Radioimmunoassay of vasopressin during pregnancy. Use and removal of cystylaminopeptidase inhibitors

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Abstract

This paper describes a radioimmunoassay (RIA) for arginine-vasopressin in which *o*-phenanthroline effectively inhibits cystyl-amino-peptidase activity in whole blood and plasma from pregnant women but in which *o*-phenanthroline is removed during the extraction of vasopressin from plasma to prevent disturbance of the RIA. Cystyl-amino-peptidase causes immediate degradation of vasopressin unless cystyl-amino-peptidase enzyme inhibitors such as *o*-phenanthroline are applied. However, *o*-phenanthroline interferes with RIA. We report an extraction procedure over octadecacyl silica-packed Sep-Pak C18 columns, by which cystyl-amino-peptidase as well as most of the cystyl-amino-peptidase inhibitor is removed from plasma with chloroform. The average *o*-phenanthroline concentration (0.25 mmol/l) found in the assay medium after extraction appeared not to interfere with the RIA. Polyacrylamide gel isoelectric focusing of extracts of platelet-rich and platelet-poor plasma from pregnant women revealed a single vasopressin immunoreactive peak in the RIA. Recovery and between-assay coefficients of variation of 3.2 ng/l vasopressin from pregnancy whole blood were comparable with non-pregnant controls (57%/8% and 59%/13%, respectively). Results with this assay compare well with those of another assay using inhibitors in pregnant subjects and with results in non-pregnant subjects.

Keywords: Vasopressin; Cystyl-amino-peptidase inhibitors; Pregnancy plasma

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1. Introduction

Because of technical problems, plasma levels of 8-arginine-vasopressin (AVP) are rarely determined during pregnancy, yet they may provide important information in disorders of water balance, e.g. transient diabetes insipidus [1–3], which may lead to severe dehydration. Moreover, platelet bound vasopressin levels might be associated with preeclampsia [4], a syndrome associated with high fetal and maternal morbidity and mortality. Since 60–90% of circulating vasopressin is bound to platelets [5,6], it could be important to establish the platelet bound fraction of vasopressin in pregnancy.

However, during pregnancy, rapid *in vitro* degradation of vasopressin and oxytocin occurs in human plasma into which the placenta releases the enzyme cystyl-amino-peptidase (CAP, EC 3.4.11.3) also referred to as oxytocinase or vasopressinase [7–12]. In the course of normal pregnancy, the plasma cystyl-amino-peptidase level rises and with it vasopressinase activity [12–15]. The K_m of cystyl-amino-peptidase for tritiated oxytocin or vasopressin in pregnancy plasma is around 10^{-5} mol/l ($V_{max} = 10^{-6}$ mol/l per min). In transient diabetes insipidus, cystyl-amino-peptidase activity may be extremely high as has been reported by Durr et al. [1]. In addition, there are differences in cystyl-amino-peptidase plasma levels depending on placental function and weight [7,9,14].

The reported variation in plasma levels of oxytocin (for discussion see Burd et al. [16]) and, to a lesser extent, vasopressin during pregnancy [14,17–20] might, at least partly, be explained by cystyl-amino-peptidase activity. In case of insufficient cystyl-amino-peptidase inhibition, oxytocin and vasopressin degradation may either increase or decrease levels of the peptides determined by radioimmunoassay (RIA) [10,14]. For a reliable RIA of vasopressin or oxytocin in human pregnancy plasma it is, therefore, crucial to inhibit cystyl-amino-peptidase activity from the very first moment of sample collection.

Vasopressin levels in platelet-rich plasma (PRP) in pregnancy have not been reported yet. Contamination of plasma with platelets may lead to high vasopressin levels and an increase in the variation of the plasma vasopressin levels [5].

Low temperature, low pH and high concentrations of EDTA and *o*-phenanthroline, among other conditions, inhibit cystyl-amino-peptidase activity. But only *o*-phenanthroline in a 10^{-3} mol/l concentration [7,16] or in combination with EDTA [16] gives immediate and total inhibition of cystyl-amino-peptidase. However, *o*-phenanthroline in this high concentration seriously disturbs the RIA [17,20–22] and is therefore either not added or is used in too low a concentration. In the present study a simple method was developed using octadecasilyl-silica packed Sep-Pak C18 columns, in order to remove the cystyl-amino-peptidase inhibitor and cystyl-amino-peptidase itself from plasma, thus minimizing disturbance of the RIA for vasopressin.

2. Materials and methods

2.1. Reagents

Synthetic 8-arginine-vasopressin (Sigma, St. Louis, MO, USA) was used for stan-

dard curves and recovery experiments. As enzyme inhibitors, 1,10 *ortho*-phenanthroline (Sigma) and Na₄-ethylenediaminetetraacetic acid (EDTA, Sigma) were tested. Vasopressin tracer was iodinated by the chloramine-T method and purified by fractionation on a Sephadex G25 column. Specific activity was more than 64.8 TBq/mmol. Polyclonal anti-vasopressin antibody (M160480) was raised in a rabbit and was used in a final dilution of 1:100 000. Cross-reactivity (ratio of pg vasopressin/peptide at $B/B_0 = 50\%$) with oxytocin was 0.009%, with vasotocin 5.8% and with lysine-vasopressin 22%.

2.2. Sample preparation

Whole blood was taken from an ante-cubital vein in silicon coated vacuum syringes (Venoject, Terumo, Leuven, Belgium) pre-cooled on melting ice. Centrifugation followed at $90 \times g$ for 15 min at 4°C to obtain platelet-rich plasma (PRP) and at $2000 \times g$ for 15 min at 4°C to obtain platelet-poor plasma (PPP).

2.3. Extraction

Extraction of vasopressin from PPP and PRP was performed over octadecasilyl silica-packed Sep-Pak C18 columns [23] (Waters Assoc. Inc., Milford, USA) using 2 ml plasma acidified to pH 1–2 with 3 ml 0.1 mol/l HCl. The columns were subsequently rinsed with 20 ml distilled water, 5 ml pure methanol (MeOH) and 20 ml distilled water before use. After the sample was placed on the column, rinsing twice with 10 ml 4% acetic acid (HAc) was followed by extraction of the sample with 4 ml 100% MeOH. Inhibitors were sufficiently removed by adding a washing step of 3 ml CHCl₃ between two washes with 10 ml 4% acetic acid. The eluate was subsequently dried overnight in a vacuum rotator with a refrigerated condensation trap (Savant Speedvac RH 40–12, RT-100A) and the residue was redissolved in 125 µl assay buffer containing 0.05 mol/l Tris–HCl (139 mmol/l NaCl, 1 mmol/l EDTA and 10 g/l BSA (Sigma, RIA-grade)), pH 8.

2.4. Assay

The assay is based upon the micro-assay described by Dogterom et al. [25] with modifications. Standard curves ranged from 0.25 to 64 pg vasopressin per 50 µl and were set up in triplicate. Samples were assayed in duplicate. Total volume in assay tubes was 110 µl and consisted of 50 µl antibody solution, 50 µl sample or standard solution (both in assay buffer) and 10 µl (2000 counts/min) tracer. After 3 days of incubation at 4°C the free label was separated from the bound by adding 50 µl of second antibody-coated cellulose suspension (SAC CEL, donkey anti-rabbit, IDS Ltd, Boldon, UK) to the assay tube followed by centrifugation at $1700 \times g$ for 5 min after which the pellet was counted in a Packard-Cobra A5005 (Packard, Meriden, USA) gamma counter. Standard curves and samples were processed using Cobra 5005 software (RIA-SMART, Packard), which includes standard curve fitting with MASS-spline and quality control analysis. The detection limit defined as B/B_0 of $<90\%$, was 0.5 pg per tube. Non-specific binding was always below 5%. In plasma from pregnant women, kept at 20°C for several minutes without cystyl-amino-peptidase inhibitors, vasopressin levels were equal to the detection limit. Standard curves extracted in pregnancy plasma were parallel to standard curves in buffer.

Intra-assay C.V. in the direct assay for 1–16 pg/tube vasopressin averages 9.7% (range 7–15%). Inter-assay C.V. in ten different assays for 2 pg vasopressin/tube is 13.4%.

2.5. Cystyl-amino-peptidase activity

Cystyl-amino-peptidase activity was measured spectrophotometrically with the substrate *S*-benzyl-L-cysteine-4-nitroanilide as described by Van Oudheusden [12].

2.6. Patients

Pregnancy plasma was obtained from healthy out-patient clinic women and patients from the obstetric ward. Control plasma was obtained from non-pregnant female volunteers working in the hospital. Informed consent was given by all subjects and the study was approved by the Ethics Committee at Amsterdam Medical Center, University of Amsterdam.

2.7. RIA-sensitivity for inhibitors

A serial dilution of *o*-phenanthroline and EDTA and a combination of both was made in assay buffer. A fixed amount of vasopressin was added to each dilution.

2.8. Removal of *o*-phenanthroline and cystyl-amino-peptidase during extraction

A wash with 3 ml CHCl_3 or 10% methanol in distilled water was added to the usual extraction procedure in order to find whether one of these solvents, which were known from earlier (unpublished) experiments not to elute vasopressin from the column, were able to elute *o*-phenanthroline.

The *o*-phenanthroline concentration in extracts from human plasma was measured spectrophotometrically with a solution of FeCl_2 (1 g Fe/l). Cystyl-amino-peptidase activity was measured in the extracts redissolved in assay buffer.

2.9. Isoelectric focusing

Isoelectric focusing (IEF) was performed using thin large pore polyacrylamide gels as described by Van der Sluis et al. [13] to detect whether immunoreactive vasopressin from PRP and PPP extracts was identical to synthetic vasopressin. Pools of 15–20 ml of PRP and PPP from (pregnant) women were extracted and dried as described above. Also, PRP was incubated with 128 pg vasopressin for 1 h and centrifuged. Subsequently the platelet pellet was washed and resuspended in buffer, extracted and dried. The residue was redissolved in 25 μl IEF medium, 10% v/v dimethylformamide (BDH, Poole, UK: for gas chromatography), 10% v/v glycerol (BDH) and 2.5% v/v Nonidet P-40 (Sigma) and 5 μl were taken for direct vasopressin assay. Another 5 μl and the same volume of 0, 10, 20, 30, 40 and 50 pg synthetic vasopressin were used for focusing. After IEF, a sheet of filter paper was applied onto the gel and 5-mm gel-paper strips of the separation tracks were cut. Each fraction was eluted by incubation for 1 h in 1 ml 1:8 diluted RIA assay buffer (see above) and dried. Each fraction was redissolved in 125 μl distilled water for vasopressin assay.

2.10. Variation including extraction and recovery

In pregnancy plasma. To determine variation with the extraction procedure included, eight 2-ml samples of pooled pregnancy plasma with 35 μmol *o*-phenanthroline and EDTA solution were extracted after adding 8, 16, 32 and 64 pg synthetic vasopressin. The extract was redissolved in 250 μl assay buffer. Also, recovery was calculated.

In whole blood. Plasma levels of a group of normal pregnant women ($n = 22$) recruited from three different hospitals were compared with a group of non-pregnant controls ($n = 9$) for recovery of 3.2 ng/l vasopressin added to whole blood. Inhibitors were put into the blood-collecting tube.

Venous blood from the forearm was drawn into two 10-ml syringes with 100 μl of 125 mmol/l solution of *o*-phenanthroline (pH 5.5), to one of which 32 pg vasopressin was added, retaining the vacuum. The blood from a tube with *o*-phenanthroline was immediately transferred to an identical tube with 100 μl of a 1 mol/l solution of EDTA, pH 7. All the tubes were pre-chilled and kept in melting ice until immediate centrifugation and extraction were carried out in the hospital the women were selected from. For PPP, 3 ml plasma were extracted to lower the detection limit to 0.3 ng/l. In each extraction run two tubes with 2 ml horse plasma were extracted, one as reference blank and one with 8 pg vasopressin added as a control for recovery. Assay was performed in one laboratory and the samples were measured in four different assays. Cystyl-amino-peptidase activity was determined in plasma and in the extracts of 11 samples. *o*-Phenanthroline concentration was measured in the same 11 extracts. To correct for loss of peptide not due to degradation, recovery of [^{125}I]AVP from whole blood collected as described above was used as reference. Iodinated vasopressin in *o*-phenanthroline solution was added to the vacuum syringe. Ten tubes were taken from a male volunteer. The average recovery of vasopressin was measured. Recovery of cold vasopressin is expressed as the ratio: % recovery of AVP/% recovery of [^{125}I]AVP.

2.11. Statistics

Unpaired *t*-test or Mann-Whitney *U* statistics were used to test differences between groups. $P < 0.05$ was considered significant. For differences in means 95% confidence intervals (95% CI) are calculated. C.V. was calculated as standard deviation divided by the mean.

3. Results

3.1. Disturbance of RIA by inhibitors

An *o*-phenanthroline concentration-dependent disturbance was found in the RIA (Fig. 1). The average concentration measured in assay buffer after extracting 2 ml horse plasma with 50 μl of 125 mmol/l *o*-phenanthroline solution added and not using CHCl_3 was 23.5 mmol/l (S.D. = 4.38, $n = 6$).

3.2. Removal of *o*-phenanthroline and cystyl-amino-peptidase

After a washing-step of 3 ml CHCl_3 in between the two washes with 10 ml 4%

Fig. 1. Disturbance of the RIA by the inhibitor *o*-phenanthroline alone and in combination with EDTA. The concentration of *o*-phenanthroline is given, that of EDTA is a factor 10^2 higher. The assay tubes contained 3 pg AVP. The length of the box corresponds with the mean *o*-phenanthroline concentration $\pm$ 3 S.D. as measured in the extracts of plasma from pregnant women with 100 μ l of a 125 mmol/l *o*-phenanthroline solution per ml blood (giving the concentration necessary to inhibit cystyl-amino-peptidase, i.e. over 3 mmol/l). No disturbance of the assay occurs within concentration range of the box.

HAc, we could no longer measure any *o*-phenanthroline in the extracts from horse plasma, the minimum detectable *o*-phenanthroline concentration being 1.59 μ mol/l. Ten percent MeOH in distilled water did not remove *o*-phenanthroline. In the extracts from pregnancy plasma obtained in the recovery experiments, *o*-phenanthroline concentration was 0.25 mmol/l (S.D. 0.13).

Cystyl-amino-peptidase levels after extraction were within the range of non-pregnancy plasma, i.e. <7 IU/l; the mean cystyl-amino-peptidase activity before extraction was 57.9 IU/l (range 11–180 IU/l).

3.3. Isoelectric focusing

Only one peak of immunoreactive vasopressin was detected either in PPP or PRP. This peak was found on the corresponding lane where the synthetic AVP standards were focused (Fig. 2).

Fig. 2. Gel isoelectric focussing of platelet-rich plasma (PRP) or platelet-poor plasma (PPP) with and without preincubation with 6.4 pg/ml AVP. Preg: pregnant. The gel is divided into 5-mm fractions that are measured in the radioimmunoassay (see Methods).

Fig. 3. Mean recovery % ($\pm$ S.D.) of AVP from whole blood of 22 pregnant (pregn) women vs. 9 non-pregnant (non-p) women and from pooled pregnancy plasma vs. horse plasma. Recovery of peptide differs significantly between whole blood and plasma ($P < 0.001$) but recovery in pregnant women does not differ from that in non-pregnant women for whole blood (95% CI for difference in recovery -6.7% to $+10.7\%$) nor from horse plasma (95% CI -1.5% to $+8.5\%$). See Discussion.

3.4. Variation and recovery

Mean recovery and C.V. from pooled third trimester plasma was 75.6% and 13.6%, respectively (Fig. 3). From horse plasma with 8 pg/ml AVP, the corresponding values were 72.1% and 14.1% (49 extraction runs and 5 assays). The 95% CI for difference in recovery between pregnant and horse plasma is -1.5 to $+8.5$.

Recovery of vasopressin from 10 ml of whole blood of pregnant women was 47%, C.V. 24.4%. As recovery of [^{125} I]AVP was 82% and C.V. for injecting peptide into the vacuum syringe 9%, and, since subtracting the endogenous vasopressin value will also increase the C.V., the actual recovery and C.V. of endogenous AVP in pregnancy whole blood will approximate to 57% and 8%, respectively. Recovery and C.V. for PRP was equal to PPP. For non-pregnant controls, corrected recovery and C.V. were 59% and 13%, respectively. The 95% CI for difference in recovery from non-pregnant and pregnant women was -6.7 to $+10.7$. The corresponding 95% CI for pregnancy plasma and pregnancy whole blood was $+13.3$ to $+18.8$ ($P < 0.001$).

3.5. AVP plasma levels

In the 22 pregnant women from whom blood was collected for recovery studies, median vasopressin levels in PPP were 0.7 ng/l (range 0–4.7), in PRP 2.6 (range 0.5–14.3), mean ratio 5.2 (S.D. 4.9) (Fig. 4). In the group of non-pregnant subjects, values were PPP: 2.2 ng/l (range 1.2–8.0), PRP: 10.2 ng/l (4.2–29.7), ratio: 5.1 (S.D. 1.5).

Both PRP and PPP differed significantly between the pregnant and non-pregnant group ($P < 0.001$).

Fig. 4. AVP levels in third trimester pregnant women (preg) and non-pregnant controls (non-preg). Values are corrected for recovery. Median (bar) and interquartile range is given. Levels in platelet-rich plasma are not corrected for platelet number. PRP and PPP differ significantly between the two groups (Mann-Whitney, $P < 0.001$)

4. Discussion

The presence of the placental enzyme cystyl-amino-peptidase is a major obstacle in the measurement by RIA of plasma levels of vasopressin in pregnant women, because it immediately cleaves the cystine-tyrosine linkage in vasopressin and oxytocin [1].

Simply to use low temperature as a cystyl-amino-peptidase inhibitor, as is done by some investigators [29], gives a recovery of only 4.5% of that from non-pregnancy plasma [12]; own data not given here). This means that either vasopressin breakdown during the blood-collection process is massive [14] or that vasopressin breakdown is not stopped at temperatures around 0°C and/or at a low pH. As cystyl-amino-peptidase activity is completely lost at pH values below 3.5 and is low below 10°C [7], degradation must occur during the time of blood sampling, which was also recognized by Iwasaki et al. [3]. Therefore, a rapid and standardized method of blood sampling, using vacuum tubes into which inhibitors are added, is necessary.

Inhibitors used, besides cold, are EDTA [14,20,22], *o*-phenanthroline [18], or a combination of both [16,21]. EDTA alone gives only 40% inhibition of cystyl-amino-peptidase activity [30] while *o*-phenanthroline in high concentration is known to interfere with the RIA [17,22] and is either not used [17,20,22,29] or used only in low concentrations [20] that do not inhibit the enzyme activity completely. *o*-Phenanthroline interferes with the antibody-antigen interaction, resulting in displacement of the standard curve. We tested two different oxytocin antibodies, THF₃ [24], kindly donated by T. Higuchi, as well as O-2-Q, raised in our laboratory and another vasopressin antibody from our laboratory (Peter). They were even more sensitive to *o*-phenanthroline (not shown here) than the vasopressin antibody. In addition, Burd and Davison found that only high concentrations of *o*-phenanthroline were effective in inhibiting cystyl-amino-peptidase activity [16,18] for oxytocin- and vasopressin-assay, but did not find displacement of the standard curve by *o*-phenanthroline in their assays. Davison et al. even put *o*-phenanthroline in the assay buffer [18]. Therefore, differences in sensitivity to *o*-phenanthroline for different antibodies are likely. As shown in Fig. 1, the disturbing effect of *o*-phenanthroline in our assay was concentration dependent: the higher the concentration of *o*-phenanthroline the higher the measured peptide level. Residual *o*-phenanthroline in the extract might therefore influence zero blank values and lead to higher detection limits, or give high recovery values because of the shift compared with the standard curve (see Fig. 2). Because we could not measure any significant cystyl-amino-peptidase activity in the extract of pregnancy plasma, no inhibitor seemed to be required during the RIA. We therefore removed *o*-phenanthroline with CHCl₃ during the extraction procedure. As a result the average *o*-phenanthroline concentration in the extract, plus 3 S.D., fell below the concentration found to disturb the RIA (Fig. 2).

Cystyl-amino-peptidase activity has apparently been inhibited effectively, since recovery in pregnant women (57%) equals that in non-pregnant women (59%). However, some non-cystyl-amino-peptidase loss of peptide may occur in whole blood since recovery is higher in plasma (75.6%) than in whole blood (59%). Binding to

platelets containing V_1 -receptors seems unlikely [31], because divalent cations necessary for binding of vasopressin to platelet receptors are bound by EDTA and *o*-phenanthroline. Irrespective of this non-pregnancy-specific loss, basal values of vasopressin plasma levels in pregnant women corrected for recovery are well in agreement with values reported by Davison et al. [28], who uses a more laborious procedure. Furthermore, vasopressin levels in non-pregnant patients agree well with values reported by Preibisz et al. [5] for PRP as well as PPP. Although vasopressin levels in platelet-rich as well as in platelet-poor plasma differed significantly between the pregnant and non-pregnant group, we realise that these values were obtained from individuals under various, not basal, conditions. Therefore, one cannot draw any conclusions from the differences found. Isoelectric focusing experiments show that immunoreactive vasopressin in PPP as well as in PRP cannot be distinguished from synthetic vasopressin. Additionally, in extracts, no other immunoreactive substances are detected and we consider purification with C18 columns, therefore, satisfactory. Another technical point not mentioned earlier is that the inhibitors EDTA and *o*-phenanthroline, in the high concentrations used, cannot be put together in the tube before blood collection. The substances react chemically and/or haemolysis occurs and this can be a reason not to use *o*-phenanthroline [32]. However, our method for blood collection with inhibitors in separate tubes is not threatened by such problems and, using vacuum tubes, very rapidly mixes the inhibitors diluted by the blood.

In conclusion we have developed a vasopressin assay suitable for human pregnancy by using rapid cystyl-amino-peptidase inhibition, yet eliminating the RIA disturbing effect of the inhibitor *o*-phenanthroline and tested it for reproducibility of sample handling and extraction in three different hospitals. Even in the case of RIA sensitivity for *o*-phenanthroline, rapid and standardized drawing of blood in vacuum syringes containing cystyl-amino-peptidase inhibitors and a simple extraction procedure over Sep-Pak C18 columns, removing cystyl-amino-peptidase and inhibitors, allow a proper RIA for vasopressin during pregnancy.

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