Journal of Chromatography, 312 (1984) 285–292
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,044

RECOVERY OF STANDARD ^{125}I-PEPTIDES AT THE PICOGRAM AND FEMTOGRAM LEVEL BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RODICA L. EMANUEL
Department of Medicine and Endocrine Hypertension Unit, Harvard Medical School and Brigham and Women's Hospital, Boston, MA 02115 (U.S.A.) and Department of Medicinal Chemistry in the College of Pharmacy and Allied Health Professions, and Barnett Institute of Chemical Analysis and Materials Science, Northeastern University, 360 Huntington Avenue, Boston, MA 02115 (U.S.A.)
GORDON H. WILLIAMS
Department of Medicine and Endocrine Hypertension Unit, Harvard Medical School and Brigham and Women's Hospital, Boston, MA 02115 (U.S.A.)
and
ROGER W. GIESE*
Department of Medicinal Chemistry in the College of Pharmacy and Allied Health Professions, and Barnett Institute of Chemical Analysis and Materials Science, Northeastern University, 360 Huntington Avenue, Boston, MA 02115 (U.S.A.)
(Received July 9th, 1984)

SUMMARY

Average recoveries of standard [^{125}I]angiotensin II are 92–103% when amounts of this peptide ranging from 10 pg to 50 fg are analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) employing a C_8-bonded silica packing, whether an "old" (one year of prior use) or previously unused column is employed. In one case this recovery is 68% on a column giving a ten-fold higher retention of this peptide with the usual mobile phase of acetonitrile–aqueous 0.01 *M* sodium perchlorate (40:60); the same mobile phase except 0.1 *M* sodium perchlorate shortens the retention ten-fold on this column and gives an average recovery of 103%. Similar recoveries are obtained for [^{125}I]bradykinin (105%) and [^{125}I]luteinizing hormone releasing hormone (107%), tested at the 1-pg level. Although two commercial preparations of [^{125}I]vasoactive intestinal peptide are recovered only to the extent of 75 and 85%, the HPLC chromatograms also display several peaks. Aside from the potential for complications in the analysis of real samples, this work establishes that RP-HPLC is a viable technique for sample clean-up/characterization in the ultratrace analysis of at least some peptides.

INTRODUCTION

Reversed-phase high performance liquid chromatography (RP-HPLC) is a useful technique for the analysis of peptides, as has been reviewed[1,2]. In some cases,

0021-9673/84/$03.00

such as the analysis of peptide hormones in physiological samples, ultratrace amounts (below the nanogram level) of peptides can be encountered. Such analyses are expected to be more difficult in several respects, one of which is peptide recovery, the subject of this paper.

It is clear from the literature that peptides can be lost on reversed-phase columns, whether high performance or classical in nature, even when the amounts analyzed are above the nanogram level. For example, Wehr *et al.*[3] reported losses of the 11 residue peptide, ranatensin, on a C_{18} HPLC column, at least when amounts less than 0.5 μg were injected. Desiderio *et al.*[4] reported losses in the range of 20–40% of neuropeptides derived from biological samples in clean-up steps involving the use of polystyrene or C_{18} bonded silica adsorbents employed as classical mini-columns.

Good recoveries (or apparently so) of trace peptides also have been reported by RP-HPLC. For example, working at the microgram level of peptide, Bennett *et al.* reported close to 100% recovery of adrenocorticotropic hormone immunoreactivity from rat anterior pituitaries throughout a clean-up sequence that included a C_{18} HPLC column. Böhlen *et al.*[6] reported a high recovery of several peptides separated by extraction from salts on a small C_{18} silica column. At a lower level, Rivier[7] observed a complete recovery of 1 ng of luteinizing hormone releasing factor on a cyanopropyl bonded silica HPLC column.

No doubt a number of considerations influence peptide recovery in reversed-phase liquid chromatography (RP-LC). For example, when Desiderio *et al.*[4] (cited above) analyzed a pure standard of one of their peptides instead of a biological extract, a quantitative recovery (amount unspecified) was obtained. Peptide size is another aspect relevant to recovery; it is well-known that larger peptides, *e.g.* greater than 20 to 30 residues (including small proteins) tend to undergo losses on RP-LC even when microgram or greater amounts are injected[1].

This paper addresses the recovery of some standard peptides analyzed at picogram and femtogram levels with RP-HPLC silica-based columns. Although we only investigate four peptides ranging in size from 8 to 28 residues using a single type of mobile phase, it is encouraging that we observe high or apparently complete recoveries in all cases, including the use of both old (prior use) and new HPLC columns.

EXPERIMENTAL

Materials and instrumentation

Iodinated peptides (2200 Ci/mmol) were obtained from New England Nuclear. HPLC water and acetonitrile were from J. T. Baker. Water was filtered through a HA 0.45-μm Millipore membrane and acetonitrile through a Rainin Ultipor-NR 0.45-μm membrane. Both solvents were degassed under reduced pressure before use. Sodium perchlorate, HPLC-grade, was purchased from Fisher.

The HPLC system consisted of two Altex Model 110 Solvent Metering Pumps, a LDC-Model 1601 Gradient Master, and a LKB-Model 2112 Redirac Fraction Collector. Separations were carried out on either Supelcosil C_8 (5 μm, 15 cm × 4.6 mm I.D., Supelco) or Microsorb C_8 (5 μm, 15 cm × 4.6 mm I.D., Rainin) RP columns. Injections were made with a 100 μl Hamilton 1710 syringe, equipped with a PTFE plunger and a 1 in. RN-80428 22 SGA needle. The syringe was cleaned as

necessary by filling and emptying seven times each with water, acetonitrile and mobile phase. The isocratic mobile phase composition is given in Tables I and II.

The injector was a manual Valco assembly model CV-6-UHPa-N60, with a 15-μl loop. The injector loop was washed before each experiment with 3 ml of mobile phase, the last 15 μl of which were injected onto the column as a blank injection. Fractions (0.5 ml) were collected for both blank and sample injections and counted on a Packard 5250 Autogamma Scintillation Spectrometer (50% counting efficiency).

Procedure

The lyophylized, iodinated peptide was dissolved in 0.2 ml of water. A volume of 5 μl of the resulting solution were measured with an Eppendorf pipette with a polypropylene tip and diluted to 2 ml with mobile phase in a 20-ml glass scintillation vial (made by Fisher). The amount of peptide injected onto the HPLC was adjusted by further dilution with mobile phase to the desired count.

Volumes of 6 μl aliquots were injected with the 100-μl Hamilton syringe into the HPLC sampling loop throughout all the experiments. The delivery precision of the Hamilton syringe was measured as follows: a solution of [^{125}I]angiotensin II was prepared as described. The syringe was first equilibrated with the solution by filling and dispensing to waste five times, followed by pumping in the solution ten more times. The syringe was filled and 6-μl aliquots were delivered manually to ten 75 × 12 mm glass tubes containing 0.5 ml of mobile phase by touching the droplet at the needle tip against the dry glass wall above the mobile phase. The tubes were vortexed for 2–3 sec, capped and counted. After subtraction of background counts, the delivery precision (mean ± standard deviation; expressed as percent) was found to be 2.4 ± 0.2% for 10,000 cpm, 4.5 ± 0.1% for 1000 cpm and 6.5 ± 0.1% for 100 cpm. This was acceptable for our purposes.

Controls for total counts from the syringe (ten injections of 6 μl each into 0.5 ml of mobile phase, followed by counting) and background counts (ten times 0.5-ml fractions of collected column eluent from a blank sample injection) were made prior to the sequence of repeated analyses for each peptide. The average total count from the syringe per injection, and the average background count from the blank injection per 0.5-ml fraction, were calculated. The latter background count was never significantly different from the normal background count for the scintillation spectrometer. For each peptide, six to ten consecutive injections were made, and 0.5-ml fractions were collected and counted until background counts were obtained (see the chromatograms in Figs. 1 and 2). The average background count was subtracted from the count for each 0.5-ml fraction. The sum of these corrected counts divided by the average total count from the syringe gave the recovery of the "peptide".

RESULTS AND DISCUSSION

Potentially RP-LC on bonded silica columns is a useful technique for sample clean-up/characterization of ultratrace, structurally-complex substances such as peptides. Both the convenience and separation characteristics of this method are attractive for this purpose. However, the recovery of such analytes has been little investigated. Towards this goal, we chose to investigate the recovery of some ^{125}I-labelled peptides at sub-nanogram levels by this technique.

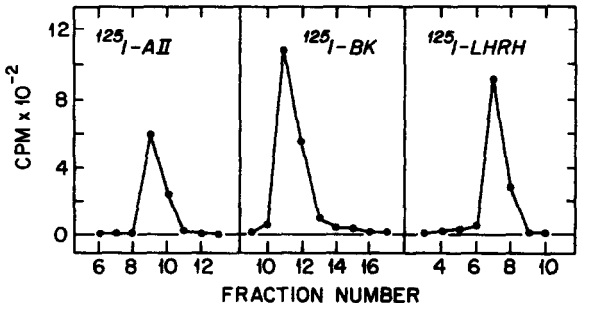

Fig. 1. Representative chromatograms of radiolabeled peptides: [^{125}I]AII (angiotensin II), [^{125}I]BK (bradykinin) and [^{125}I]LHRH (luteinizing hormone releasing hormone). The "old" C_8 Supelcosil column was employed, and 0.5-ml fractions were collected for counting. The mobile phase conditions were those cited in Tables I and II for the "old" C_8 Supelcosil column.

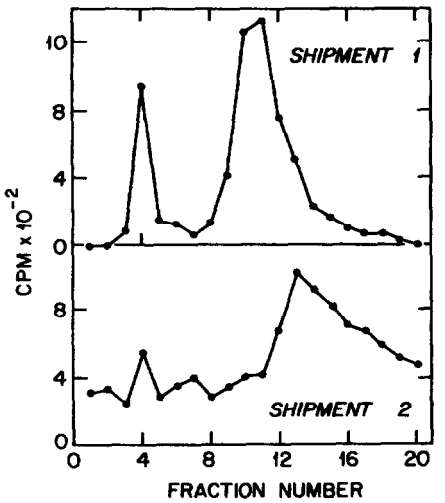

Fig. 2. Representative chromatograms for two shipments of [^{125}I]vasoactive intestinal peptide, with the column and conditions the same as in Fig. 1.

[^{125}I]Angiotensin II

We began with [^{125}I]angiotensin II, anticipating that recovery would be a problem for this substance below the nanogram level because this peptide is reported to readily undergo adsorption losses[8]. A low pH perchlorate–acetonitrile (60:40) mobile phase was selected given the results and characteristics of this solvent for RP-LC reported[9,10]. This choice was nevertheless somewhat arbitrary since several mobile phases give high-performance separations of small peptides on reversed-phase silica HPLC columns[1].

Recovery from the injector

It was first necessary to determine the amount of sample delivered to the LC column by the injection valve. According to the manufacturer, the injector retains 1.12 μl of the sample in its port passage upon injection. We determined this volume

for our valve by repeatedly (eight times) injecting 6 μl of a solution of [^{125}I]angiotensin II (10,000 cpm) into the injection loop (variable-volume type) that had previously been flushed clean (based on blank injections) with mobile phase prior to each injection. This was followed by activation of the valve to inject the sample directly into a counting vial (0.5-ml collected volume). The recovery of [^{125}I]angiotensin II counts was 81.9 ± 2.7% (mean ± standard deviation; expressed as percent), corresponding to a retained volume in the valve of 1.08 μl. Thus, in our subsequent LC analyses, we corrected the counts obtained from the corresponding initial injection by multiplying by 1.22 (100/81.9).

Replenishing the retained volume of 1.08 μl with fresh sample immediately prior to injection of a given 6-μl volume gave an effective recovery of the latter volume of 99.5 ± 4.5%, because the 1.08-μl retained volume was compensated for by the 1.09 μl added in from the previous injection. However, in the course of our experiments, 10–12 min elapsed between injections, allowing some diffusion loss of sample from the 1.08-μl retained volume. We determined, based on nine injections directly into collection vials under these conditions, that 93.3 ± 2.6% of such samples were delivered to the column. Thus, we corrected the counts obtained from the second *etc.* injections by multiplying by 1.08 (100/93.3).

Recovery from the column

Our results on the recovery of [^{125}I]angiotensin II at the picogram and femtogram level by HPLC are shown in Table I. As defined in the Experimental section, these recovery values reflect all of the radioactivity eluting from the column, but appropriately corrected with an average blank, for each injection. As will be shown later, essentially single peaks were obtained in all cases except for [^{125}I] vasoactive intestinal peptide.

We began using an "old" C_8-silica column that had been in use for one year prior to this work, including the analysis of ng and μg amounts of angiotensin II.

TABLE I

REVERSED PHASE HPLC OF [^{125}I]ANGIOTENSIN II (MW 1170) AS A FUNCTION OF THE COLUMN, MOBILE PHASE, AND QUANTITY OF THE PEPTIDE INJECTED

C_8 Column		*Aq. sodium perchlorate (M)**	*Amount of [^{125}I]-angiotensin II injected (pg)*	*Number of injections*	*Retention time (min)*	*Recovery (%)*	
Type	*Age*					*Average*	*Range*
Supelcosil	"Old"**	0.01	5 0	9	4.5	96	91–101
		0.01	0.5	8	4.5	96	90–100
		0.01	0.05	8	4.5	92	68–105
	New***	0.01	0.5	5	43.0	68	62–79
		0.1	0.5	5	4.5	103	98–111
Microsorb	New***	0.01	0.5	5	23.0	101	97–107
		0.1	0.5	7	5.0	103	98–111

* Isocratic acetonitrile–aq. sodium perchlorate (40:60) at the concentration cited adjusted (the aqueous part) to pH 2.1 with phosphoric acid

** The column was previously used for one year mainly for the analysis of angiotensin II and related samples, involving mostly the mobile phase reported here.

*** The column was washed with the mobile phase for 2 h before this analysis.

Starting with an injection of 5.0 pg of [^{125}I]angiotensin II, we were somewhat surprised, given the reported adsorptive properties of this peptide[8], that its recovery at this level was 96%. This encouraged us to investigate lower levels. As seen, a high recovery (92%) is maintained even when 50 fg of this peptide is analyzed on this column.

Potentially the prior use of this column for angiotensin II analyses at nanogram and microgram levels had saturated loss-sites on the column. Thus we proceeded to test the recovery of [^{125}I]angiotensin II on a new column of the same type. When the analysis was repeated at the 0.5-pg level on a new column from the same manufacturer that had only been washed for a few hours with mobile phase prior to the injections of [^{125}I]angiotensin II, the recovery of this analyte dropped to 68% and the retention time increased ten-fold to 43 min. Variations in retention, especially for more "complex" solutes analyzed by RP-LC, are not uncommon when the column is changed[11]. Increasing the concentration of the sodium perchlorate from 0.01 to 0.1 *M* gave the "old column" retention time of 4.5 min, and then a high recovery (103%) once again was obtained. We did not investigate the mechanism behind the lowered retention with this higher sodium perchlorate concentration, *e.g.* whether it is related to the chaotropic or ion-pairing properties of this reagent, or some other aspects.

The lower recovery in 0.01 *M* sodium perchlorate was shown to not arise merely from the longer retention of the peptide on the column. Stopping the flow for 44 min after the peptide was midway in the column (2.5 min after injection), followed by resumption of the flow, gave recoveries of 97 and 95% on two occasions when [^{125}I]angiotensin II was analyzed on this column in 0.1 *M* sodium perchlorate mobile phase (data not shown). This suggests that the recovery of this peptide can be dependent on the sodium perchlorate concentration in this column.

A similar type of column, also new, from another manufacturer, was investigated in the same way. As shown in Table I, we also see a longer retention time for [^{125}I]angiotensin II on this column in a 0.01 *M* sodium perchlorate mobile phase that is similarly overcome at a higher concentration of this salt. In this case, however,

TABLE II

REVERSED-PHASE HPLC OF ^{125}I-PEPTIDES

LHRH = Luteinizing hormone releasing hormone, and VIP = vasoactive intestinal peptide.

^{125}I-peptide			*Aq. sodium perchlorate (M)*	*Recovery (%)**	
Type	*Amount injected (picograms)*	*Molecular weight (including ^{125}I)*		*Average*	*Range*
Bradykinin	1.0	1184	0.01	105	98–108
LHRH	0.7	1305	0.01	107	95–114
VIP	1.4	3450	0.10	75**	68–83
	1.4	3450	0.10	88**	83–93

* Each recovery represents the average of six analyses on the "old" C_8 Supelcosil column with the mobile phase that was previously used as described in Table I.

** Two different shipments were determined.

the recovery is high (101 and 103%) irrespective of the sodium perchlorate concentration.

Other peptides and chromatograms

Encouraged by these high recoveries of ultratrace [^{125}I]angiotensin II, we proceeded to analyze the ^{125}I-derivatives of three other peptides: bradykinin, luteinizing hormone releasing hormone (LHRH) and vasoactive intestinal peptide (VIP). The amounts injected (*ca.* 1 pg of each), molecular weights and recoveries are shown in Table II. Although good recoveries are seen for both [^{125}I]bradykinin and [^{125}I]LHRH (105 and 107%, respectively), both shipments of [^{125}I]VIP from the manufacturer gave an incomplete recovery (75 and 88%).

Representative chromatograms from these and the prior analyses are shown in Figs. 1 and 2. Single, relatively sharp peaks (taking the method of detection into account) are seen for [^{125}I]angiotensin II, -bradykinin and -LHRH in Fig. 1. For [^{125}I]VIP, complex chromatograms were obtained for both shipments, as seen in Fig. 2. This complexity, the nature of which is not defined here, may be related to the lower recovery obtained for this latter peptide. Perhaps some of this complexity is due to partial oxidation of the methionine to methionine sulfoxide, or placement of the iodine atom on either tyrosine 10 or 22, in [^{125}I]VIP[12].

In these recovery studies, the potential role of the Trasylol, glycine and albumin carriers in the iodinated peptides, as supplied by the manufacturer, was not investigated.

CONCLUSIONS

Our data establish that RP-HPLC has attained a high-performance level in the ultratrace recovery of [^{125}I]angiotensin II, [^{125}I]bradykinin, and [^{125}I]luteinizing hormone releasing hormone, all having molecular weights in the range of 1000, when analyzed as standards under the isocratic conditions employed here. The overall average recovery is 100%, and the corresponding range is 92–107%, for these three peptides under the best conditions in Tables I and II. Although the corresponding recovery for ultratrace [^{125}I]VIP, having a molecular weight of 3450, is complicated by uncertainty about sample integrity, the average yields of 75 and 88% of the injected counts in two experiments with different samples of this peptide are also high.

At least in the case of [^{125}I]angiotensin II, we also have data indicating that its good recovery persists in the analysis of a physiological extract containing angiotension II at the 50–100-pg level[13]. Thus, RP-HPLC, even with simple mobile phase conditions, is a high recovery technique potentially useful for sample clean-up/characterization in the ultratrace analysis of at least some peptides.

ACKNOWLEDGEMENT

This work was supported by DARPA Contract N00019-82-K-0811 administered by the Office of Naval Research, and NIH Grants HL16821, HL29950, and RR00888. We are grateful to Russel Garlick for helpful discussions. Contribution number 212 from the Barnett Institute of Chemical Analysis.

REFERENCES

1 M. T. W. Hearn, in Cs. Horváth (Editor), *High Performance Liquid Chromatography*, Vol. 3, Academic Press, New York, 1983, p. 87.
2 F. E. Regnier, *Anal. Chem.*, 55 (1983) 1298A.
3 C. T. Wehr, L. Correia and S. R. Abbott, *J. Chromatogr. Sci.*, 20 (1982) 114.
4 D. M. Desiderio, S. Yamada, F. S. Tanzer, J. Horton and J. Trimble, *J. Chromatogr.*, 217 (1981) 437.
5 H. P. J. Bennett, C. A. Browne and S. Solomon, *Biochemistry*, 20 (1981) 4530.
6 P. Böhlen, F. Castillo and R. Guillemin, *Int. J. Pept. Protein Res.*, 16 (1980) 306.
7 J. E. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343.
8 T. L. Goodfriend and D. L. Ball, in G. E. Abraham (Editor), *Handbook of Radioimmunoassay*, Marcel Dekker, New York, 1977, Ch. 16, p. 511.
9 J. L. Meek and Z. L. Rossetti, *J. Chromatogr.*, 211 (1981) 15.
10 J. L. Meek, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1632.
11 R. W. Giese, *Clin. Chem.*, 29 (1983) 1331.
12 J. C. Marie, C. Boissard and G. Rosselin, *Peptides*, 5 (1984) 179.
13 R. L. Emanuel, R. Joppich-Kuhn, G. H. Williams and R. W. Giese, in preparation.