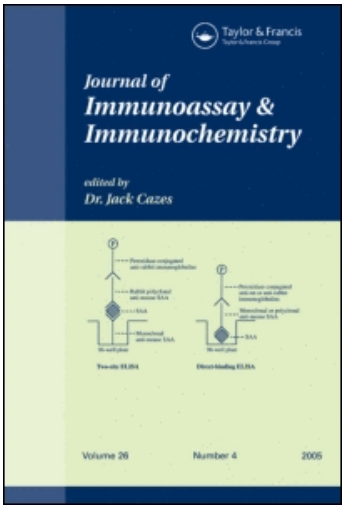

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Development of an Immunoassay for Glypressin, an N-Terminal Extended Vasopressin Analogue

Mats Ohlin[a]; Ulf Alkner[a]
[a] Immunochemical Dept., Ferring Pharmaceuticals, Malmö, Sweden

To cite this Article Ohlin, Mats and Alkner, Ulf(1988) 'Development of an Immunoassay for Glypressin, an N-Terminal Extended Vasopressin Analogue', Journal of Immunoassay and Immunochemistry, 9: 1, 19 — 36
To link to this Article: DOI: 10.1080/01971528808053208
URL: http://dx.doi.org/10.1080/01971528808053208

JOURNAL OF IMMUNOASSAY, 9(1), 19-36 (1988)

DEVELOPMENT OF AN IMMUNOASSAY FOR GLYPRESSIN, AN N-TERMINAL EXTENDED VASOPRESSIN ANALOGUE

Mats Ohlin and Ulf Alkner
Ferring Pharmaceuticals, Immunochemical Dept.,
P.O. Box 30561, S-200 62 Malmö, Sweden

ABSTRACT

The development and evaluation of a radioimmunoassay for N^{α}-triglycyl-lysine^{8}-vasopressin is described. The site of hapten conjugation of the immunogen has been controlled and the use of various radiolabelled tracers has been evaluated with special reference to the site of iodination. The most extensively studied antiserum showed specificity for the N-terminal triglycyl-extension as well as for several aminoacid residues of the vasopressin ring. It crossreacted 27%, 28% and 0.3% with Lys^{8}-vasopressin, arg^{8}-vasopressin and oxytocin respectively, and it was used to quantify triglycyl-lysine^{8}-vasopressin in human plasma after SepPak C18 extraction. The sensitivity of the assay was 5 pg/tube with an intra-assay CV of 5-6% at 17 and 70 pg/tube. The identity of the immunoreactivity was studied by reversed phase chromatography.

INTRODUCTION

Tri-glycyl-lysine^{8}-vasopressin (TGLVP) has been reported to act as an hormonogen to lysine^{8}-vasopressin (LVP) (1,2) but has little antidiuretic and pressor activity by itself (3). The N-terminal triglycyl-extension is also believed to extend the biological half-life of the molecule (2). These facts indicate a potential therapeutic use of this synthetic peptide, and it has been used in the clinic as an agent to control hemorrhage (4).

Previous reports on the pharmacokinetics of this compound have described antisera that crossreact appreciably with the proposed bioactive metabolite (1,2), since no specific antibody for this hormonogen has been available. To be able to quantify the mother compound as well as N-terminal modified metabolites thereof by immunoassay with or without chromatography it is important to obtain more specific antibodies for these compounds. To improve the exposure of the vasopressin ring the hapten has been coupled to a carrier protein through the lysine residue while the N^{α}-amino group has been temporarily protected.

MATERIALS

Peptides were synthesized by liquid or solid phase techniques and purified by reversed phase chromatography at Ferring AB (Malmö, Sweden) except N^{α}-Boc-glypressin (N^{α}-Boc-TGLVP) which was synthesized by Dr. G. Kupryszewski and Dr. P. Rekowski at the University of Gdansk (Gdansk, Poland). Carrier-free $Na^{125}I$ was obtained from Amersham Int. (Amersham, Buckinghamshire, UK) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 3-(p-hydroxyphenyl)-propionic acid-N-hydroxysuccinimide ester (PHPP-NOSu; Bolton-Hunter reagent) and mono- and diiodinated tyrosine from Sigma Chemical Company (St. Louis, MO, USA). Human serum albumin (HSA) was supplied by Behring Institut (Marburg, West Germany), Bovine serum albumin (BSA) by Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex, UK), pronase (Streptomyses griseus) by Boehringer-Mannheim (Mannheim, West Germany) and Freund's complete adjuvent by Difco (Detroit, MI, USA). TLC-plates (Kiselgel 60) were

obtained from Merck (Darmstadt, West Germany). Other reagents were of analytical grade or HPLC grade whenever available.

The HPLC-system was obtained from Waters (Milford, MA, USA) as were μBondpak C18 (3.9 x 300) columns and Sep-Pak C18 minicolumns.

METHODS

Succinylation of bovine serum albumin was performed as previously described (5).

Modification of glypressin (TGLVP, triglycyl-lysine-vasopressin) with the Bolton-Hunter reagent (6) at Lys^8 position was performed by incubating 76 μg PHPP-NOSu with 257 μg N^{α}-Boc-TGLVP in 200 μl dimethylformamide for 3 h at room temperature. The N^{α}-protecting group was removed by incubation with 50% trifluoroacetic acid (TFA) in methylene chloride for 15 minutes at room temperature and the $(Lys\text{-}PHPP)^8$-TGLVP product purified by reversed-phase chromatography and stored at -20°C until used for iodination.

Iodination of peptides was performed by the chloramine T method (7). 2-5 μg peptide was incubated with 0.5-1 mCi carrier free $Na^{125}I$ and 2 μg chloramine T for 60 s and then immediately purified by isocratic reversed phase chromatography using acetonitrile in 0.1% TFA as the eluant. The monoiodinated peptide was diluted with 10 volumes of assay buffer and stored at -20°C.

N^{α}-Boc-TGLVP was conjugated to succinylated BSA (sBSA) using EDC at a molar ratio of 25:1:25 in 2 ml H_2O for 24 h at room tem-

perature. The product was dialyzed against water, freeze-dried and the N^{α}-protecting group removed as described above. The conjugate was once again dialyzed against water and freeze-dried.

Four rabbits were immunized with 500 µg TGLVP-sBSA conjugate each in 1 ml 0.9% NaCl emulsified with 1 ml Freund's complete adjuvant by multiple subcutaneous injections. Booster doses of 400 µg conjugate were given 4 weeks later and thereafter when required. The animals were bled fortnightly and separated serum was stored at -20°C.

Radioimmunoassay was carried out in 0.1 M sodium phosphate, 0.05 M NaCl, 0.02% NaN_3, 0.1% HSA pH 7.6 in polystyrene tubes (11x55 mm). 200 µl of standard or extracted plasma was incubated with 100 µl of tracer (1-2 fmole) and 100 µl of diluted antiserum for 40-50 h at +4°C. Standards were run in triplicates while samples were run in duplicates. Separation of bound and free tracer was carried out with 500 µl 5 mg charcoal/ml assay buffer supplemented with 2.5% human plasma. The supernatant was collected after centrifugation and counted in a LKB Wallac 1272 gamma counter. Standard curves were on-line computer calculated using a spline-function methodology and subsequently used to evaluate sample results.

Extraction of peptides from plasma was performed by the use of Sep-Pak C18 minicolumns (8). Briefly acidified plasma (1 volume 1 M HCl and 10 volumes of plasma) was added to an activated and washed column. Contaminants were removed with 2x5 ml 0.1% TFA and 5

ml 10% ethanol in 0.1% TFA. Peptides were eluted with 4 ml 75% acetonitrile in 0.1% TFA. Extracts were evaporated in a Savant Speed Vac Concentrator.

Antiserum A:8 with C-terminal specificity obtained against an AVP-thyroglobulin conjugate has been previously described (9). Crossreactivity data (relative to TGLVP) was Arg^8-vasopressin (AVP) 1000%, Lys^8-vasopressin 100%, AVP(1-8)-OH 7%, oxytocin 0.4%, Arg^8-vasotocin 600%, desamino^1-AVP 1000%, D-Arg^8-vasopressin 5%.

Scatchard plots to determine antibody affinity constants were done as described earlier (10). HPLC of plasma extracts was performed using an analytical μBondpak C18 column eluted with 16% CH_3CN in 0.1% TFA followed by a rapid gradient to 50% acetonitrile. 100 μl of each fraction was evaporated in duplicates in a Savant Speed Vac Concentrator and assayed for TGLVP-LI using antisera K8509-3 and A:8.

Pronase digestion of iodinated peptides was carried out in 50 mM sodium phosphate buffer pH 7.5 with 2 mg HSA/ml and 1 mg pronase/ml for 24-48 h at 37°C. Elution of amino acids on silica gel TLC-plates was done by 1-butanol:acetic acid:water 4:1:1. Radioactivity was detected and compared with nonradioactive iodine modified tyrosine standards.

Errors are given as standard deviation (SD) unless stated otherwise.

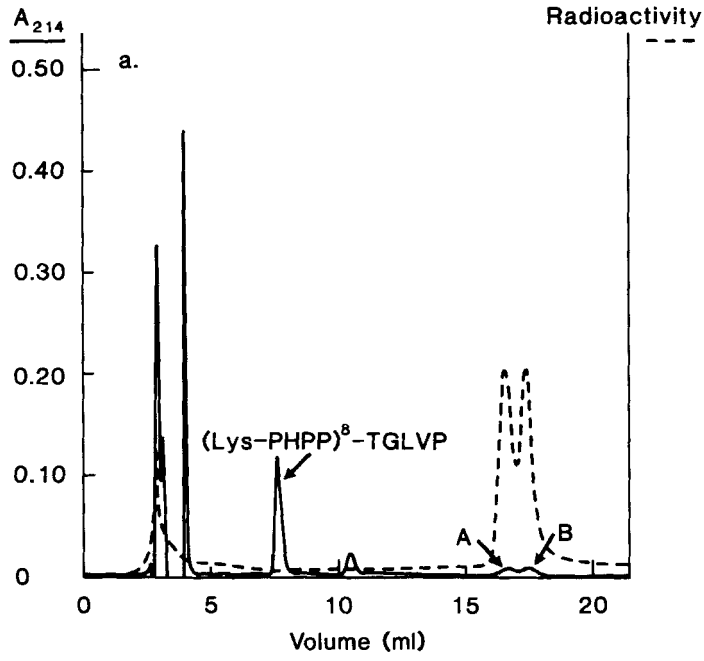

FIGURE 1. Elution profile of the iodinated products of (Lys-PHPP)^8-TGLVP(a) or TGLVP(b) by reversed phase HPLC. 24.8% and 22.8% respectively of acetonitrile were used in the mobile phase. The identity of peaks A and B is discussed in the text.

RESULTS

Iodination of (Lys-PHPP)^8-TGLVP, which was homogeneous as determined by isocratic reversed phase chromatography, produced two chromatographically incompletely resolved (R_S=0.9) iodinated components in approximately equal yield (Figure 1). After pronase cleavage radioactivity of peak B coeluted with monoiodinated tyrosine on TLC while radioactivity of pronase cleaved peak A did not migrate appreciably using this system. Iodination of TGLVP resulted in one monoiodinated (as determined by TLC after pronase digestion)

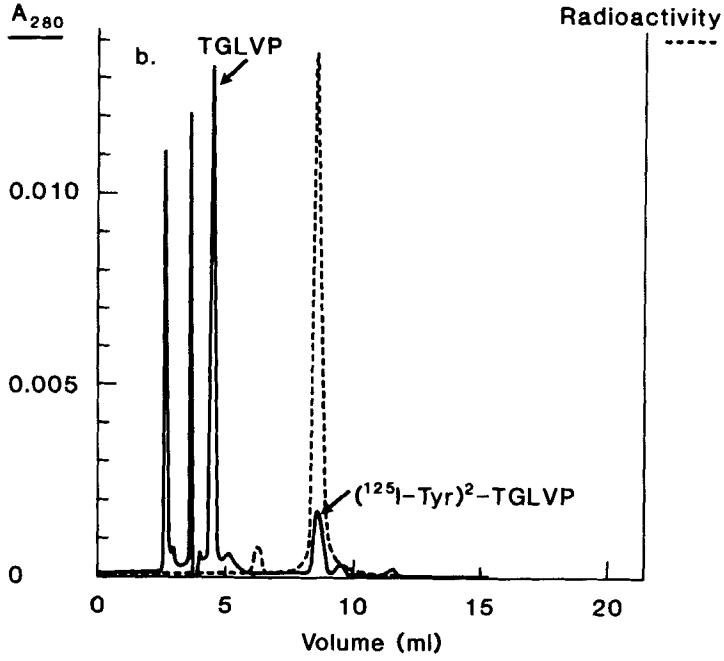

FIGURE 1 (continued)

component well separated from excess nonradioactive peptide (Figure 1).

Two out of four rabbits immunized with the water-soluble TGLVP:sBSA conjugate produced hapten specific antibodies after two booster injections as determined by radioimmunoassay. These antibodies reacted with different affinities (K_A=(0.3-1.1)•10^{11} M^{-1} using K8509-3) with the tracers being tested in that (^{125}I-Tyr)^2-TGLVP and ^{125}I-(Lys-PHPP)^6-TGLVP peak B showed similar behaviour in contrast to ^{125}I-(Lys-PHPP)^6-TGLVP peak A (Figure 2). The cross-

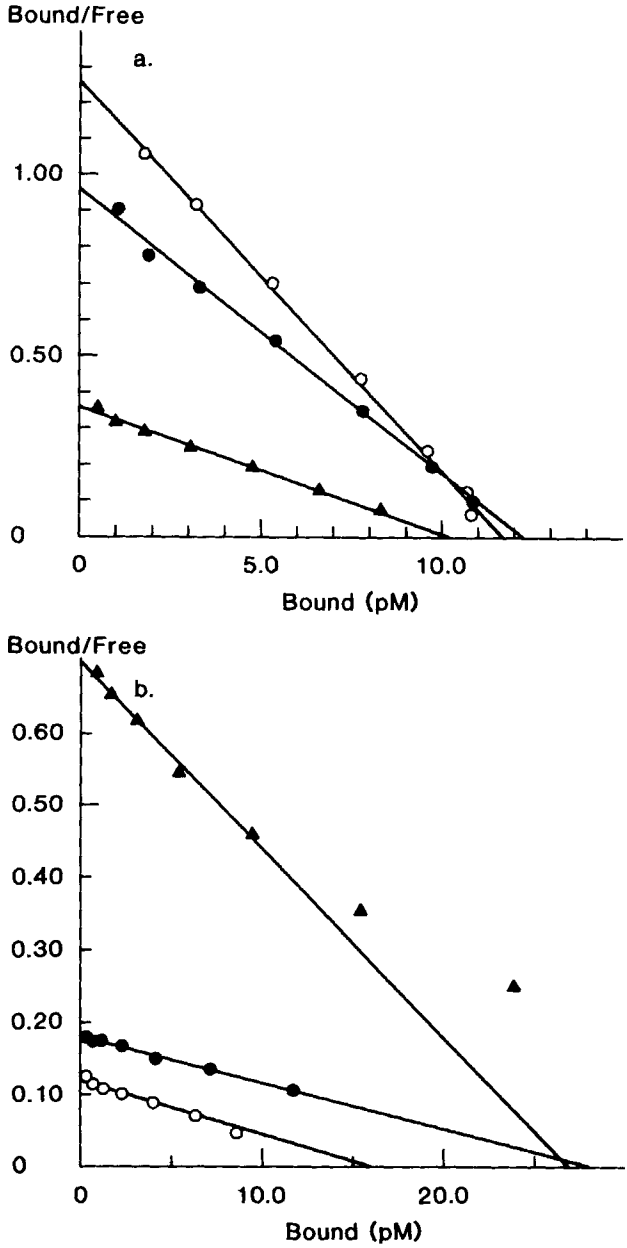

FIGURE 2. Scatchard plot for antisera K8509-3(a) and K8512-3(b) using (^{125}I-Tyr)^{2}-TGLVP (o), ^{125}I-(Lys-PHPP)^{6}-TGLVP peak A (▲) and ^{125}I-(Lys-PHPP)^{6}-TGLVP peak B (•) as standard.

TABLE I

Crossreactivity of antiserum K8509-3 using (^{125}I-Tyr)^{2}-TGLVP as determined by the weight of TGLVP required to displace 50% of radioligand x 100% / weight of crossreacting substance required to displace 50% of radioligand. mpa=mercaptopropionic acid. -=deleted amino acid. tyr(OEt)=O-ethyl-tyrosine. Amino acids are in the L configuration unless otherwise stated. OH and NH_2 indicate a free and amidated carboxyl terminal respectively.

Peptide	-2	-1	0	1	2	3	4	5	6	7	8	9	Cross-reactivity
				⌜					⌝				
TGLVP	gly	gly	gly	cys	tyr	phe	gln	asn	cys	pro	lys	$glyNH_2$	100 %
gly-gly-LVP	-												85 %
gly-LVP	-	-											56 %
LVP	-	-	-										27 %
desamino^{1}-LVP (dLVP)	-	-	-	mpa									21 %
Dtrp-dLVP	-	-	-	mpa	Dtrp								0.1 %
AVP	-	-	-								arg		28 %
AVP (free acid)	-	-	-								arg	glyOH	21 %
desglycinamide-AVP	-	-	-								argOH	-	12 %
desamino^{1}-AVP(dAVP)	-	-	-	mpa							arg		21 %
tyr^{3}-dAVP	-	-	-	mpa		tyr					arg		2 %
tyr(OEt)^{2}-dAVP	-	-	-	mpa	tyr(OEt)						arg		18 %
dDAVP	-	-	-	mpa							Darg		11 %
asn^{4}-dDAVP	-	-	-	mpa			asn				Darg		5 %
asn^{4},gln^{5}-dDAVP	-	-	-	mpa			asn	gln			Darg		<0.2 %
val^{4}-dDAVP	-	-	-	mpa			val				Darg		0.1 %
gly^{5}-dDAVP	-	-	-	mpa				gly			Darg		<0.2 %
arg^{8}-vasotocin	-	-	-			ile					arg		5 %
oxytocin	-	-	-			ile					leu		0.3 %

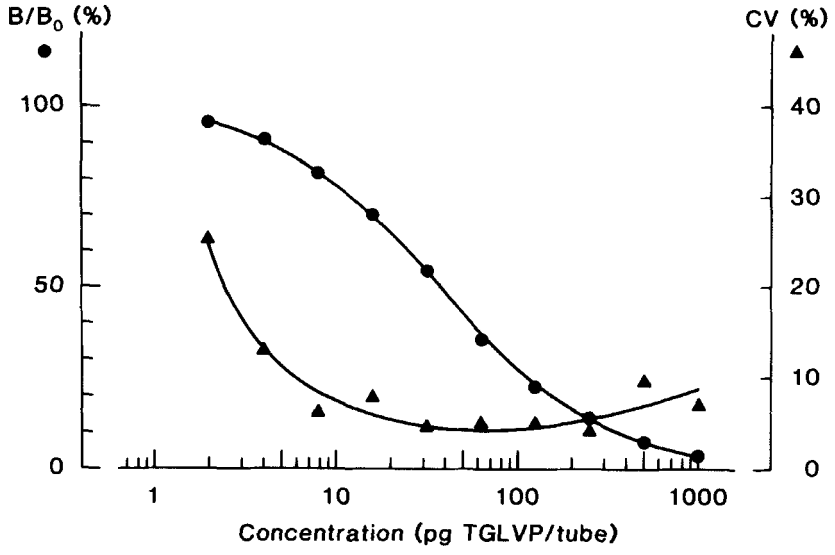

FIGURE 3. Standard curve (•) of TGLVP using antiserum K8509-3 and (^{125}I-Tyr)^{2}-TGLVP. The precision profile (▲) indicates the CV% of each standard point as determined from triplicates in 7 assays.

reactivity of antiserum K8509-3 for a number of peptides were similar using either (^{125}I-Tyr)^{2}-TGLVP (Table I) or ^{125}I-(Lys-PHPP)^{8}-TGLVP peak B.

Using antibody K8509-3 at a final antiserum dilution of 1/160000, Bo/T was 45.7 ± 1.4% using (^{125}I-Tyr)^{2}-TGLVP. 50% inhibition of binding was obtained at 36.3 ± 1.1 pg TGLVP/tube and the useful working range was 5-500 pg TGLVP/tube (Figure 3). Intra- and interassay coefficient of variation was 6.0% and 7.5% respectively at 17 pg TGLVP/tube and 4.7% and 8.2% at 70 pg TGLVP/tube (n=12). Extraction recovery of 4 ng TGLVP/ml added to normal human male plasma by Sep-Pak C18 extraction was estimated to be 86.8 ± 3.7%.

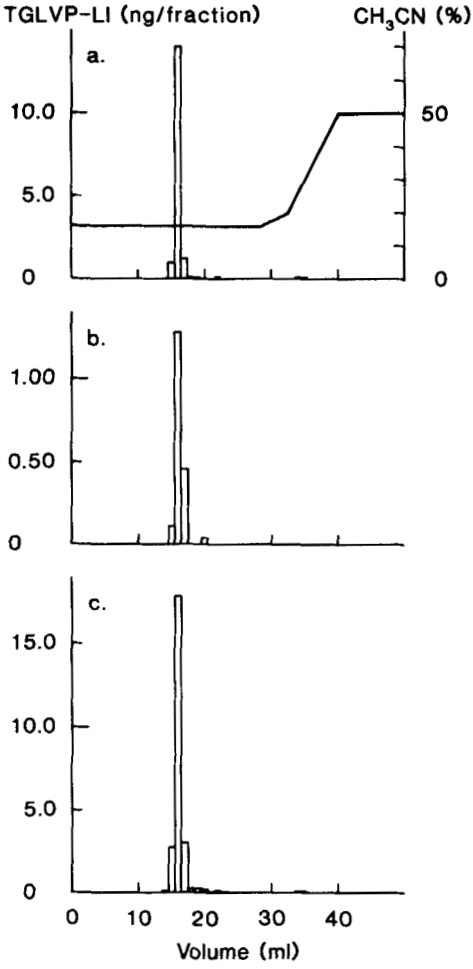

FIGURE 4. Reversed phase chromatography of TGLVP-LI (determined with K8509-3) in 270 µl extracted plasma from a healthy volunteer who had received a single intravenous injection of 20 µg TGLVP/kg b.w. 5(a) and 40(b) minutes after administration. The elution of standard TGLVP is shown in c. Recovery of TGLVP-LI was 72±3%. A plasma sample obtained prior to administration of the peptide showed no detectable TGLVP-LI (detection limit 40 pg TGLVP-LI/fraction).

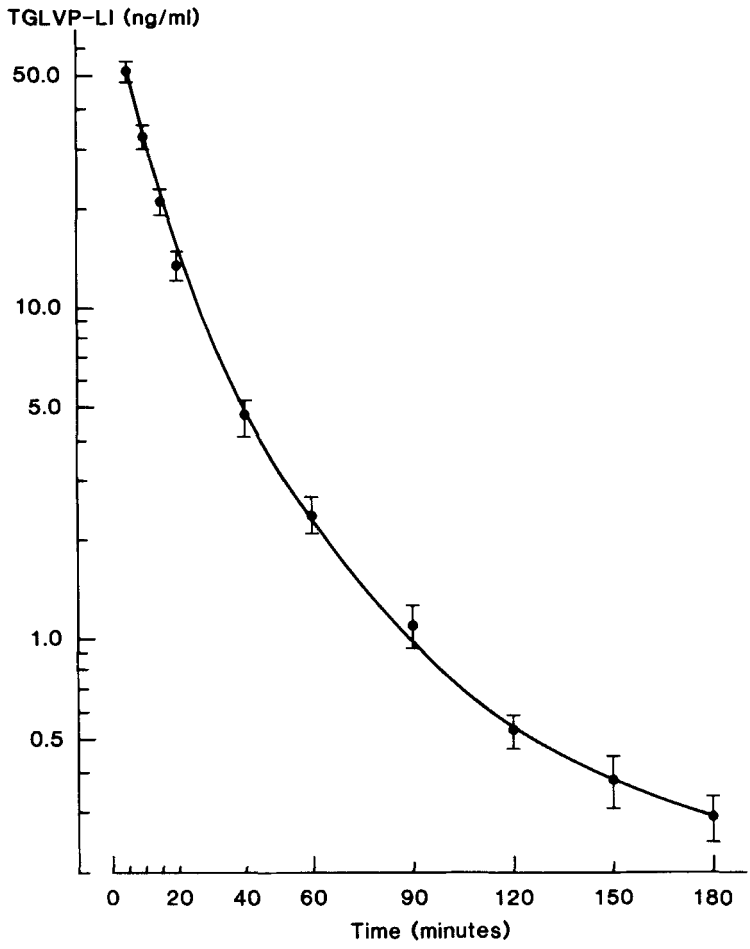

FIGURE 5. Elimination of TGLVP-LI (determined with K8509-3) from plasma after a single intravenous injection of 10 µg TGLVP/kg b.w. as determined by K8509-3 (mean ±SEM, n=6).

Extractable TGLVP-LI in plasma obtained from a healthy volunteer who had received a single intravenous injection of TGLVP diluted in parallel to standard TGLVP but not to standard LVP or standard AVP in the assay and eluted as standard TGLVP using

reversed phase chromatography (Figure 4). After 3 hours less than 1% of TGLVP-LI remained in plasma (Figure 5).

DISCUSSION

Antibodies produced against vasopressin and related peptides are often specific for the C-terminal part of the molecule due to the conjugation of the hapten to the carrier through the α-amino group. In order to obtain an antibody reacting mainly with the N-terminal ring structure a novel approach to the coupling of the hapten to a carrier had to be taken. The use of partially protected haptens for coupling to carrier proteins has previously been described (11). The coupling of a hapten to succinylated BSA could be performed directly with equimolar amounts of carbodiimide as shown by the incorporation of Arg^8-vasopressin using an initial molar ratio of AVP: carbodiimide: sBSA 25:25:1. The use of excess carbodiimide may modify the Tyr residue that possibly will be a part of the antigenic epitope (12). More than 80% of this hapten was coupled to the carrier as determined by the incorporation of trace amounts of iodinated AVP (data not shown). However, this does not mean that haptens having only ε-amino groups available for coupling would be as effectively coupled as AVP. Also the presence of unprotected carboxyl groups will most likely affect the coupling efficiency using certain haptens. Succinylated BSA was also suitable as carrier protein because, contrary to keyhole limpet hemocyanin (11) and thyroglobulin, it tolerated the reagents used to remove the N-terminal protecting group. This hapten-carrier conjugate proved useful for immunization. Other similar conjugates

have also been used successfully in our laboratory to elicit a hapten specific response in rabbits which further indicate the usefulness of this coupling procedure.

Any antibody which is specific for the ring of the vasopressin molecule may show a reduced affinity for an iodinated radioactive tracer due to the modification of the tyrosine residue. A (Lys-PHPP)^{8}-TGLVP conjugate was thus synthesized. This compound was homogenous as determined by reversed phase chromatography but when iodinated it separated into two components. Our data suggested that the first eluting radioactive peptide was identical to (Lys-PHPP-^{125}I)^{8}-TGLVP and that the second compound was monoiodinated at the Tyr^{2}-residue as judged by TLC-data of pronase digested tracer as well as by their antibody reactivity compared to (^{125}I-Tyr)^{2}-TGLVP and by the fact that both compounds appeared to be monoiodinated when comparing the amount of radioactivity being present per absorbance unit. The fact that the radioactivity of pronase treated ^{125}I-(Lys-PHPP)^{8}-TGLVP peak A did not migrate using TLC may be due to incomplete cleavage of this part of the molecule due to the modification of the lysine residue. The differences in reactivity of antiserum K8509-3 with these tracers seemed to be generally more related to the affinity constant than to the concentration of reactive antibodies as determined by Scatchard plot analysis (Figure 2). In the case of K8509-3 these data indicated that the antibody tolerated the iodination of Tyr^{2} as well as a PHPP-moiety at Lys^{8} but not an iodinated PHPP-moiety at Lys^{8} while antiserum K8512-3 showed a reduced affinity for a peptide iodinated at Tyr^{2}.

Antiserum K8509-3 was chosen for further use in RIA due to its superior sensitivity as well as a slightly improved specificity for TGLVP as related to LVP as compared to K8512-3. The preferable use of (^{125}I-Tyr)^{2}-TGLVP which is easier to prepare than (Lys-PHPP-^{125}I)^{8}-TGLVP also makes K8509-3 a suitable choise.

The behaviour of antiserum K8509-3 is somewhat pH sensitive in that the selectivity of the various tracers is reduced at pH 5.7 and the crossreactivity of LVP but not of desamino^{1}-LVP is reduced relative to the assay performed at pH 7.6 (data not shown). This may indicate that the presence of a positive charge in this part of the antigen is not well accommodated within the antigen binding site. The specificity of antiserum K8509-3 was thus related to the N-terminal triglycyl extension as well as to amino acid residues in the ring structure especially gln^{4} and asn^{5}. The crossreactivity of oxytocin and AVP was negligible at physiological concentrations. The reduced crossreactivity of oxytocin relative to arginine-vasotocin may be due to conformational changes induced in the molecule by the hydrophobic leucine residue rather than to a direct involvement of this amino acid in the antigenic epitope. When compared in analysis for TGLVP-LI in extracts of plasma these anti-TGLVP antisera gave similar results as did the C-terminal specific antiserum A:8 (data not shown), indicating that this assay measured the intact molecule in plasma after intravenous administration of the peptide.

Extraction of vasopressin from plasma has been performed by a variety of methods (8,13-16). The use of octadecasinyl minicolumns

generally gave consistent and high recovery of standard TGLVP and no (<5 pg/200 µl extracted plasma) non-specific background immunoreactivity was present in these extracts using K8509-3. Extracted TGLVP-LI after intravenous injection also showed identity with standard TGLVP as determined by parallel dilution and reversed phase chromatography. The elution profile of TGLVP-LI appeared similar when using the C-terminal specific antiserum A:8 (data not shown) except that this antibody detected a somewhat larger amount of hydrophobic immunoreactive components (<10% of total TGLVP-LI) eluting with the gradient.

In cases where an antibody more specific for the N-terminal extension of a vasopressin analog is necessary, a different approach has to be taken. This is further supported by results obtained by similar antibodies specific for N^{α}-diglycyl-lysine^{8}-vasopressin (data not shown). It is possible that the use of another carrier may affect the specificity of hapten antibodies even though no thorough investigation has been made. Another possibility would be to use glu^{4} or asp^{5} amino protected peptide analogues to synthesize the hapten-protein conjugate, in which the N-terminal amino acid may be optimally exposed. For our purposes this antibody is an important complement to the usual C-terminal specific vasopressin antibodies especially when used in metabolic studies. The antiserum K8509-3 may be used for the analysis of GVP without significant interference of endogenous AVP at physiological concentrations or LVP, which is a proposed metabolite of GVP being present at low concentration (2).

ACKNOWLEDGEMENTS

We wish to thank Dr. Gunnar Nilsson, Dept. of Anaesthesiology, Malmö General Hospital, Malmö, Sweden for providing human plasma samples obtained after i.v. administration of TGLVP and Ann-Christin Flyving for secretarial assistance.

REFERENCES

1. Pliška, V., Chard, T., Rudinger, J. and Forsling, M.L. In vivo activation of synthetic hormonogens of lysine vasopressin: N^{α}-glycyl-glycyl-glycyl-[8-lysine]vasopressin in the cat. Acta Endocrinol. 1976;81:474-81.

2. Forsling, M.L., Aziz, L.A., Miller, M., Davies, R. and Donovan, B. Conversion of triglycyl vasopressin to lysine-vasopressin in man. J. Endocr. 1980;85:237-44.

3. Pliška, V. Synthetic hormonogens of neurohypophyseal hormones: Relationships between structure and pharmacokinetic parameters. In: Dearden, J.C., ed. Quantitative Approaches to Drug Design. Amsterdam: Elsevier Science Publishers B.V., 1983:193-201.

4. Vosmik, J., Jedlička, K., Mulder, J.L. and Cort, J.H. Action of the triglycyl hormonogen of vasopressin (glypressin in patients with liver cirrhosis and bleeding esophageal varices. Gastroenterology 1977;72:605-9.

5. Samokhin, G.P. and Filimonov, I.N. Coupling of peptides to protein carriers by mixed anhydride procedure. Anal. Biochem. 1985;145:311-14.

6. Bolton, A.E. and Hunter, W.M. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem. J. 1973;133:529-39.

7. Greenwood, F.C., Hunter, W.M. and Glover, J.S. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. J. 1963;89:114-23.

8. LaRochelle, F.T., Jr., North, W.G. and Stern, P. A new extraction of arginine vasopressin from blood: The use of octadecasilyl-silica. Pflügers Arch. 1980;387:79-81.

9. Stoneham, M.D., Everitt, B.J., Hansen, S., Lightman, S.L. and Todd, K. Oxytocin and sexual behaviour in the male rat and rabbit. J. Endocr. 1985;107:97-106.

10. Scatchard, G. The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 1949;51:660-72.

11. Morley, J.S., Capper, S.J. and Miles, J. New methodology in the raising of antibodies to small peptides leading to a highly specific radioimmunoassay for enkephalins. Neuropeptides 1984;4:477-82.

12. Carraway, K.L. and Koshland, E.D. Reaction of tyrosine residues in proteins with carbodiimide reagents. Biochim. Biophys. Acta 1968;160:272-4.

13. Beardwell, C.G., Geelen, G., Parmer, H.M., Roberts, D. and Salomonson, L. Radioimmunoassay of plasma vasopressin in physiological and pathological states in man. J. Endocr. 1975;67:189-202.

14. Skowsky, W.R., Rosenbloom, A.A. and Fisher, B.A. Radioimmunoassay measurement of arginine-vasopressin in serum: development and application. J. Clin. Endocrinol. Metab. 1974;38:278-86.

15. Sequeira, R.P., Raghavan, K.S. and Chaudhury, R.R. Development and validation of radioimmunoassay of antidiuretic hormone in plasma. Indian J. Med. Res. 1980;72:365-73.

16. Dogterom, J., van Wimersma Greidanus, T.J.B. and de Wied, D. Vasopressin in cerebrospinal fluid and plasma of man, dog and rat. Am. J. Physiol. 1978;234:E463-67.