized an RIA for measurement of arginine-vasopressin (AVP) in plasma by use of 100-mg Isolute C18 columns for extraction, addition of a preincubation step, and use of maximal dilution of a commercially available antiserum. The detection limit was 0.06 ng/L when 0.5 mL of acidified plasma was extracted. The within- and between-run CVs (n = 16) at physiological concentrations were 5.8–10.2% and 6.5–11.7%, respectively. Prolonged storage of blood at 25 °C, but not at 4 °C, led to a significant increase in measured plasma AVP concentrations. When plasma samples were prepared at several centrifugation speeds, plasma AVP was significantly correlated with the platelet count (r = 0.899; P < 0.001). This emphasizes the need for careful sample preparation to avoid contamination of plasma with platelet-bound AVP. Basal plasma AVP in 203 children and adolescents (105 males and 98 females; ages, 1 day to 18 years) averaged 1.1 ± 0.6 ng/L. There was no significant difference between males and females and no correlation with age. In 16 healthy adult controls, plasma AVP averaged 1.0 ± 0.5 ng/L.

Interfering factors and low plasma AVP concentrations necessitate extraction and concentration of AVP before RIA. Several extraction and RIA procedures have been reported (9–13). However, there is still a lack of methods combining high sensitivity, small sample volume, and an appropriate physiological detection range. We sought to develop a procedure that optimizes AVP measurement, to identify possible causes for the variations in reported AVP mean values (i.e., whether the premeasurement treatment of blood samples, such as storage and separation, had an influence on plasma AVP concentrations), and to provide reference values for children. We therefore developed an AVP measurement procedure that included certain premeasurement steps, extraction and concentration of AVP from plasma with a 100-mg Isolute C18 column, and the use of a commercially available antigen and antiserum for the RIA. This method combines a very low detection limit and small sample volumes. In addition, we demonstrated the influence of blood storage and centrifugation speed on plasma AVP and provided reference values for children close to those published recently for adults.
**Materials and Methods**

**Sample preparation.** Blood was collected into polyethylene tubes containing K₂EDTA as anticoagulant and cooled immediately at 4 °C. The plasma was separated by centrifugation (3600g at 4 °C for 20 min) within the next 15 min, and then frozen and stored at −20 °C. Before analysis by RIA, within a period of 6 weeks at the maximum, the thawed plasma was centrifuged once again.

**Sample extraction.** Isolute C₁₈ columns (100 mg; Jones Chromatography) were attached to a vacuum manifold (Vac Elut SPS 24; Analytichem International), and were activated with 2 mL of methanol (>99.8%, analytical grade) and equilibrated with 2 mL of deionized water to prevent the columns from running dry. The vacuum manifold allowed us to use as many as 24 columns simultaneously. Plasma (0.5 mL) was acidified with 50 μL of 1 mol/L HCl to pH 3.5. A 0.5-mL aliquot of this acidified plasma was loaded onto a column and allowed to pass through at a rate of 50 μL/min. The columns were then washed with 3 mL of acetic acid (0.67 mol/L) and allowed to run dry by means of suction for 15 min. Elution was carried out by leaving 0.5 mL of methanol containing 1.0 g/L trifluoracetic acid in contact with the sorbent for at least 6 min. The eluates were evaporated to dryness by a vacuum centrifuge (Univapo 150 H; Uniequip).

AVP RIA. Residues were reconstituted in 250 μL of 0.05 mol/L phosphate buffer (pH 7.5) containing 2.5 g/L bovine serum albumin, 0.01 mol/L Na₂EDTA, and 1 g/L neomycin sulfate. Calibrators were assayed in triplicate; samples were assayed in duplicate. Polyclonal AVP antiserum (25 μL) in an eightfold higher final dilution than that recommended by the manufacturer (Amersham) was added to 100 μL of calibrator or sample. All samples and calibrators were incubated in polyethylene tubes for 24 h at 4 °C. Diluted 125I-labeled AVP (25 μL; 1500 cpm/25 μL; specific activity, 74 TBq/mmol), purchased from Amersham, was then added, and the mixture was incubated for 16 h at 4 °C. To separate the AVP, 0.5 mL of 2.5 g/L activated charcoal coated with 0.25 g/L dextran dissolved in 0.05 mol/L phosphate buffer (pH 7.5) were added to the calibrators and samples, and the mixture was centrifuged immediately (3600g at 4 °C for 30 min). Supernatants were removed, and the radioactivity of the pellets was measured for 20 min (Cobra II-Counting-Systems; Packard Instruments; >74% counting efficiency). All values obtained were corrected for recovery.

**Premeasurement Sample Treatment**

**Effect of centrifugation on plasma platelet counts and plasma AVP concentrations.** A 20-mL blood sample was taken from each of five healthy adult volunteers (ages, 23–50 years; all nonsmokers) and divided into five aliquots. One aliquot per volunteer was centrifuged at 200g, 1000g, 1850g, or 6200g (at 4 °C), and one aliquot per volunteer was centrifuged once at 1250g and then twice at 2100g (15 min at 4 °C each time). The resulting plasma AVP concentrations and plasma platelet counts, measured by a platelet counter (Sysmex 2000; TOA Medical Electronics), were determined.

**Effect of delayed blood preparation on plasma AVP concentrations.** A 30-mL blood sample was collected from each of six healthy adult volunteers (ages, 22–32 years) and divided into 16 aliquots. Individual aliquots were stored at either 4 or 25 °C for time periods ranging from 0.5 to 48 h. The plasma was subsequently separated, and plasma AVP concentrations were determined.

**AVP Reference Values for Children**

A total of 203 children and adolescents (105 males and 98 females; ages, 1 day to 18 years) were studied after routine physical activity and unrestricted food and water intake. Only subjects in good physical condition without any obvious disturbance of water or electrolyte metabolism, nausea, or vomiting were included. Neonates studied during the first days and weeks of life had a mild degree of hyperbilirubinemia. The majority of the older children were patients who were either consulting our general outpatient unit for minor disorders, reexamination after minor illness, or basic evaluation before intended surgery, or coming to our specialized unit for disturbances of sexual development, such as constitutional delay of growth and puberty, gynecomastia, obesity, or idiopathic precocious puberty.

Five patients with clinically diagnosed nephrogenic diabetes insipidus (ages, 1.5–2.5 years), and two patients with clinically diagnosed neurogenic diabetes insipidus (ages, 1.5 and 13 years) were studied by way of comparison. Sixteen healthy adults (ages, 23–40 years) served as controls. Blood (1.0–1.5 mL) was obtained from each subject by venipuncture in combination with routine blood collection between 0800 and 1200. Informed consent was obtained from all subjects and/or their parents, and the guidelines of the Helsinki Declaration of 1975 were followed.

**Statistics**

Data are given as means ± SD. Within- and between-run coefficients of variation (CV) and SD were calculated as described by Krouwer and Rabinowitz (14). The slope of the calibration curve was calculated after logit transformation of B/B₀ as described by Rodbard (15). The Student t-test was used for comparison. P <0.05 was considered significant. The correlation coefficient (r) was determined, and linear regression analysis was performed for determining relations.

**Results**

**RIA Performance**

**Stability, slope, and range of calibration curve.** The mean (± SD) AVP calibration curve calculated from 16 consecutive
calibration curves is shown in Fig. 1. The average SD over the entire curve was 3.4%. The slope of the logit-log transformed calibration curve was $-2.1 \pm 0.1$. The range of the 80% effective dose ($ED_{80}$) to the 20% effective dose ($ED_{20}$) was 0.045–0.93 pg AVP/tube, which was equal to 0.25–5.1 ng AVP/L plasma when the 0.5-mL samples were and extraction procedure described in Materials and Methods were used. The 50% intercept ($ED_{50}$) was at 0.2 ± 0.017 pg AVP/tube (1.1 ± 0.1 ng AVP/L plasma).

**Limit of detection and nonspecific binding.** The minimum detectable concentration of the assay, defined as the concentration corresponding to a signal 3 SD above the mean for a calibrator free of AVP, was 0.06 ng/L when a 0.5-mL sample was extracted. Nonspecific binding, determined by performing the RIA without antibody, was 1.8% ± 0.3% of the total counts ($n = 30$).

**Within- and between-assay precision.** AVP-free plasma was made by treating outdated plasma from the blood bank with activated charcoal (16). Three plasma pools were prepared by adding 0.4, 1.1, and 3.1 ng/L synthetic AVP (Sigma Chemical Co.) to AVP-free plasma. Intra- and interassay CVs were assessed by repeated analysis ($n = 16$) of samples from these plasma pools. The intraassay CV was 10.2%, 5.8%, and 8.5%, and the interassay CV was 11.7%, 6.5%, and 6.6% for the three plasma pools.

**Recovery.** Recovery of cold (unlabeled) AVP in low, medium, and high physiological concentrations was deter-

**Dilution test.** Outdated plasma from a blood bank was pooled and supplemented with 4 mg/L synthetic AVP. Subsequently, it was further diluted with AVP-free plasma and assayed. As depicted in Fig. 1, the dilution curve obtained paralleled the calibration curve.

**Effect of centrifugation on resulting plasma platelet and plasma AVP concentrations.** The negative relationships between centrifugation and plasma AVP concentration, and cen-
trifugation and platelet count, respectively, are shown in Fig. 2. The significant linear correlation between platelets and AVP concentration is shown in Fig. 3 ($r = 0.899; P < 0.001$). However, it is noteworthy that the slopes of the individual correlation curves differ considerably (Fig. 3).

**Effect of delayed blood preparation on measured plasma AVP concentrations.** Storage of blood at ambient temperature (25 °C) led to an increase in plasma AVP that was significant after 2 h and reached virtually 100% after 24 h. In contrast, plasma AVP concentrations of blood stored at 4 °C did not change significantly within a 48-h storage period (Fig. 4).

**AVP reference values for children**

In 203 fully hydrated infants, children, and adolescents, plasma AVP averaged 1.1 ± 0.6 ng/L. There was no correlation with age and no significant difference associated with sex. Plasma AVP concentrations in 16 adult volunteers averaged 1.0 ± 0.5 ng/L and did not differ significantly from those in children and adolescents. Plasma AVP of five patients with nephrogenic diabetes insipidus was substantially increased (7.7 ± 4.5 ng/L). Plasma AVP of two patients suffering from hypophysial diabetes insipidus was below the detection limit of this RIA (Fig. 5).

**Discussion**

Development of a simple and sensitive RIA for determination of AVP in plasma has proven more complicated than for other polypeptide hormones. Difficult extraction procedures, lack of sensitivity, and time-consuming incubation periods limit the clinical application of many methods.

The extraction procedure with the 100-mg Isolute C$_{18}$ columns that we used is technically simple. With sample volumes of 0.25–1 mL, the recovery was comparable to that reported for Sep-Pak C$_{18}$ columns (Waters Corp.) loaded with two- or fourfold larger sample volumes (17–20). However, when loaded with just 0.5 mL of plasma, 360-mg C$_{18}$ Sep-Pak columns showed markedly
lower and more variable recoveries than Isolute C$_{18}$ columns (data not shown) in our laboratory. These findings are consistent with reports by Ysewijn Van Brussel and De Leenheer (17) of clearly decreased extraction efficiency in Sep-Pak C$_{18}$ columns when smaller sample volumes (1 mL instead of 2 mL) were processed. One reason for this may be a disproportion between the sorbent and sample volumes. In our laboratory, the extraction efficiency of 100-ng Isolute C$_{18}$ columns was higher at low volumes and did not differ significantly from 0.25 to 1 mL.

To improve the detection range and assay sensitivity, we tested nonequilibrium conditions and different dilutions of antiserum with different quantities of tracer. When extracted plasma was incubated with antiserum for 24 h before being incubated with antigen for an additional 16 h, the detection limit was 0.06 ng/L when 0.5 mL of plasma was extracted, as is done routinely in our laboratory. This, to our knowledge, is lower than any detection limit reported previously (Table 1). In addition, the steep, stable calibration curve, as indicated by its low SD value, facilitated low intra- and interassay CVs. The most precise part of the detection range, between ED$_{80}$ and ED$_{20}$ (−0.25–5.1 ng/L), coincided excellently with physiological AVP plasma concentrations (Fig. 5).

It is well known that contamination of plasma by platelet-bound AVP is considerably greater in platelet-rich than in platelet-poor plasma (5, 21–24). We ascertained that plasma platelet counts and plasma AVP concentrations are linearly correlated ($r = 0.899$; $P < 0.001$) and thus confirmed earlier findings by Preibisz et al. (21), Bichet et al. (23) and Inaba et al. (24). We also demonstrated the crucial role of centrifugation speed during blood preparation in the final outcome of AVP measurement. These results emphasize the need for careful plasma preparation because platelets present in plasma cause overestimation of plasma AVP and might be one of the most important reasons for the great differences in basal AVP concentrations observed in different laboratories (21). To produce virtually platelet-free plasma for the assay described, we centrifuge blood samples at 3600 g for 20 min at 4 °C.

When blood was stored at 25 °C before separation, plasma AVP concentrations increased significantly; however, they did not change significantly after storage at 4 °C. The increase in AVP at room temperature cannot be explained by alterations in platelet counts because it is known that platelets are stable at room temperature (25); in addition, there was no change in platelet counts during our study of sample storage temperature (data not shown). Therefore, AVP probably was released from blood cells in vitro during storage at room temperature.

Anfossi et al. (26) reported a release from platelets after aggregation. Thus, a possible explanation for the increase in AVP is aggregation of platelets caused by the release of platelet aggregation-inducing factors (such as adenosine 5′-pyrophosphate or arachidonate) from hemolyzed red blood cells.

AVP concentrations in our children (1.1 ± 0.6 ng/L) were very similar to those reported recently by other groups (5, 21–24) in adults when platelet-free plasma was extracted (0.7–1.7 ng/L). However, the AVP concentrations in our children are substantially lower than the values reported by Rascher et al. (3) in 145 children, and their study was the only one on reference values in a large number of children up to now. We were not able to confirm the tendency toward higher plasma AVP concentrations in infants (ages, 1–12 months) reported by Rascher et al. (3). In addition to the influence of platelets on measured AVP concentrations, the hydration status of patients or characteristics of the antiserum used may differ, and thus may be additional reasons for diverging results.

### Table 1. Characteristics of several RIAs for determination of AVP in plasma.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Detection limit, a ng/L</th>
<th>Sample volume, mL</th>
<th>ED$_{20}$ b ng/L</th>
<th>Incubation time, h</th>
<th>Intraassay CV, c, %</th>
<th>Interassay CV, c, %</th>
<th>Plasma AVP, d ng/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bichet and co-workers (23, 27)</td>
<td>0.5</td>
<td>1</td>
<td>1.2</td>
<td>120</td>
<td>5–13</td>
<td>20 (0.5–18 ng/L)</td>
<td>1.1 ± 0.3; n = 9</td>
</tr>
<tr>
<td>Bodola and Benedict (18)</td>
<td>0.4</td>
<td>2</td>
<td>4.6</td>
<td>32</td>
<td>4.3 (n = 4)</td>
<td>7.6 (n = 4)</td>
<td>2.6 ± 0.3; n = 7</td>
</tr>
<tr>
<td>Crawford and Gyory (20)</td>
<td>0.45</td>
<td>0.5</td>
<td>?</td>
<td>44</td>
<td>11.6 (3–10 ng/L)</td>
<td>7.6–11.3 (3–10 ng/L)</td>
<td>2.2 ± 0.3; n = 11</td>
</tr>
<tr>
<td>Gerbes et al. (12)</td>
<td>0.3</td>
<td>1</td>
<td>3.5</td>
<td>40</td>
<td>9.4 (n = 8)</td>
<td>15 (n = 8)</td>
<td>3.5; n = 13*</td>
</tr>
<tr>
<td>Husain et al. (9)</td>
<td>0.1</td>
<td>1</td>
<td>0.8</td>
<td>120</td>
<td>16.7 (4 ng/L; n = 23)</td>
<td>17.2 (10 ng/L; n = 7)</td>
<td>1.1 ± 0.6; n = 203</td>
</tr>
<tr>
<td>Kluge et al. (present study)</td>
<td>0.06</td>
<td>0.5</td>
<td>1.1</td>
<td>40</td>
<td>5.8 (1.1 ng/L; n = 16)</td>
<td>6.6 (1.1 ng/L; n = 16)</td>
<td>3.3 ± 1.8; n = 64*</td>
</tr>
<tr>
<td>Rascher and co-workers (3, 28)</td>
<td>1</td>
<td>1</td>
<td>3.5</td>
<td>96</td>
<td>10.3</td>
<td>13.1</td>
<td>3.3 ± 1.8; n = 64*</td>
</tr>
<tr>
<td>Robertson et al. (10)</td>
<td>0.5</td>
<td>1</td>
<td>1.1</td>
<td>168</td>
<td>13 (1–5 ng/L; n = 20)</td>
<td>17 (2.5 ng/L; n = 16)</td>
<td>2.7 ± 1.4; n = 12</td>
</tr>
<tr>
<td>Van de Heijning et al. (13)</td>
<td>1</td>
<td>1</td>
<td>3.7</td>
<td>72</td>
<td>10–16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Ysewijn Van Brussel and De Leenheer (17)</td>
<td>0.3</td>
<td>2</td>
<td>25</td>
<td>88</td>
<td>7.7 (4.8 ng/L; n = 12)</td>
<td>6.3 (4.1 ng/L; n = 14)</td>
<td></td>
</tr>
</tbody>
</table>

a Different definitions for “detection limit” have been applied.

b If absolute concentrations were not available, data have been adopted as specified in cited reference.

c Concentration and number of samples are in parentheses if derivable from reference cited; if CVs were determined for more than one concentration, the CV determined for the concentration closest to the ED$_{20}$ is shown.

d Mean (±SD) of normally hydrated adults (9, 10, 12, 18, 23) or children (3) and this study, respectively.

* After overnight fast.

* Children, ages 1–18 years.
In conclusion, this optimized procedure for determining AVP in plasma provides an extremely low detection limit even when small sample volumes are extracted, a detection range adapted to physiological AVP concentrations, and a high degree of reproducibility. Therefore, it may serve as a tool for determining AVP in infants and children. The simple extraction procedure, short incubation periods, and the commercial availability of reagents permit the easy establishment of this method in any suitably equipped laboratory.

We thank Gabriele Wendtlandt for skillful technical assistance.

References