Journal of Chromatography, 565 (1991) 159–171
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5732

Solid-phase extraction of plasma vasopressin: evaluation, validation and application

BERT J. M. VAN DE HEIJNING*, INEKE KOEKKOEK-VAN DEN HERIK, TIBOR IVÁNYI and TJEERD B. VAN WIMERSMA GREIDANUS

Department of Pharmacology, Rudolf Magnus Institute, University of Utrecht, Vondellaan 6, 3521 GD Utrecht (The Netherlands)

(First received August 14th, 1990; revised manuscript received October 29th, 1990)

ABSTRACT

A new solid-phase extraction method using octyl-silica columns to extract vasopressin-like immunoreactivity from plasma has been developed. The extraction was followed by a radioimmunoassay on the vacuum-dried extracts, which were reconstituted in assay buffer. The total recovery of synthetic vasopressin was *ca.* 100%. Based on co-elution with synthetic vasopressin after separation by reversed-phase high-performance liquid chromatography of plasma extracts from normal Wistar and Brattleboro rats, and the cross-reactivity of the antiserum used in the radioimmunoassay system, the extracted material was found to be indistinguishable from authentic vasopressin. Unknown experimental samples were interpolated on a standard curve established in "zero" plasma (plasma derived from rats subjected to waterload) spiked with known amounts of synthetic vasopressin, and not on a standard curve established in assay buffer. The limit of detection was 1 fmol of vasopressin equivalent per millilitre. The intra- and inter-assay coefficients of variance were 10–16% and 16%, respectively. The procedure reliably showed that osmotic challenge and 24-h dehydration increased, whereas ethanol ingestion decreased vasopressin-like immunoreactivity plasma levels in the rat, compared with normally hydrated controls.

INTRODUCTION

[Arg^8]-vasopressin (VP), a cyclic nonapeptide, is released from neurosecretory terminals in the posterior pituitary gland, and from this neuro-haemal organ it reaches the blood circulation to be transported to its target organ(s). Estimation of VP plasma levels by radioimmunoassay (RIA) is complicated by the low concentration of endogenous VP in blood (fmol/ml) and by the presence of interfering factors in plasma. Therefore, the determination of plasma VP levels requires a highly efficient and reproducible extraction procedure, as well as a very sensitive RIA system.

Extraction of VP from biological fluids such as plasma, urine and cerebrospinal fluid is laborious, and poor or variable recoveries have been reported: the formerly used Vycor-glasspowder extraction was very time-consuming, and had a recovery of only 60–80% [1]. In order to get a more consistent, simpler and better

0378-4347/91/$03.50

recovery, new extraction methods have been developed. Solid-phase extraction using silica-based column materials is commonly applied. This technique results in higher recoveries and in fewer interfering impurities co-eluting with the analyte from the sorbent. Particularly, the use of C_{18} silica is well established, and solid-phase extraction protocols for synthetic VP from plasma yielding maximally 70–85% recovery have been reported [2–5]. Other workers reported a very high recovery (>96%) using C_{18} columns [6].

We have developed a new solid-phase extraction for VP from plasma, using C_8 silica packed columns. The method gives a higher recovery than those previously reported, and can also be used to extract oxytocin (OT) from both rat and human plasma. In combination with our RIA system, it provides an accurate and reproducible method for the determination of VP levels in blood.

EXPERIMENTAL

Animals and sample collection

Male rats (180–200 g) from an inbred Wistar strain (TNO, Zeist, The Netherlands) were kept under standard laboratory conditions with free access to food and water. The dehydrated group of animals were deprived of water for 24 h. Brattleboro rats (300 g; homozygous for diabetes insipidus) were kept under the same conditions and drank 150–200 ml water per day.

In all experiments, saline was used as the vehicle and served as control treatment.

For osmotic stimulation experiments, rats were given an intraperitoneal (i.p.) injection (10 ml/kg) of hypertonic saline (9% NaCl) 30 min prior to decapitation.

For ethanol ingestion experiments, at $t = 0$ rats were given 10 ml of an ethanol solution (10%) in saline intragastrically (i.g.) by means of an oral cannula. The rats were decapitated at $t = 50$ min.

For water-loading experiments conscious rats (200 g) were injected i.g., at 1-h intervals, with 10-ml (= 5% body weight) ethanol solutions using a cannula: firstly 2%, then 10% followed by 2% again. The rats were killed by decapitation 30 min after the last injection.

Trunk blood was collected in chilled polypropylene tubes that contained 200 µl of anticoagulant (125 m*M* Na_2EDTA in saline). The tubes were centrifuged (4°C, *ca.* 2000 *g* for 20 min), and plasma of individual animals was either immediately assayed for VP-like immunoreactivity (VP-LI) or stored at −80°C after the addition of 12 µl of 8 *M* acetic acid per ml plasma.

Extraction and RIA

The RIA for [Arg^8]-VP was preceded by a solid-phase extraction using Bond Elut® C_8 silica columns (LRC series, Analytichem) containing 100 mg of sorbent. Solutions were aspirated through the column by vacuum; a vacuum manifold column processor was used, connected to a small vacuum pump.

The column was firstly conditioned ("activated") with 4 ml of methanol, and subsequently washed with 4 ml of distilled water; care was taken not to let the column run dry. Acidified (acetic acid) plasma (1 ml) was added to 4 ml of 0.1 *M* hydrochloric acid, and this mixture was applied to the column. At least 5 min were allowed for the mixture to run through the column (less than 1 mmHg vacuum). Subsequently, 4 ml of 10% (v/v) acetonitrile in 0.1% trifluoroacetic acid (TFA) were applied to wash impurities from the column, followed by elution with 1.5 ml of 60% acetonitrile in 0.1% TFA. The column was then cleaned with 4 ml of 100% acetonitrile, 4 ml of 8 *M* urea and 4 ml of distilled water. A column cleaned in this way could be re-used up to 20 times.

Elution fractions were dried in a Speed Vac concentrator (Savant), and the residues were reconstituted in 125 μl of assay buffer for subsequent determination in an RIA system.

In order to establish an "internal" standard curve, standard amounts ("VP equivalents") of synthetic VP (Organon International) were added to VP-free "zero plasma" obtained from rats subjected to water-loading (see above). Pooled VP-free "zero-plasma" [7] was left at room temperature for at least 1 h and was subsequently divided into aliquots and stored at −20°C. These "standard samples" were subjected to the same extraction procedure as all experimental plasma samples.

The assay buffer contained 0.02 *M* 5,5-diethylbarbituric acid (Veronal, Merck), 0.01 *M* Na_2EDTA and 0.067 m*M* L-cystine (Sigma), 0.14 *M* NaCl and 5% human serum albumin (HSA, Sigma); the pH was adjusted to 8.0 with NaOH. The RIA system used an antiserum coded W1E. W1E was characterized by cross-reaction with synthetic peptides, and was found to recognize the C-terminus of VP. It had almost 100% cross-reactivity with vasotocin, and 50, 25 and 13% with VP fragments [Cyt^6]VP-(3–9), [$pGlu^4$,Cyt^6]VP-(4–9) and [Cyt^6]VP-(5–9), respectively. No significant displacement of tracer (<0.01%) was found with des-amino-[D-Arg]VP (DD-VP), VP-(1–8), VP-(1–7) or oxytocin (see ref. 8). A disequilibrium system was used: equal amounts of standard/sample (50 μl) and antibody (diluted 1:240 000 in assay buffer) were pre-incubated for 48 h at 4°C. Next, 10 μl of tracer (*ca.* 10 kcpm) were added; tracer was prepared according the Iodogen method [9], using $Na^{125}I$ (NEN). After another 24 h 4°C, the antigen–antibody complex (Ag–Ab) was precipitated using a second antibody (donkey anti-rabbit) coated with cellulose (SacCel®, IDS). After allowing 0.5–1 h of binding at 4°C, 0.5 ml of ice-cold water was added and tubes were spun for 20 min at *ca.* 3500 *g* (4°C). The radioactivity in the pellets was counted for 5 min in a Cobra® γ-counter (Packard).

A standard curve established in assay buffer, the "external", always accompanied the standard curve in "zero plasma", the "internal", to check for parallelism. The intra-assay coefficient of variance (C.V.) for the "internal" curve was determined by repeated measurements of "zero plasma" spiked with known amounts of VP (5 and 10 fmol/ml). The inter-assay C.V. was estimated in the same way,

except that the spiked "zero plasma" was divided into aliquots and measured in different RIAs (stored frozen at −20°C until extraction).

For determination of oxytocin-like immunoreactivity (OT-LI) in the plasma extracts and in the HPLC fractions, we carried out an RIA using an antiserum, coded THF3, that was kindly donated to us by Dr. Higuchi (Matsuoka, Japan). For details about the antiserum, see Higuchi *et al.* [10]. The detection limit was 0.15 pg OT-LI per assay tube (*i.e.* 3 fmol/ml); the intra-assay C.V. was 7% and the inter-assay C.V. 10%.

The OT assay buffer consisted of 0.01 *M* phosphate buffer (pH 7.5) containing 5% BSA (Sigma, fraction V) and 1% sodium azide (Merck). Equal amounts of standard/sample (50 μl) and antibody (1:300 000 dilution) were pre-incubated for 24 h at 4°C, whereupon tracer was added (50 μl, 10 000 cpm) and the tubes were incubated for another 48 h before bound and free tracer were separated and pellets counted (as for VP).

Evaluation of mobile phase

Plasma from rats or humans, or assay buffer, containing VP and OT, radiolabelled with either ^{3}H or ^{125}I, were applied to several types of column, and the elution efficiency (recovery) was evaluated using various protocols (published or personally communicated from other laboratories). The eluted amount of radioactivity was compared with the amount applied ("totals").

To determine which washing step was to be used when extracting with C_8 and phenyl (PH) columns, increasing percentages of acetonitrile were applied to a column to which assay buffer containing labelled peptide was applied.

Recovery of VP in plasma

Known amounts of synthetic VP added in quadruplicate to 1-ml aliquots of "zero plasma" were extracted as described above and subsequently assayed.

Characterization of extraction products by HPLC

Reversed-phase HPLC was performed on μBondapak C_{18} columns (300 mm × 4 mm I.D., 10 μm) using a Waters Assoc. chromatographic system. A 1-ng amount of synthetic VP or OT dissolved in 0.5 ml of 10 m*M* ammonium acetate–0.1% BSA was used as a standard. Speed Vac-dried plasma extracts were dissolved in 10 m*M* ammonium acetate containing 0.1% BSA and pooled. After centrifugation (10 min, 3500 *g*), 500 μl of pooled plasma extract were applied to the column. The UV absorption of the eluate was monitored at 210 nm, and separation was performed using a linear 15–55% methanol gradient in 10 m*M* ammonium acetate (pH 4.15) at a flow-rate of 1.0 ml/min; 30-s fractions were collected in polystyrene tubes containing 100 μl of 0.1% BSA in 10 m*M* ammonium acetate. The individual (0.5 ml) fractions were vacuum-dried in a Speed Vac concentrator (Savant) and reconstituted in assay buffer for subsequent RIA for VP-LI and OT-LI content.

Standards were run prior to the pooled extracts. Before each run, both standard and extracts, a blank run (500 μl of 10 mM ammonium acetate in 0.1% BSA were applied to the column) was carried out to check that the column was clean (*i.e.* no VP-LI or OT-LI in the fractions).

Statistics

Data are presented as means ± S.D. Data were evaluated by one-way ANOVA, followed by the least-significance differences test (LSD). A p-value of 0.05 was considered significant.

RESULTS

Evaluation of mobile phase

Various sorbents and extraction protocols were tested on the retention and elution of radiolabelled synthetic OT and VP dissolved in either assay buffer or human or rat plasma. The results are summarized in Table I. Based on these tests, we chose to optimize the extraction on the Bond Elut C_8 and PH columns.

As depicted in Fig. 1B, ^{3}H labelled OT elutes in considerable amounts (22%) from PH columns using 10% acetonitrile in 0.1% TFA, whereas it does not from C_8 columns (<1%). ^{3}H labelled VP does not elute at this percentage of acetonitrile in a significant amount from either type of column (<2%). Both sorbents retain VP and OT to the same extent and both peptides are eluted with the same efficiency at 60% acetonitrile. Similar recoveries were obtained only at 60% using ^{125}I-labelled VP and OT (Table I and Fig. 1).

It was decided to use C_8 columns for the extraction rather than PH columns for two reasons. First, because the chromatographic profile of VP looks more consistent (saturated elution level) in C_8 columns (Fig. 1A), and second, because a washing step with 10% acetonitrile preceding the elution step is possible for both VP and OT (Fig. 1A and 1B). Such a washing step is desirable for the potential removal of plasma matrix factors that might interfere in the RIA system.

Extraction and RIA

Fig. 2 shows that the standard curve established in assay buffer (the "external" curve) and the one established in "zero plasma" (the "internal" curve) run parallel to each other. The "internal" standard curve is superimposed on the "external" curve: the amounts of VP (in pg per tube) which cause a 50% displacement of the tracer (*i.e.* the ED_{50} values) were 6.17 ± 0.20 (n = 12) and 3.07 ± 0.29 (n = 10), respectively.

The detection limit obtained with the "external" standard curve was 0.25 fmol per tube, and intra- and inter-assay coefficients of variance (C.V.) at 32 fmol per tube were 3–4% (n = 4) and 13.5% (n = 16 in three RIAs), respectively.

The detection limit obtained with the "internal" standard curve was 0.5 fmol/

TABLE I

ELUTION EFFICIENCY OF RADIOLABELLED VP AND OT ON VARIOUS SORBENTS

Known amounts of radioactively labelled VP or OT were added to assay buffer (Buffer), rat plasma (r-Pl) or human plasma (h-Pl). Sorbents from Analytichem unless stated otherwise. Average value of at least two determinations in each case.

Sorbent	Peptide	Carrier (1 ml)	Elution solution (2 ml)	Elution efficiency (%)
^{125}I label				
C_2	VP	r-Pl[c]	60% acetonitrile–0.1% TFA	94
C_8				96
C_8[a]				96
PH				95
C_{18}				88
C_{18}[b]	VP	r-Pl[d]	80% methanol-4% HAc[e]	68
C_{18}[b]		Buffer		76
C_{18}[b]		r-Pl	60% ethanol-0.1 *M* HCl	78
C_8	OT	r-Pl	60% acetonitrile-0.1% TFA	84
PH		r-Pl		80
C_8		h-Pl[f]	30% acetonitrile-0.1% TFA	98
^{3}H label				
C_8	VP	r-Pl[c]	60% acetonitrile-0.1% TFA	85
C_8[a]				91
PH				89
C_{18}				63
C_{18}[b]			80% methanol-4% HAc[e]	86

[a] Amersham.
[b] Sep-Pak (Waters Assoc.).
[c] 1 ml of r-Pl + 4 ml of 0.1 *M* HCl.
[d] 2 ml of r-Pl.
[e] After Bevilacqua *et al.* [3].
[f] 1 ml of h-Pl + 4 ml of 0.1 *M* HCl.

ml. An intra-assay C.V. of 9.95% was obtained by measuring eight times 10 fmol/ml in one assay (9.64 ± 0.36 fmol/ml), and a C.V. of 15.9% by measuring twelve times 5 fmol/ml in one assay (5.02 ± 0.23 fmol/ml). The inter-assay C.V. was 16%, from quadruplicate measurement of four pooled plasma samples containing 10 fmol/ml: 10.03 ± 0.39 fmol/ml (n = 16).

The recovery of various amounts of VP from plasma was *ca.* 100% (Table II). The material extracted from plasma and measured in the RIA yielded a dilution curve parallel to that of synthetic VP (Fig. 3). Interpolation of assayed serial dilutions on the "internal" standard curve also showed a quantitatively match to the expected fall-off (Table III).

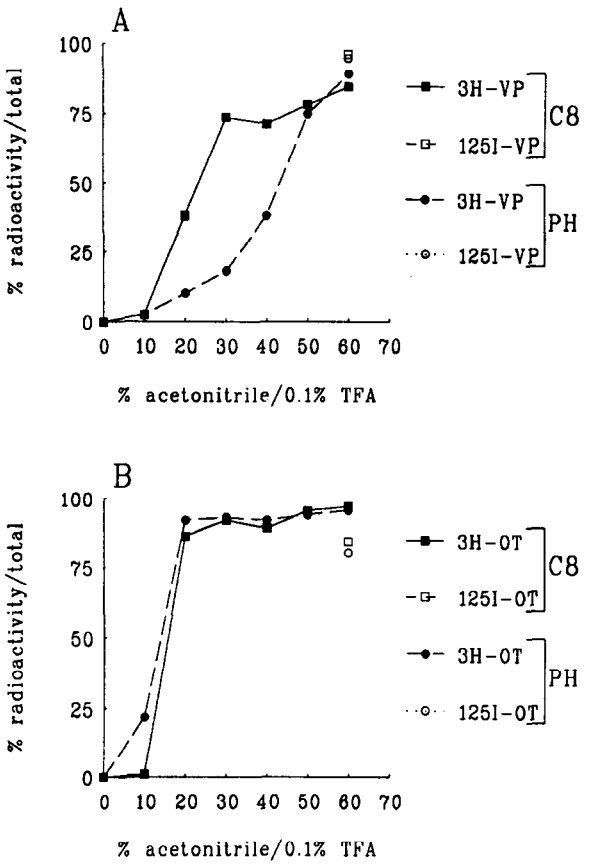

Fig. 1. Acetonitrile elution of (A) radiolabelled vasopresin (VP) and (B) radiolabelled oxytocin (OT) from octyl- and phenyl-silica columns, C_8 and PH, respectively. A 1-ml volume assay buffer containing ^{3}H-labelled OT or VP was applied to the columns. After a washing step with 4 ml of 0.1% TFA, the peptides were eluted with increasing concentrations of acetonitrile (2 ml). Elution with 60% acetonitrile was also carried out with ^{125}I label. The radioactivity recovered in the eluate was determined.

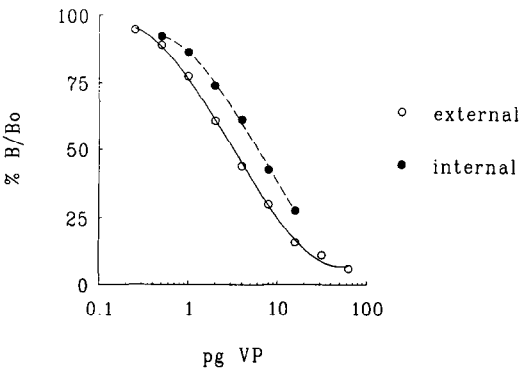

Fig. 2. Representative standard curves for synthetic vasopressin (VP) established in assay buffer ("external") and in "zero plasma" from rats subjected to water-loading ("internal"). B is the specific binding (cpm) observed, and B_0 is specific binding (cpm) of the blank.

TABLE II

VP RECOVERY TEST

Known amounts of VP were added to rat "zero plasma" containing no VP; B/B_0 = 98.22 ± 0.9%; data represent mean ± S.D. (n = 4).

VP added (pg/ml)	VP obtained (pg/ml)	Recovery (%)
1.00	1.07 ± 0.02	107
4.00	4.35 ± 0.46	109
16.00	15.92 ± 1.02	99.5

The extracted VP immunoreactivity was examined by HPLC. The VP-LI in pooled plasma extracts of water-deprived rats eluted as a single immunoreactive peak at the same position as synthetic VP (see Fig. 4). Pooled plasma extracts of VP-deficient Brattleboro rats did not show any VP-LI. As depicted in Fig. 4B, the OT-LI in plasma extracts of both Wistar and Brattleboro rats co-eluted with synthetic OT; the amount of OT-LI was found to be higher in the Brattleboro rats.

Application

Table IV shows the usefulness of the extraction procedure by documenting the effects on plasma VP-LI levels of treatments known to affect VP release. Compared with normally hydrated controls, rats subjected to 24-h water-deprivation or an i.p. injection of hypertonic saline showed significantly elevated VP-LI plasma levels. On the other hand, ethanol ingestion resulted in a decrease of VP-LI plasma levels.

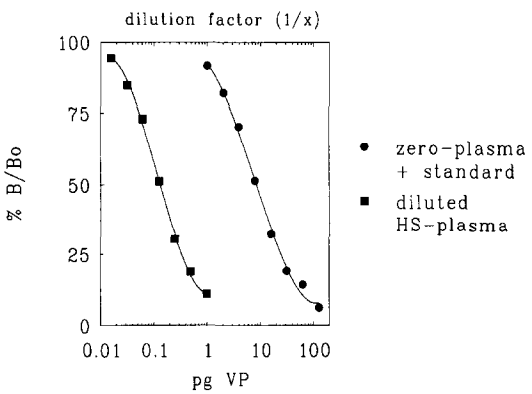

Fig. 3. Parallelism between the standard curve for synthetic vasopressin (VP) established in "zero plasma" and the dilution curve (reciprocal dilution factor) of plasma from rats subjected to an i.p. injection of hypertonic saline (9% NaCl = 1.5 *M*) 30 min prior to decapitation (HS).

TABLE III

VP DILUTION TEST

Plasma from rats injected i.p. with hypertonic saline was serially diluted with "zero plasma"; data represent mean ± S.D. (n = 4).

Dilution factor	Concentration of VP (pg/ml)
1	192.8 ± 27.8
2	90.9 ± 16.6
4	45.4 ± 4.4
8	19.8 ± 0.8
16	8.1 ± 0.6
32	3.9 ± 0.4
64	1.6 ± 0.2

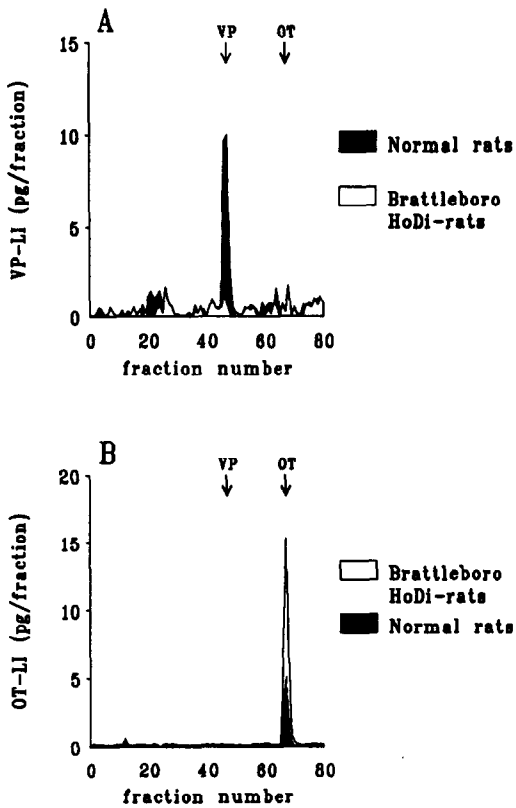

Fig. 4. HPLC elution profile showing that the VP-LI and OT-LI material from normal rat plasma extracts co-eluted with synthetic VP and OT, respectively. Extracts from Brattleboro rat plasma contain no VP-LI, only OT-LI.

TABLE IV

EFFECT OF VARIOUS TREATMENTS ON PLASMA VP LEVELS

Values are mean ± S.D.; N.D. = not determined.

Treatment	Concentration (mean ± S.D.) (pg/ml)	
	Normally hydrated rats	24-h Water-deprived rats
Control	4.23 ± 1.32 (n = 5)	19.07 ± 7.69 (n = 5)[a]
Ethanol ingestion	2.74 ± 1.38 (n = 4)	7.67 ± 5.88 (n = 4)[b]
Hypertonic saline	192.8 ± 27.8 (n = 4)[c]	N.D.

[a] $p < 0.01$ *versus* normally hydrated rats.
[b] $p < 0.01$ *versus* water-deprived controls (see also ref. 11).
[c] $p < 0.001$ *versus* all other groups.

DISCUSSION

Our data prove the usefulness of solid-phase extraction as a practical sample preparation technique to be carried out prior to the analyte measurement by RIA, for example. Typically, disposable columns filled with a certain amount and type of sorbent are used to reach the two main goals of extraction: sample clean-up and analyte concentration (see also ref. 12). A wide variety of sorbent-packed columns is now commercially available. Using a vacuum manifold column processor, many columns can be processed simultaneously and sample handling time is reduced considerably.

This paper reports a novel solid-phase extraction for VP-LI from plasma. We used reversed-phase chromatography, and tested various types of silica-based columns as well as different extraction protocols. The reversed-phase sorbents octadecyl (C_{18}), octyl (C_8), ethyl (C_2) and phenyl (PH) were tested for retention from an aqueous matrix (blood plasma diluted with 0.1 *M* hydrochloric acid) and elution of the synthetic peptide (VP).

Use of 60% acetonitrile gave the best elution efficiency (recovery) using the C_8 or the PH columns; solvents of even higher eluotropic strength towards silica (*i.e.* methanol and ethanol) did not improve the elution efficiency of the peptide from C_{18} columns (Table I). Somehow, the interaction between the peptide and the sorbent is too strong in the latter case and is difficult to disrupt with a relatively non-polar solvent such as acetonitrile. Use of a bonded phase with a shorter alkyl chain (C_8 or C_2) solved this problem and reduced the retention. We furthermore found that plasma extracts from C_{18} columns displayed a higher non-specific binding of the tracer in our RIA system (results not shown), and produced an "internal" standard curve that did not parallel the "external" one (*cf.* Fig. 2).

Apparently, the long-chain octadecyl group is relatively less selective in its attraction for hydrocarbon moieties of solutes/analytes. In this way, impurities that might interfere in the RIA may be retained after the washing step and co-extracted with the analyte.

Nevertheless, several groups [2–6] have used C_{18} columns to extract VP-LI from plasma employing different protocols. In a range from 10 to 200 fmol of synthetic VP added to rat plasma, the recovery was 85 ± 10% [2]; apparently the sorbent-bound VP did not elute completely even though it was eluted with 6 ml of 75% acetonitrile containing 4% acetic acid. These authors stated furthermore that their extraction procedure removes interfering factors from the plasma [2]. Others [3] used a total volume of 4 ml of methanol to recover *ca.* 80% of 1–10 fmol of (presumably) synthetic VP added to human plasma. More recently, Carman *et al.* [4] reported a method for the simultaneous extraction of OT-LI and VP-LI from ewe serum and also found an "overall VP recovery" of *ca.* 80% in the femtomole range (eluting with 3 ml of 80% acetone–0.02 *M* hydrochloric acid).

Larose *et al.* [5] attempted to improve the retention and elution of VP-LI on C_{18} columns by using triethylamine to occupy the free silanol groups of the sorbent, which tightly bind the peptide. Furthermore, they reported a 13% increase in VP-LI recovery by passing the plasma sample four times through the same column. After elution with 3 ml of methanol, their mean recovery of synthetic VP added to human plasma was *ca.* 75%. We recovered all synthetic VP added to 1 ml of rat plasma (see Table II) in our elution step, as measured by RIA. The determination of the elution efficiency using radioactive labels showed some discrepancies, depending on the label used (see Table I). However, iodinated VP is a different molecule from tritiated VP and hence their retentions might be different.

However, there is one other report of very high recoveries of synthetic VP from human plasma using C_{18} columns [6]. α-Human ANP-LI and VP-LI were co-extracted from 5 ml of EDTA-plasma by 4 ml of 90% methanol in 0.5% TFA, with an efficiency of more than 95%. These authors advise pretreatment of the columns with one complete "extraction cycle" before application of the plasma. In this cycle, small particles of column material that bleed from the column and interfere with the RIA are removed. The authors state that this pretreatment produced better recoveries.

Recently, it has been shown that also the flow-rate influences the retention/elution efficiency [12]. The recovery was found to be optimal when the sample was applied at a very low flow-rate. We applied the sample at a flow-rate of less than 1 ml/min.

Our results show that the extraction procedure isolates VP-LI and OT-LI that, after separation by HPLC, co-elutes with synthetic VP and OT, respectively. Based on the cross-reactivity of the antisera employed in the RIA systems used to detect VP-LI and OT-LI, we conclude that the immunoreactive material present in the extracts is indistinguishable from the authentic peptides.

In plasma extracts from Brattleboro rats that served as negative control, no VP-LI could be detected (see Fig. 4). Plasma extracts of both Wistar and Brattleboro rats, however, contained OT-LI. The reported higher plasma OT-LI level in Brattleboro rats [13] might explain the higher peak in the elution profile.

In addition, we demonstrated that the VP-LI material in the extracts is indistinguishable from synthetic VP by showing that the plasma dilution curve is parallel to the "internal" standard curve (Fig. 3).

In order to correct for possible non-specific effects introduced by the composition of extracted samples, interpolation on a standard curve established in "zero-samples" spiked with known amounts of analyte is preferred to a standard curve in the assay buffer. In the case of plasma hormone levels, one thus needs plasma devoid of the hormone to be determined. To obtain such plasma is not possible in most cases, and often other solutions are found: pre-extracted plasma (the effluent), plasma passed through an affinity column or surrogate plasma (often albumin added to the assay buffer). It is also possible to spike normal plasma or serum with additional synthetic hormone. Afterwards the standard curve can be corrected for the endogenously (but variously extracted) hormone [3,4].

In the case of VP, however, it is quite possible to acquire "zero-plasma" by water-loading the animals [7], or to use plasma from Brattleboro rats [1]. We currently obtain "zero plasma" by water-loading as described above. The superimposition of the two standard curves, which was also found by others [4], implies that interpolation on the "internal" curve renders almost twice the hormone concentration compared with the "external". This could explain the big differences that exist in the literature on VP-LI plasma levels. Assuming that the synthetic hormone added to "zero plasma" behaves like the endogenous hormone during the extraction, there is no need to correct for recovery or efficiency.

We also established standard curves for OT-LI in both rat and human plasma (from normal males), as the C_8 columns were found to be perfectly suited to the extraction of synthetic OT-LI as well. After correction for the endogenous OT-LI, standard curves that paralleled the "external" were obtained (results not shown). As we currently measure both VP-LI and OT-LI in our laboratory we can determine plasma levels of both hormones by this extraction protocol. By applying 2 ml instead of 1 ml of plasma to the column, simultaneous extraction for both hormones should be possible.

We used treatments known to affect VP-LI plasma levels to demonstrate the usefulness of the extraction procedure (Table IV).

Solid-phase extraction using C_8 silica columns is very suitable for preparing plasma samples for subsequent VP-LI and or OT-LI determination by RIA: it gives very high recovery and is therefore to be preferred above the use of C_{18} columns. Apart from immediate acidification after plasma preparation (in order to reduce protease activity), it also seems preferable to interpolate the samples on a standard curve established in "zero plasma". Better estimates of hormone content are obtained and there is no need for recovery correction.

REFERENCES

1 J. Dogterom, Tj. B. Van Wimersma Greidanus and D. De Wied, *Am. J. Physiol.*, 234 (1978) E463.
2 F. T. LaRochelle Jr., W. G. North and P. Stern, *Pflügers Arch.*, 387 (1980) 79.
3 M. Bevilacqua, R. Meroni, R. Dagani, E. Renesto, C. Baruto and G. Norbiato, *J. Endocrinol. Invest.*, 8 (1985) 97.
4 F. S. Carman III, Ch. E. Dreiling and D. E. Brown, *Clin. Biochem.*, 21 (1988) 265.
5 P. Larose, H. Ong and P. du Souich, *Clin. Biochem.*, 18 (1985) 357.
6 E. Hartter and W. Woloszczuk, *J. Clin. Chem. Clin. Biochem.*, 24 (1986) 559.
7 D. De Wied, *Acta Physiol. Pharmacol. Neerlandica*, 9 (1960) 69.
8 B. Liu and J. P. H. Burbach, *Peptides*, 8 (1987) 7.
9 P. R. P. Salacinski, Ch. McLean, J. E. C. Sykes, V. V. Clement-Jones and P. J. Lowry, *Anal. Biochem.*, 117 (1981) 136.
10 T. Higuchi, K. Honda, T. Fukuoka, H. Negoro and K. Wakabayashi, *J. Endocrinol.*, 105 (1985) 339.
11 B. J. M. Van de Heijning, I. Koekkoek-Van den Herik, N. Y. Rots and Tj. B. Van Wimersma Greidanus, *J. Neuroendocrinol.*, 3 (1991) in press.
12 T. Higa and D. M. Desiderio, *Int. J. Peptide Protein Res.*, 33 (1989) 250.
13 J. Dogterom, Tj. B. Van Wimersma Greidanus and D. F. Swaab, *Neuroendocrinology*, 24 (1977) 108.