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Targeted tyrosine iodination in a multi-tyrosine vasopressin analog

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Abstract: Iodination of the conserved 2-tyrosine (Tyr^2) residue in the pressin and tocin rings of arginine- or lysine-vasopressin (AVP or LVP), and oxytocin, respectively, impairs binding to their respective receptors. Synthetic antagonists that have their Tyr^2 either replaced by another amino acid or irreversibly blocked by an *O*-methyl or *O*-ethyl ether, but have, instead, an iodinatable phenol moiety outside the pressin/tocin ring, are used for radiolabeling. We explored another approach to avoid iodinating Tyr^2 by capping this residue with a reversible *O*-acetyl group, incorporated during peptide synthesis. The *O*-acetyl-Tyr^2 LVP peptide, with a free iodinatable tyrosine attached to the ε-amine of 8-lysine, is iodinated at a neutral pH and purified by reverse-phase high-pressure liquid chromatography (HPLC) at an acidic pH, conditions under which the *O*-acetyl groups are stable. Deacetylation with hydroxylamine is selective, and leaves intact the disulfide bridge. The marked shortening of the HPLC retention time after deblocking produces a chemically homogeneous label, iodinated exclusively on the free tyrosine residue attached to the ε-amine of LVP. Hitherto, this ^{125}I labeled vasopressin agonist could be obtained only in low yield, via conjugation labeling with iodinated *N*-*t*-Boc-tyrosine succinimidyl ester. This fully reversible tyrosine protection strategy does not require special equipment, and retains the conserved Tyr^2, typical of vasopressin and oxytocin agonists. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: iodination; tyrosine modification; *O*-acetyl; hydroxylaminolysis

INTRODUCTION

'Carrier free' ^{125}I or ^{131}I permits iodination of peptides to high specific radioactivities. However, since iodination methods indiscriminately label all accessible tyrosine and, at higher pH, also histidine residues, the reversible protection of selected tyrosine(s) in peptides with additional iodinatable tyrosine or histidine residue(s) may be desirable. Indeed, the incorporation of a radioisotope larger than the aromatic ring of tyrosine may hamper receptor binding. For example, iodination of the conserved tyrosine of the pressin or tocin ring of neurohypophyseal hormones abolishes their affinities for the arginine vasopressin (AVP) and oxytocin receptors, respectively, precluding their use in receptor research [1]. Thus, while the high specific radioactivity iodinated AVP and oxytocin peptides serve as radioimmunoassay tracers, the low specific radioactivity tritium-labeled peptides are still used in receptor research [1,2]. However, ^{3}H detection is less efficient than ^{125}I or ^{131}I, making ^{3}H-peptides less suited for autoradiography [3] or other studies. To overcome these limitations, peptide chemists have synthesized a variety of vasopressin and oxytocin analogs bearing an iodinatable moiety outside the pressin or tocin ring, but with the conserved tyrosine within this ring either replaced by another amino acid, or irreversibly blocked by an *O*-methyl or *O*-ethyl ether [4–7]. For the renal V_2 AVP receptor, the peptides of interest are all derivatives of the AVP analog [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)]AVP, $d(CH_2)_5$AVP, with an iodinatable group at the carboxy end. In the case of the vascular V_1 receptor, linear AVP antagonists also exist that can be iodinated directly [8]. One such antagonist [8] has an *O*-methylated tyrosine in position 2, but an iodinatable OH-phenylacetyl group in position 1. Of the two cyclic V_2 antagonists $d(CH_2)_5$[D-$Tyr(Et)^2$, Val^4, Tyr-$NH_2{}^9$]AVP and $d(CH_2)_5$[D-Ile^2, Ile^4, Tyr-$NH_2{}^9$]AVP designed for direct iodination on the 9-tryrosineamide, the iodinated version of the former retains enough receptor affinity to be of interest [4,5]. However, the consequence of modifying the conserved 2-tyrosine (Tyr^2) in neurohypophyseal hormones is that these iodinatable AVP analogs are all competitive AVP antagonists, and hence cannot distinguish the high (R^*) from the low (R) affinity conformations of the receptor. Agonists, on the other hand, have higher affinity for the active conformation R^* of the receptor. The only known high-affinity iodinated AVP agonist is obtained by conjugating an iodinated tyrosine to the ε-amino group of 8-lysine, in lysine vasopressin (LVP) [9].

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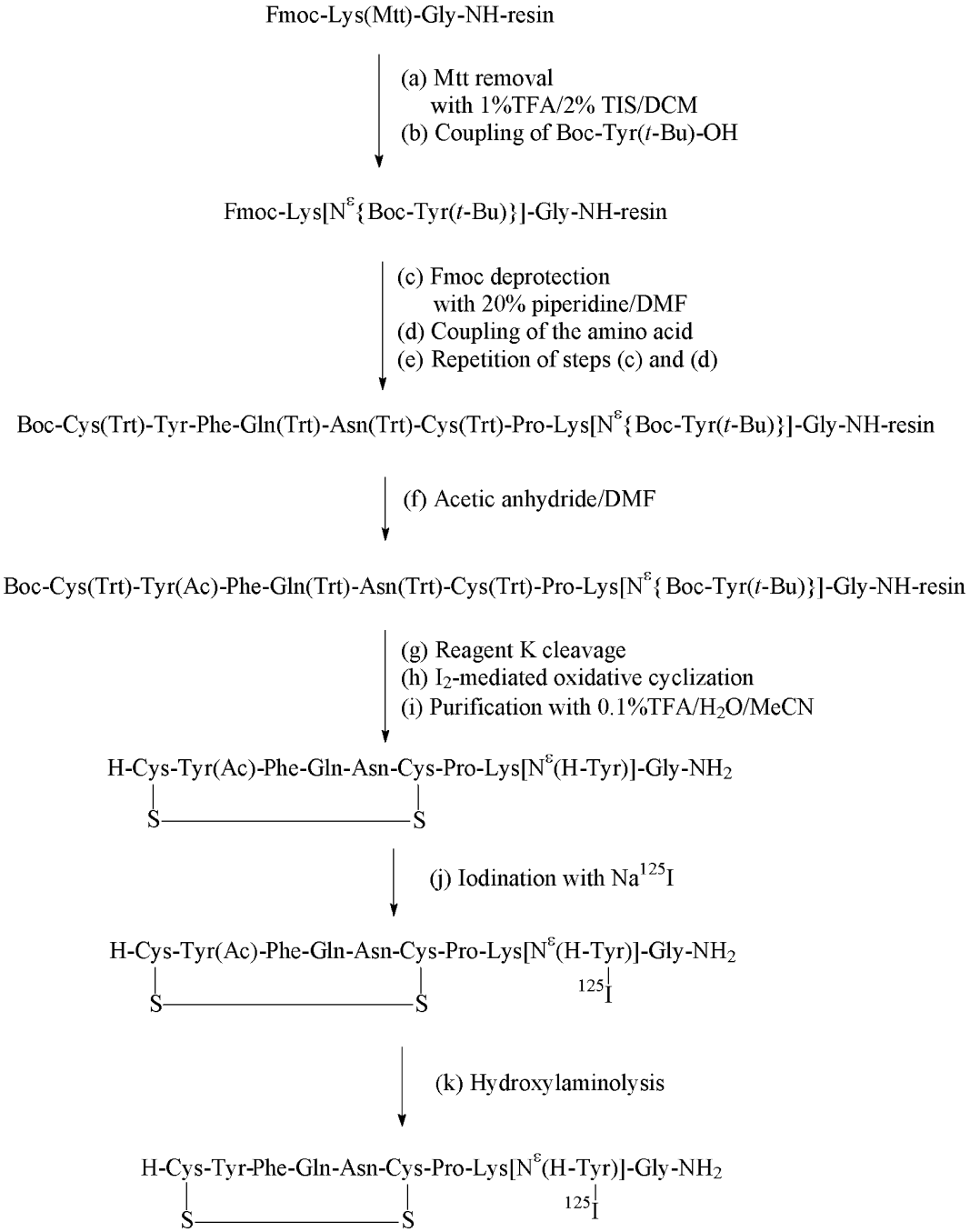
The finding that 2-*O*-acetyl-oxytocin derivatives remain stable under acidic to neutral conditions, but that the *O*-acetyl moiety readily comes off the tyrosine hydroxyl in the presence of hydroxylamine [10], led us to explore a novel, selective iodination method that could be of broad utility. We designed [*O*-acetyl-Tyr^2, N$^\varepsilon$(Tyr)-Lys^8]LVP (Scheme 1), which is essentially LVP with the conserved Tyr^2 in the pressin ring reversibly acetylated (*O*-acetyl-Tyr^2) but with a free tyrosine residue conjugated to the ε-amine of 8-lysine. Direct iodination of this precursor, followed by deacetylation yields [N^ε(^{125}I-Tyr)-Lys^8]LVP, the high-affinity LVP agonist [9] that is iodinated exclusively in the tail of the peptide, and which has an intact Tyr^2 in the pressin ring, typical of high-affinity AVP agonists.

RESULTS

Direct iodination of [*O*-acetyl-Tyr^2, N$^\varepsilon$(Tyr)-Lys^8]LVP, followed by deacetylation produces [N^ε(^{125}I-Tyr)-Lys^8] LVP, a vasopressin analog exclusively iodinated outside



Scheme 1 Synthesis of selective ^{125}I labeled Tyr vasopressin peptide analog.

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the pressin ring, with exactly the same retention time as the authentic agonist obtained by directly conjugating ^{125}I-tyrosine to the ε-amine of the Lys^8–LVP peptide [9]. Indeed, the peptide from the major radioactive peak (Figure 1, upper HPLC profile) produced, upon deblocking, a label that emerged with the same retention time as this agonist, hitherto obtained in our laboratory by conjugating ^{125}I-tyrosine to the ε-amine of Lys^8-LVP (Figure 1, single peak, lower HPLC profile).

This peptide binds extensively to an excess AVP antibody ($B_o \sim 96\%$) and/or to the V_2 receptor (>35%, Table 1), consistent with the finding that hydroxylaminolysis leaves intact the disulfide bridges [10]. The label from the minor peak in the first HPLC profile (Figure 1, upper panel) also binds to the AVP antibody, and probably corresponds to the fraction of peptide di-iodinated on the same free tyrosine 8-N^{ε}(Tyr)-Lys.

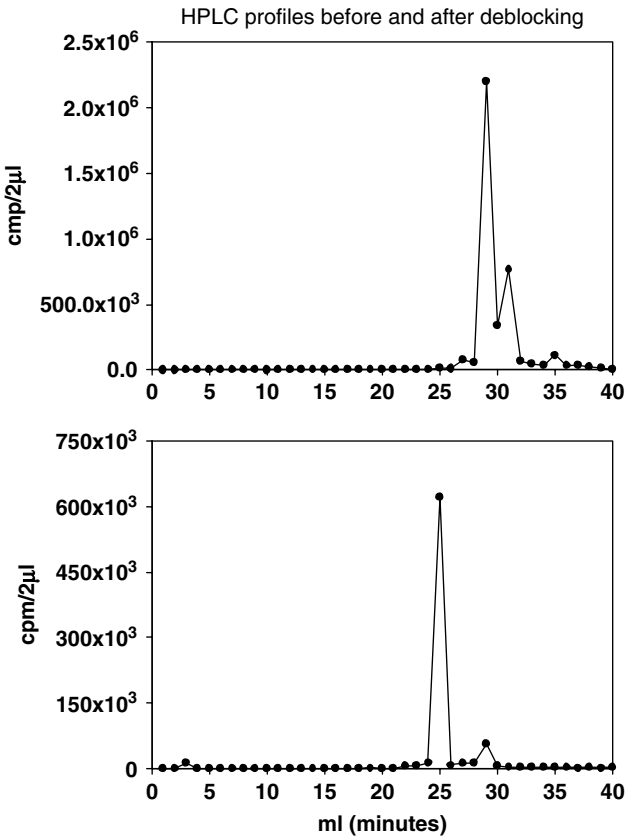

Figure 1 The HPLC elution profiles of the peptide after iodination (upper panel) and deblocking (lower panel). The RP-HPLC was performed on a C-18 column with a gradient of MeCN in 0.1% TFA, starting at 5% MeCN, and increasing the MeCN fraction by 1% per min, and run at a flow rate of 1 ml/min. Radioactivity was counted in a gamma spectrometer on 2 µl samples taken from each successive 1 min collection. The peptide from the major peak (upper panel, fraction 29) was extracted and deacetylated (see text) prior to repeat chromatography (lower panel).

Table 1 Binding of the Iodinated Peptide to the Human V_2 Receptor[a]

Counts				cpm/min /tube (±SD), (24.0 ± 1.4 µg protein)	% bound (±SD)
Total[b] counts/tube,				138 130 ± 3800	
Bound	"	"	*wt* V_2R	49 170 ± 1900	35.60 ± 1.38[c]
NSB	"	"	*wt* V_2R	1500 ± 50	1.09 ± 0.04
Bound	"	"	no V_2R	850 ± 50	0.62 ± 0.04
NSB	"	"	no V_2R	840 ± 40	0.61 ± 0.03

[a] Plasma membranes from COS-7 cells transfected (*wt* V_2R), or mock-transfected (no V_2R), nonspecific binding (NSB) obtained in the presence of 1 µM V_2R antagonist SR-121 463.
[b] Counts per minute (cpm) in 150 µl incubate, 30 min incubation, data in mean ± SD.
[c] >35% binding is due to high specific activity (~2000 Ci/mmol), and receptor density.
The K_d is ~1 nM, (Note, that for the ~100 000 cpm/tube, 'free' ligand concentration is <2 nM).

DISCUSSION

Hitherto, the only known method to generate the high-affinity [N^{ε}(^{125}I-Tyr)-Lys^8]LVP agonist was by conjugation labeling, using the iodinated *N*-hydroxysuccinimidyl ester of *N*-*t*-Boc-tyrosine to the Lys in LVP [9]. This conjugation method, which resembles that described for the labile Bolton–Hunter reagent [11], is cumbersome and of very low yield [9]. This explains why the low specific radioactivity ^{3}H-AVP analogs are still used in receptor research [1,2,12]. The 'iodine exchange reaction' is an alternative method that also permits a targeted labeling of the tyrosine of choice in peptides with several tyrosine residues [13]. This method would require synthesizing [Tyr^2, N^{ε}([^{127}I]-3,5-diiodo-Tyr)-Lys^8]LVP, and then exchange the stable ^{127}I isotope with ^{125}I from carrier free Na^{125}I. However, low specific radioactivity (~10 Ci/mmol) and poor yield [13] render tritiated peptides more attractive (30–70 Ci/mmol). The direct iodination of [Tyr^2, N^{ε}(Tyr)-Lys^8]LVP, followed by isolation of the label of interest by HPLC, would increase both the labeling yield and specific radioactivity. Unfortunately, the direct iodination of peptides with several tyrosine residues produces iodinated species that are difficult to separate [14], in addition to the reduced yield of the labeled species of interest [15,16]. Moreover, since the incorporation of a first iodine atom into the phenolic ring of tyrosine facilitates incorporation of a second one [13], even under conditions of excess peptide, for each mono-iodinated peptide peak by HPLC a lesser one will be found corresponding to the di-iodinated version for that same tyrosine residue (Figure 1, upper panel). The strategy adopted here for

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obtaining in high yield the [N^{ε}(^{125}I-Tyr)-Lys^{8}]LVP vasopressin receptor analog [9] was through the direct iodination of [*O*-acetyl-Tyr^{2}, N^{ε}(Tyr)-Lys^{8}]-LVP, followed by deacetylation and HPLC purification.

The alternative, 'stoichiometric labeling' method [17], which requires the addition of Na^{127}I, is pointless since HPLC separation of iodinated peptides from the unlabeled fraction can be so efficient that the purified peptides essentially acquire the specific radioactivity of the carrier free radioiodine used for labeling [18]. The shortening in the HPLC retention time after deblocking not only produces chemically homogeneous monoiodinated peptides but also indicates that the iodinated peptide corresponds to the anticipated one. Indeed, any accidental loss of the *O*-acteyl-protecting group, which would allow iodination of the alternative tyrosine, would produce a labeled peptide with unchanged HPLC retention time before and after deblocking. As shown in Figure 1 (upper *vs* lower trace), there was no overlap between both HPLC peaks, and the deblocking was nearly complete after only 30 min incubation under very mild conditions at pH 6.4 and <40 °C in the presence of 100 mM buffered hydroxylamine.

Although many other easily removable *O*-tyrosine protecting groups could be used for the selective single tyrosine iodination strategy proposed here, a scan of the literature did not produce any reports. A differential iodination method was proposed for the detection of peptides with sulfated tyrosine residues [19]. This HPLC detection method takes advantage of the fact that *O*-sulfated tyrosine is readily desulfated under acidic conditions. Thus, the peptide extract is first iodinated with nonradioactive ^{127}I on the free tyrosine and histidine residues, before removing the acid labile sulfate groups and iodinating the unmasked tyrosine residues with radiaoctive ^{125}I. The judicious use of azidomethyl (Azm) protection of tyrosine hydroxyls has recently made possible the synthesis of peptides sulfated on selected tyrosine residues [20], and hence that could be used as alternatives to peptides with *O*-acetyl-protected tyrosine residues. In fact, the Azm-tyrosine protection employed to generate these peptides could also serve to synthesize selective Azm-tyrosine protected peptides as alternative substrates for the site-directed iodination strategy proposed here. Finally, peptides containing photo-labile protecting groups on selected tyrosine residues [21] could also be used. This diversity in orthogonal tyrosine protecting groups, removable under either reducing or oxidizing, alkaline or acidic, or other conditions, provides a spectrum of alternatives, which can be matched with the specific characteristics of a given peptide to be iodinated. Our *O*-acetyl-tyrosine peptide is very stable; we have used the same working dilution stored at −20 °C for more than one year without any noticeable loss of the *O*-acetyl protecting group, as assessed by HPLC following iodination. Studies on *O*-acetylphenol (phenyl acetate), used as the model compound, have shown that the *O*-acetyl linkage does not hydrolyze under acidic to neutral conditions [10].

CONCLUSION

The [*O*-acetyl-Tyr^{2}, N^{ε}(Tyr)Lys^{8}]LVP peptide was synthesized using Fmoc chemistry. The reversible *O*-acetyl phenolic hydroxyl protecting group can be used advantageously for the direct, yet targeted iodination on one single tyrosine or additional histidine residue, in synthetic peptides that contain more than one tyrosine residues, or that have an additional iodinatable histidine residue.

METHODS

Materials

All chemicals, unless specified, were from Sigma Chemical Company, St Louis, Missouri, USA (www.sigmaaldrich.com). Protected amino acids and resin were obtained from BACHEM AG, Bubendorf, Switzerland(www.bachem.com).

Peptide Synthesis

The [*O*-acetyl-Tyr^{2}, N^{ε}(Tyr)Lys^{8}]LVP precursor peptide was synthesized by solid-phase methodology on a Symphony parallel synthesizer using Fmoc chemistry (Scheme 1). The Ramage amide linker resin was used as the solid support. *N*-[(1*H*-Benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methyl methanaminium hexafluorophosphate *N*-oxide (HBTU) was used as the activating agent. The α-amino function was protected with the Fmoc group, and the reactive side-chain functional groups were protected as follows: trityl (Trt) for Cys, Gln, and Asn; *tert*-butyl (*t*-Bu) for Tyr; and 4-methyltrityl (Mtt) for Lys. A 20% piperidine solution in dimethylformamide (DMF) was used to deprotect the Fmoc group, and a 1% trifluoroacetic acid (TFA) solution in dichloromethane (DCM) was used to remove the Mtt group from Lys. Coupling protocol utilized three equiv. of amino acid, 2.85 equiv. of HBTU, and six equiv. of diisopropylethyl amine (DIEA) in DMF for 40 min. The peptide synthesis began by loading Fmoc-Gly-OH on to the resin followed by Fmoc deprotection and coupling of Fmoc-Lys(Mtt)-OH. The Mtt group was selectively removed with 1% TFA/DCM. The free $^{\varepsilon}NH_2$ group was reacted with Boc-Tyr(*t*-Bu)-OH. The Fmoc group from the α-amino function of Lys was removed with 20% piperidine/DMF and the synthesis was continued. Tyr^{2} was coupled without any side-chain protecting group. The *N*-term amino acid was introduced as Boc-Cys(Trt)-OH. Fully assembled peptide thus obtained was treated with acetic anhydride (2 × 30 min) to acylate the hydroxyl function of Tyr [2]. The peptide was cleaved from the resin and the side-chain protecting groups were removed using Reagent K as the cleavage cocktail. The crude peptide was taken in 20% acetic acid and oxidatively cyclized with 0.1 M solution of iodine in methanol using standard procedure. The cyclized peptide was purified by RP-HPLC on a C-18 column using standard 0.1% TFA/H_2O/MeCN buffer system and the fractions with desired

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purity were pooled and lyophilized. The lyophilized peptide was obtained as a white fluffy solid with 91% purity. MALDI-MS analysis of the peptide showed a molecular ion peak $[M]^+$ at m/z 1259.8 ($C_{57}H_{74}N_{14}O_{15}S_2$). The amino acid analysis showed a peptide content of 75% and the amino acid values were in the expected range.

The peptide was stored at −20 °C under dry conditions. A working dilution of this peptide (1 mg/ml in H_2O), used for the direct iodination, was also stored at −20 °C. Under these conditions, the peptide has been stable for more than one year, as judged by the reproducible, high labeling yield and consistent elution pattern in our HPLC system (Figure 1).

Iodination

The chloramin T and Iodogen methods are both appropriate. We made two modifications to the standard iodination procedure for the labeling of this peptide. First, the iodination with carrier free $Na^{125}I$ (about 1 mCi $Na^{125}I$, MS300, Amersham Biosciences, GE Healthcare, for 5–10 μg (5–10 μl) of peptide in 75 μl 0.1 M sodium phosphate buffer) is performed under neutral conditions, at pH 7.0, rather than 7.4, to avoid hydrolyzing the *O*-acetyl moiety during labeling. Second, 100 μl of 4% acetic acid is used to quench the reaction, instead of the standard sodium metabisulfite, to avoid reductive opening of the [Cys^1–Cys^6] disulfide bridge of the peptide ring. The first HPLC pattern developed in a gradient of MeCN in 0.1% TFA at a flow rate of 1 ml/min, starting with 5% MeCN and increasing by 1% MeCN per min, produces one major radioactivity peak (Figure 1, upper panel). The labeled peptide from this peak, which binds to our AVP antibody, was deblocked.

Deblocking

Hydroxylaminolysis removes the 2-*O*-acetyl protecting group from tyrosine residues under mild conditions [10]. Before deblocking, the labeled peptide is first extracted from the HPLC peak (Figure 1, upper panel). This is accomplished by diluting the peak with 4% acetic acid, followed by loading on a C-18 Sep-Pack cartridge (Waters Co, Milford MA), previously activated with 10 ml methanol and equilibrated with 4% acetic acid. The peptide is then eluted with 2 ml methanol and the solvent is evaporated in a warm water bath under a stream of N_2. A 100-mM aqueous solution of hydroxylamine hydrochloride (Pierce, #26 103, www.piercenet.com) is prepared and the pH adjusted to 6.4 by adding powdered sodium bicarbonate under constant stirring. Deblocking is then performed by adding 200 μl of this hydroxylamnine solution to the evaporated peptide and incubating at 30–38 °C in a water bath for 30 min, with occasional stirring. This 200 μl sample was then directly rechromatographed in the same HPLC system, without attempting to recover all the material from the syringe or tube using solvents such as MeCN or DMF. The label extracted from the peak of the first chromatogram (Figure 1, fraction 29, upper panel) after deblocking emerges 4 min earlier (Figure 1, lower panel, fraction 25). The radiolabel from this second HPLC peak illustrated in Figure 1 displayed 95.8% binding to an excess AVP antibody, but only 1.3% binding in the absence of antibody (nonspecific binding).

Binding of the Label to the AVP Antibody and the V_2 Receptor

Binding to a 1/1500 final dilution of our AVP antibody #10 169 is allowed to proceed for 30–60 min after mixing 100 μl tracer with 200 μl antibody, both diluted in phosphate buffered saline (PBS), pH 7.4, containing 0.1% BSA and 0.1% sodium azide. The antibody-bound fraction is precipitated by adding 50 μl diluted inactivated NRS and 100 μl of an appropriately titrated goat anti rabbit IgG antibody, followed 90 min later by the addition of 3.5 ml of an ice cold 5% polyethylene glycol (MW 8000) solution in PBS, and centrifugation (3200 rpm) at 4 °C for 30 min. All the tubes are decanted by inversion, prior to counting. Binding of this ligand to the wild type (*wt*) human V_2R was also tested on plasma membranes prepared from transiently transfected COS-7 cells (American Type Culture Collection (ATCC), Manassas, VA). Membrane preparations and bacitracin-containing binding buffer are both described elsewhere [9]. The COS-7 cells were transfected with the mammalian expression vector pCMV6-XL4 harboring the *wt* human V_2R cDNA using the S-lipid reagent (both from OriGene: http://www.origene.com/). Tubes containing 100 μl (24.0 ± 1.4 μg protein) of a membrane suspension were incubated with 50 μl ligand (138 130 ± 3800 cpm), both in binding buffer, for 30 min at room temperature, prior to the addition of 4 ml ice cold binding buffer and centrifugation (3200 rpm) for 30 min at 4 °C and decanting. The NSB was determined in the presence of 1 μM of the V_2R antagonist SR-121 463 (Sanofi Recherche, France). The results are shown in Table 1.

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