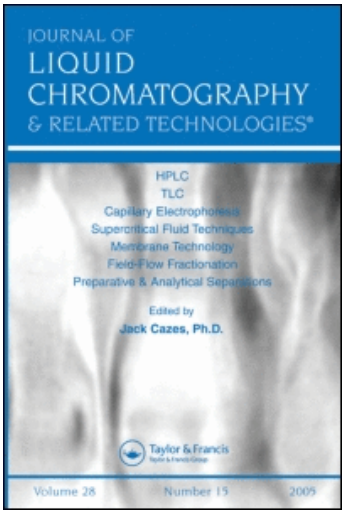

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Sertl, D. C. , Johnson, R. N. and Kho, B. T.(1981) 'An Accurate, Specific HPLC Method for the Analysis of a Decapeptide in a Lactose Matrix', Journal of Liquid Chromatography & Related Technologies, 4: 7, 1135 — 1156
To link to this Article: DOI: 10.1080/01483918108068802
URL: http://dx.doi.org/10.1080/01483918108068802

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(7), 1135-1156 (1981)

AN ACCURATE, SPECIFIC HPLC METHOD FOR THE ANALYSIS OF A DECAPEPTIDE IN A LACTOSE MATRIX

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method is described for the determination of an analog of the hormone LH-RH in lyophilized vials at the low parts-per-million level. The peptide (Schally analog) is quantitatively recovered from the glass lyophilization vials after reconstitution with mobile phase. The peptide solution is eluted on a reversed-phase, C_{18} column and monitored with ultraviolet (UV) detection at 220 nm. The chromatography resolves Schally analog from a number of synthetic impurities and decomposition products.

INTRODUCTION

The decapeptide LH-RH (Luteinizing Hormone-Releasing Hormone) is an important hormone in the human body. Excreted by the hypothalamus, LH-RH activates the release of pituitary hormones which control reproductive development. Its structure and function were determined by Schally and co-workers (1,2) in 1971.

[D-Trp^6]-LH-RH (Figure 1) is an analog of LH-RH where the 6th amino acid (glycine) is replaced with D-Trptophan. This analog was first

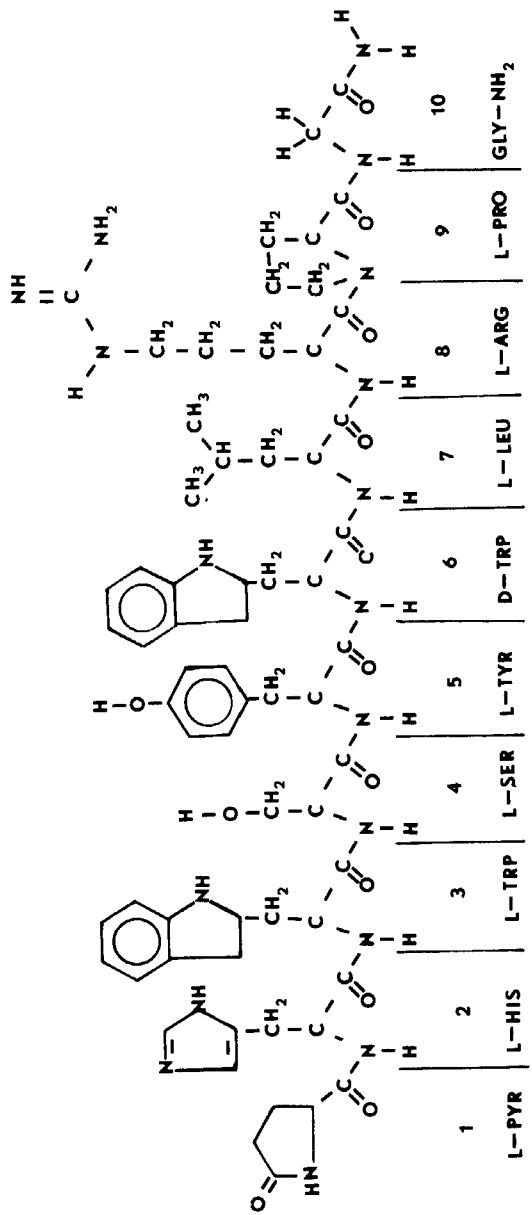

Figure 1

synthesized by Schally et al. and has been shown to be more potent and longer-acting than LH-RH. This would offer the advantages of lower dosage size and frequency.

Until recently, the analysis of small polypeptides (up to 20 amino acids) relied on such techniques as radioimmunoassay, ion exchange and gel chromatography and electrophoresis. The development of high performance liquid chromatography (HPLC) has provided a more specific, rapid and sensitive technique for analysis.

Reversed-phase HPLC, in particular, offers the advantages of being able to work at low detection wavelengths (210-220 nm) and directly inject aqueous samples (simplifying sample preparation). Many of the early reversed-phase HPLC methods for small polypeptides employed the use of solvent gradients to gain the necessary selectivity (3,4,5). Improvements in the columns packings over the years have made gradients unnecessary. Isocratic mobile phases of acetonitrile or methanol and an aqueous buffer (phosphate or acetate) are common in the literature (6,7,8,9,10).

This paper describes an HPLC method for the assay of Schally analog (D-Trp^6 -LH-RH) in lyophilized vials at the low parts-per-million level (0-50 μg/vial).

MATERIALS AND METHODS

The isocratic liquid chromatograph consisted of a Model 6000 A Solvent Delivery System, a Model 710 A WISP® autoinjector, a Model 730 Data Module (Waters Associates, Milford, MA) and a Model SF-770 Spectroflow Monitor (Schoeffel Instruments, Westwood, NJ).

The column was slurry-packed in-house using ODS Hypersil® (Shandon Southern, Sewickley, PA) and had the dimensions 15 cm x 4.1 mm. The mobile phase was 30 percent acetonitrile and 70 percent phosphate buffer (0.05M KH_2PO_4 at pH = 6.5). The columns packed in our laboratory routinely yielded baseline resolution of the four impurity peaks in the Schally analog reference standard (Figure 2).

Reagents

All reagents were of analytical grade. Acetonitrile (HPLC grade) was purchased from Burdick and Jackson Laboratories (Muskegon, MI). Potassium Dihydrogen Phosphate (KH_2PO_4) and Lactose powder (USP) were

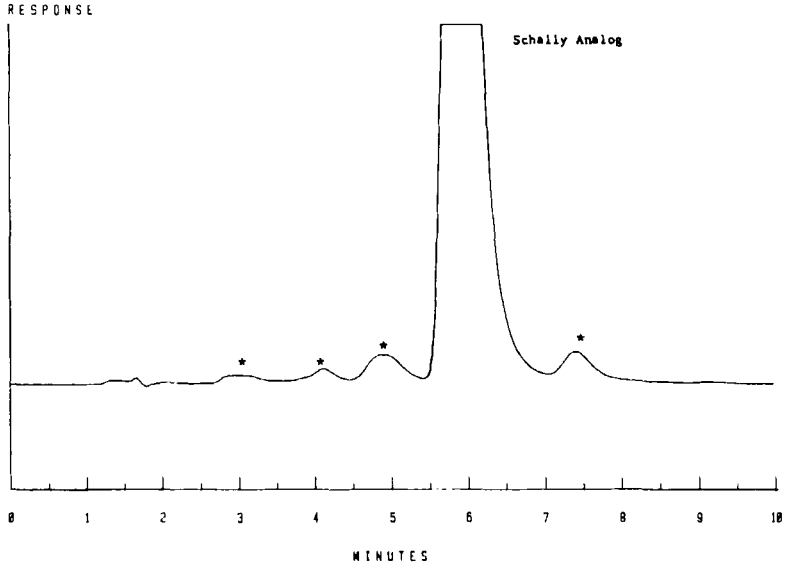

Figure 2: HPLC Chromatogram of Schally Analog Reference Standard

* - Impurity

Chromatographic Conditions

Column:	ODS Hypersil®, 15 cm x 4.1 mm
Mobile Phase:	30% acetonitrile
	70% 0.05M KH_2PO_4 (pH = 6.5)
Detection:	Ultraviolet at 220 nm

reagent grade (J. T. Baker Chemical Co., Phillipsburg, NJ). Distilled water was used to make all solutions. The phosphate buffer and the acetonitrile were filtered using membrane filters MF Millipore® (0.45 μm) and Fluoropore (0.50 μm) respectively (Millipore® Corporation, Bedford, MA). Mobile phase containing 5% lactose was prepared by first dissolving the lactose in the buffer followed by addition of the acetonitrile. Schally analog reference standard and lyophilized vials were obtained from Ayerst Laboratories (Montreal, Canada).

Methods

A flow rate of 1 ml/minute was used in eluting the peptide. All equipment was at room temperature. Injections of 10 to 100 microliters were made (depending on the peptide concentration) and the eluate monitored with UV detection at 220 nm. The main peptide peak elutes in 4-6 minutes.

To minimize adsorptive loss of the Schally analog on the vial surface, the lyophilized vials were reconstituted with 1 ml of mobile phase. Ten vials per sample were reconstituted and two 5-vial pools formed. Since each lyophilized vial contained 50 mg of lactose, the sample solutions contained 50 mg/ml of lactose. Therefore, the standard solutions were prepared in mobile phase containing 5% lactose.

The column was equilibrated with mobile phase at a flow of 1 ml/minute. Three multilevel standards were injected throughout their respective sample injections. The sample concentrations were calculated from the sample areas and the linear regression of the standard concentrations and areas (11). A correction of 0.03 ml is included for the increase in volume due to the 50 mg of lactose dissolving in 1.0 ml of mobile phase.

RESULTS

The inherent linearity of the HPLC response of Schally analog in the 5-75 μg/ml range was measured by injecting serial dilutions of a solution of Schally analog in 5% lactose/water. The HPLC response is (Figure 3) very linear. In an actual assay, three, separately-weighed standards (at 80, 100 and 120% of input claim) are used to form a calibration line. The correlation coefficient for these lines range from .999 (at 50 μg/ml) to 0.995 (at 5 μg/ml).

The use of mobile phase to reconstitute the vials provides for essentially complete recovery of the peptide (see Figure 4 and the DISCUSSION section). Instrument settings used in the assay allow for detection of Schally analog down to approximately 5% of the expected concentration range.

Each assay result is an average of the results from two 5-vial pools. The assay precision ranges from about 2% for 50 μg/vial to 5% for 5 μg/vial samples. Since the precision of replicate injection areas is 0.5 to 2%, much of the assay imprecision is due to vial-to-vial non-uniformity in peptide content.

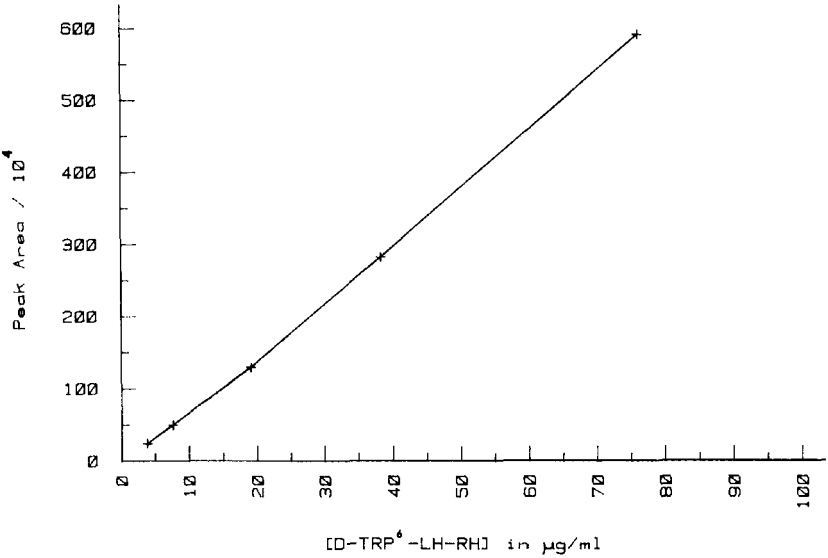

Figure 3: Linearity of HPLC Response (UV - 220 nm)
Schally Analog in 5% lactose/water

Correlation Coefficient = .9996

DISCUSSION

Development of Chromatography

Three 5-6 µm, reversed-phase packings (Zorbax ODS, Spherisorb ODS and Hypersil ODS) were evaluated as chromatographic supports for Schally analog. Hypersil ODS gave the best selectivity for the impurity peaks in the Schally raw material. The ODS Hypersil columns were packed in-house and gave very reproducible chromatograms from column to column. A particular column was judged suitable for the assay if the impurity peak eluting just before the main peak was resolved to at least one half of its peak height.

The effect of buffer pH on the chromatography of Schally analog (AY-25,650) was investigated and the results are shown in Table I. Lowering the buffer pH decreases the relative retention time of the main peptide but the resolution of the impurity peaks is compromised as a result. It appears that a pH of 6.5 is best, affording baseline resolution of Schally analog and its four impurities in less than 10

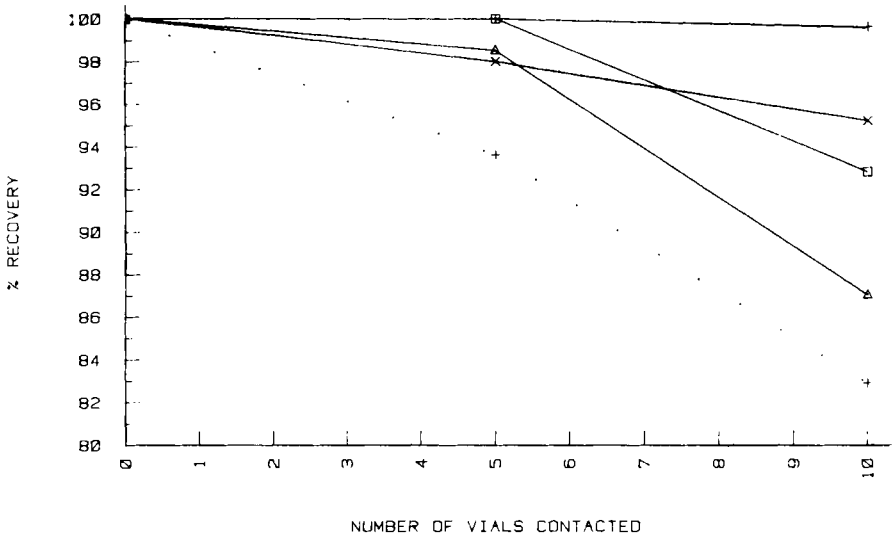

Figure 4: Adsorption of Schally Analog on Glass Vials
Dependence on Reconstitution Solvent
Schally = 5.4 μg/ml in 5% Lactose

Line	Symbol	Solvent
———	+	Mobile Phase
....	+	Distilled Water
———	X	pH = 2.5
———	□	pH = 4.5 } 0.05M KH_2PO_4
———	Δ	pH = 6.5

TABLE I

OPTIMIZATION OF BUFFER pH IN MOBILE PHASE

Buffer (0.05M KH_2PO_4) pH	Retention Time (minute)	Resolution of Impurities
2.5	4.0	partial
4.5	4.4	partial
6.5	5.1	baseline
6.5 (0.005M Bu_4N^+)	2.4	partial

minutes. The presence of tetrabutylammonium ion in the mobile phase greatly decreased the retention of the peptides. This effect was probably due to the tetrabutylammonium ions blocking hydrophobic binding sites on the reversed-phase packing.

Specificity of Chromatography

Method specificity deals with the ability of the chromatography (column and mobile phase) and/or the detection system to distinguish the compound of interest from any impurities, excipients or decomposition products that might interfere with the assay. The method must be specific if it is to produce accurate and precise results.

In the case of Schally Analog, the chromatography provides the needed specificity. Evidence for the chromatographic specificity falls into five categories:

1. Synthetic Impurities

   As shown in Figure 2, the chromatography separates four synthetic impurities from the main peptide peak in the raw material. Only the main peptide peak is quantitated in actual assays, however. The baseline resolution of the impurities allows for very accurate quantitation of the main peak.

2. Purity of the Main Chromatographic Peak: Spectral Discrimination

   A new technique called Spectral Discrimination was recently introduced (12) to aid the chromatographer in characterizing species eluting from the column. In this technique detector absorbance values at nine different wavelengths are measured on a stopped-flow portion of the column eluate. The absorbance values are corrected at each wavelength for absorbance due to the chromatographic solvent itself. We thus have a corrected series of absorbance values characteristic of the solute in that particular stopped-flow portion of the chromatogram. If a similar series of absorbance measurements is collected for a different portion of the same or other chromatographic peak, we can compare the two portions spectrally. If the ratio (Q) of absorbance values (at each wavelength) between two portions is relatively constant ($D = Q_{max}/Q_{min} <$ approximately 1.5), we have strong evidence that the two portions contain the same molecular species.

If different (beginning, middle, end) portions of a chromatographic peak are thus indicated to contain the same molecular species, we have strong evidence that the chromatographic peak is homogeneous and contains a single molecular species.

If peaks in different (e.g. sample, standard) chromatograms are compared and shown to contain the same sample molecular species, we have added evidence (along with retention time) for the identity of the sample peak.

The Spectral Discrimination technique was used to test the purity and homogeneity of the main peaks in the chromatograms of the reference standard (Figure 2) and a decomposed sample (Figure 5). The data and results are shown in Tables II and III. Both peaks were evidenced to be homogeneous and contain a single molecular sample type.

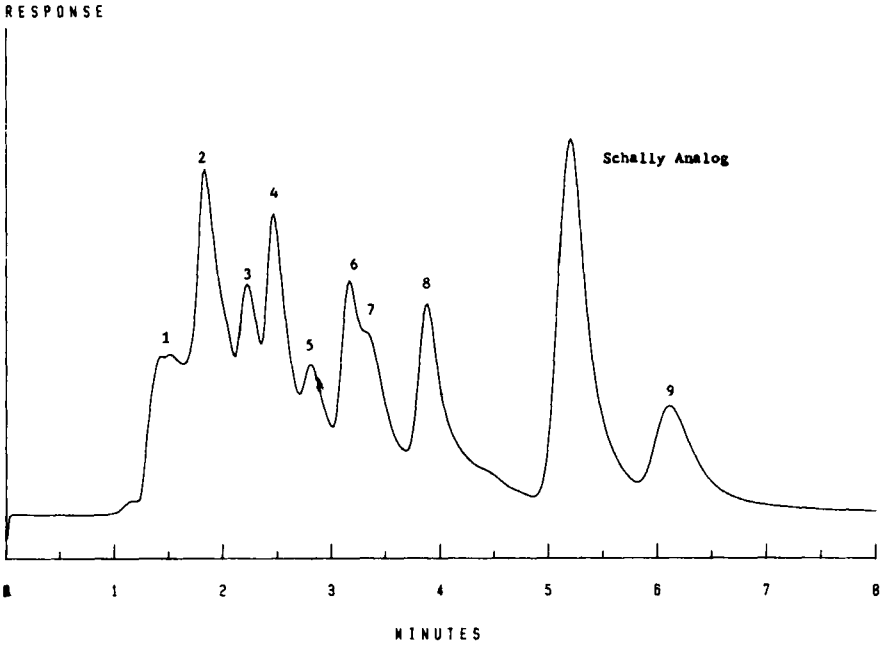

Figure 5: Decomposition of Schally Analog in Water (1 mg/ml)
(17 months at room temperature, dark)
Peaks #1-9 are aqueous decomposition products

TABLE II.

SPECTRAL DISCRIMINATION DATA

EXPERIMENT 1.

Purity of Main Peak of Reference Standard Chromatogram.

EXPERIMENT 2.

Comparison of Reference Standard and Dosage Form Peaks.

Wavelength (nm)	ABSORBANCE VALUES			ABSORBANCE VALUES	
	Beginning	Middle	End	Ref. Standard	Dosage Form
220	.336	1.141	.520	.122	.128
230	.148	.499	.232	.055	.055
240	.025	.085	.040	.010	.011
250	.019	.065	.029	.009	.008
260	.032	.107	.048	.012	.013
270	.047	.158	.074	.019	.020
280	.050	.176	.080	.019	.019
290	.035	.121	.057	.014	.015
300	.006	.020	.009	Too small to use.	-

	Q (Beg./Mid.)	Q (Beg./End)	Q (Mid./End)	Q
220	.294	.646	2.19	.953
230	.297	.638	2.15	1.000
240	.294	.625	2.13	.909
250	.292	.655	2.24	1.13
260	.299	.667	2.23	.923
270	.297	.635	2.14	.950
280	.284	.625	2.20	1.000
290	.289	.614	2.12	.933
300	.300	.667	2.22	-
$D = \frac{Q\ Max.}{Q\ Min.}$	1.06	1.09	1.06	1.24
CONCLUSION:	The main peak in the reference standard chromatogram appears to contain a single, intact molecule.			The main peaks of the standard and sample chromatograms appear to contain the same molecule.

TABLE III.

SPECTRAL DISCRIMINATION DATA

Wavelength (nm)	EXPERIMENT 1. Purity of main peak in decomposed Schally figure. ABSORBANCES			EXPERIMENT 2. Comparison of decomposed Schally and Reference Standard. ABSORBANCES	
	Beginning	Middle	End	Ref. Standard	17-Month
220	.333	.874	.364	.874	2.513
230	.112	.290	.124	.290	0.821
240	.024	.060	.026	.060	0.171
250	.021	.052	.022	.052	0.147
260	.033	.086	.036	.086	0.250
270	.048	.126	.054	.126	0.368
280	.051	.134	.057	.134	0.391
290	.031	.083	.035	.083	0.248
300	.005	.011	.004	.011	0.033

	Q (Beg./Mid.)	Q (Beg./End)	Q (Mid./End)	Q
220	.381	.915	2.40	.348
230	.386	.903	2.34	.353
240	.400	.923	2.31	.351
250	.404	.955	2.36	.354
260	.384	.917	2.39	.344
270	.381	.889	2.33	.342
280	.381	.895	2.35	.343
290	.373	.886	2.37	.335
300	-	-	-	.333
$D = \frac{Q\ Max.}{Q\ Min.}$	1.08	1.08	1.04	1.06

CONCLUSION: The Schally peak in the decomposed sample appears to contain a single, intact molecule.

The "Schally" peaks in the reference standard and decomposed samples appear to contain the same molecule.

This technique was also used to identify the main peaks in the chromatograms of a dosage form and the decomposed Schally solution. As shown in Table I and II, comparison with the main peak of the reference standard chromatogram indicates that the main peaks in both sample chromatograms contain the same single, sample molecular species found in the reference standard peak.

3. Purity of the Main Chromatographic Peak: Increased Column Length
As an additional test of the chromatographic selectivity, the sample in Figure 2 is eluted on two columns in series (in effect, doubling the column length). The goal is to see if any hidden, co-eluting peaks can be "pulled out" of the main peak.

As shown in Figure 6, no additional peaks or shoulders from the main peak are seen using the two columns. Given that the detection

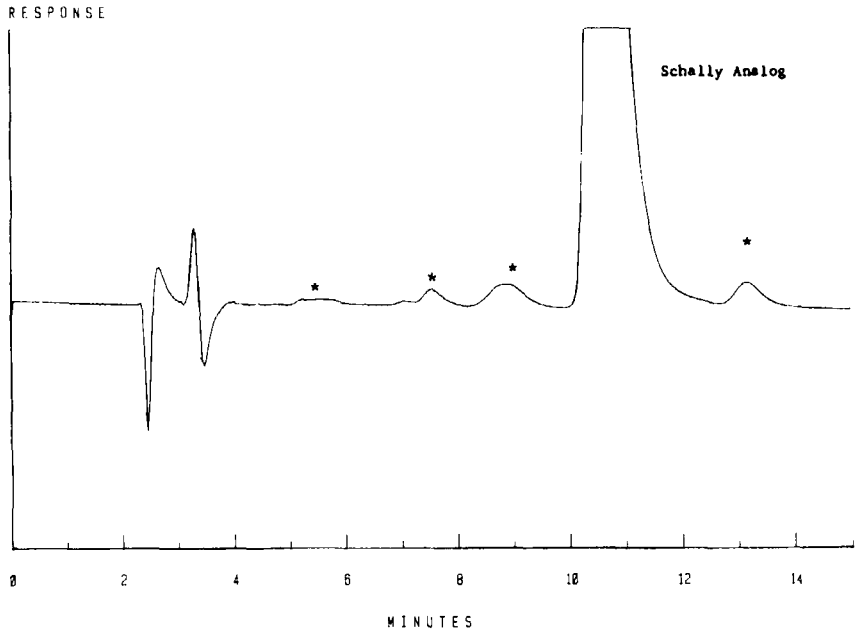

Figure 6: Chromatographic Specificity: Increased Column Length
Schally Analog Reference Standard eluted on two columns connected in series

* - Impurity

is UV at 220 nm (quite non-selective), this is additional evidence of the specificity of the chromatography.

4. Decomposition Products

Schally Analog (200 μg/ml in 5% lactose/water) in solution was decomposed under various conditions and eluted using the chromatography described in the assay methods. The intent was to see if the decomposition products were resolved from the main Schally peak. The results are shown in Table IV and Figures 5, 7-11.

The chromatogram of a stability sample of a 50 μg/vial lyophilized vial is shown in Figure 12. The resolution of a decomposition product from the main Schally peak indicates that the methods are stability-indicating.

Also, a solution of Schally Analog in water and 1 mg/ml was stored 17 months at room temperature in the dark. The chromatogram of the resulting solution (Figure 5) shows nine decomposition products resolved from the main peak. In Table III we see the Spectral Discrimination data supports the purity of the main peak.

TABLE IV

DECOMPOSITION OF SCHALLY ANALOG SOLUTION

(SCHALLY ANALOG AT 200 μg/ml IN 5% LACTOSE/WATER)

SOLUTION STORAGE CONDITION (7 DAYS)	% SCHALLY REMAINING (VS. INPUT)	# RESOLVED, DECOMPOSITION PRODUCTS	CHROMATOGRAM (FIGURE #)
4°C (Control)	96%	0	7
Light Cabinet*	84%	2	8
62°C	87%	0	9
pH = 2*	93%	1	10
pH = 12*	10%	7	11

*Room Temperature

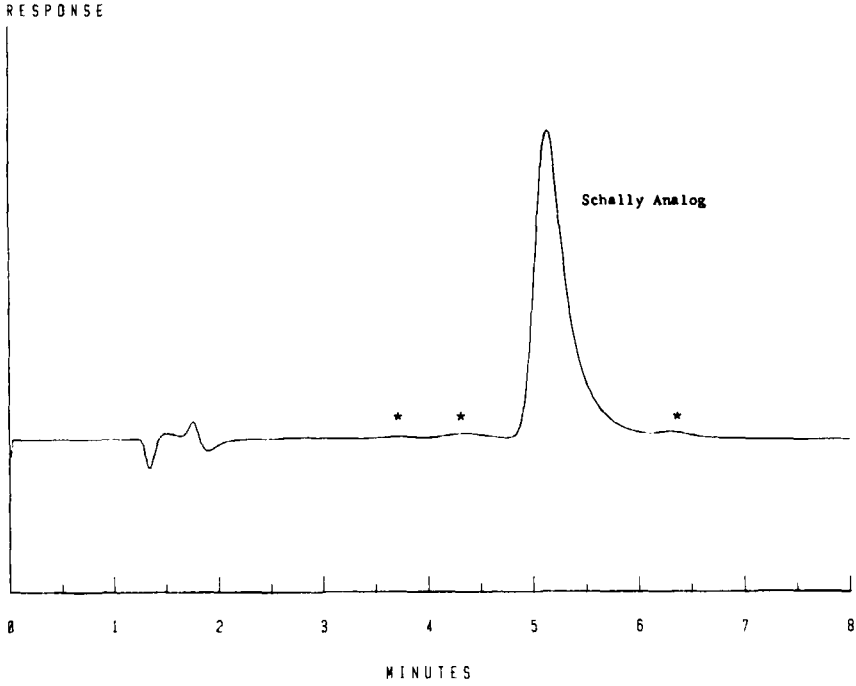

Figure 7: Solution Stability: 7 Days at 4°C
Schally Analog - 200 μg/ml in 5% lactose/water, dark

* - Impurity in Raw Material

5. Similar Decapeptide

LH-RH is a decapeptide which is identical to Schally Analog except for one amino acid (the 6th amino acid tryptophan in Schally Analog is replaced with glycine). However, as seen in Figure 13, LH-RH is well resolved from Schally Analog using the chromatography of the assay methods. O'Hare and Nice (13) have suggested that the main mechanism governing the reversed-phase chromatography of polypeptides is hydrophobic interaction

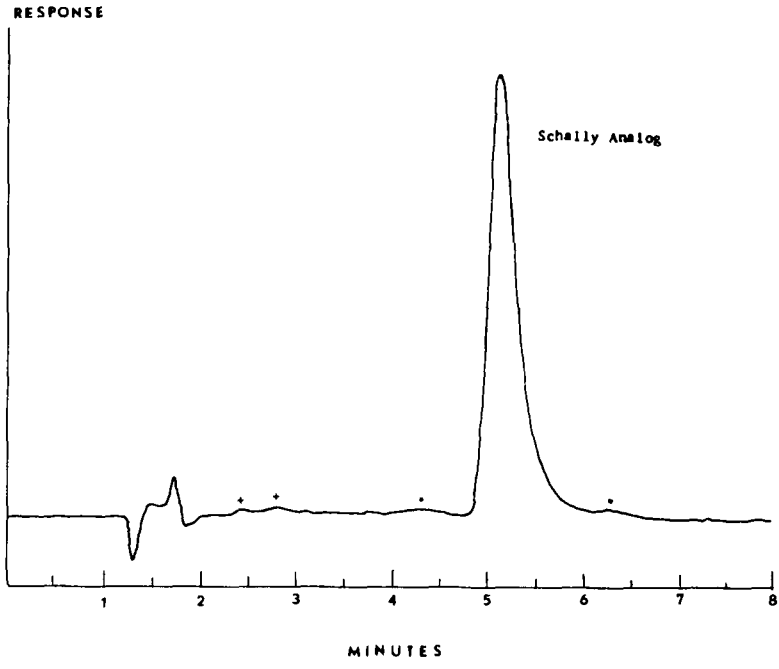

Figure 8: Solution Stability: 7 Days in Light Cabinet
Schally Analog - 200 μg/ml in 5% lactose/water, room temperature

* - Impurity in Raw Material
\+ - Solution Decomposition Product

with the stationary phase. The observed elution order of LH-RH and Schally Analog supports this proposal since the Schally Analog, containing a much more hydrophobic amino acid in the 6th position, elutes much later than does the LH-RH.

Effect of Lactose on the HPLC Response (UV at 220 nm) of Schally Analog

Since the reconstituted dosage form contains 5% lactose by weight, the question was raised as to whether the standard solutions should

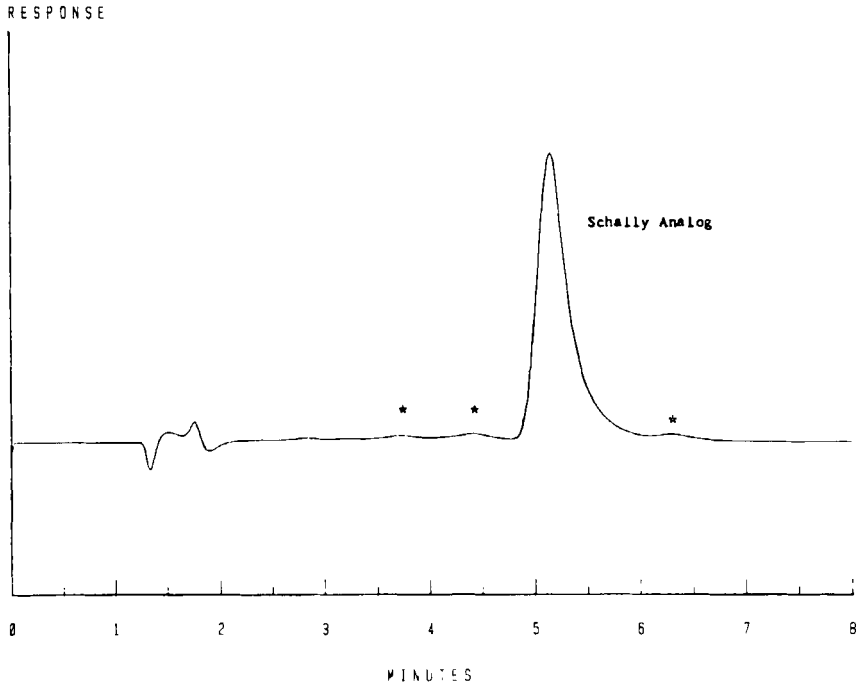

Figure 9: Solution Stability: 7 Days at 62°C
Schally Analog - 200 μg/ml in 5% lactose/water, dark

* - Impurity in Raw Material

contain 5% lactose as well. So, the effect of 5% lactose on the HPLC response (area/concentration) of Schally Analog was investigated. When dissolved in water, it was found that the presence of 5% lactose caused up to an 8% increase in the response. In mobile phase, the presence of 5% lactose increases the response of Schally Analog by only 1-2%. Thus, in the actual assay, the standard solutions are made 5% in lactose to avoid any bias in the results.

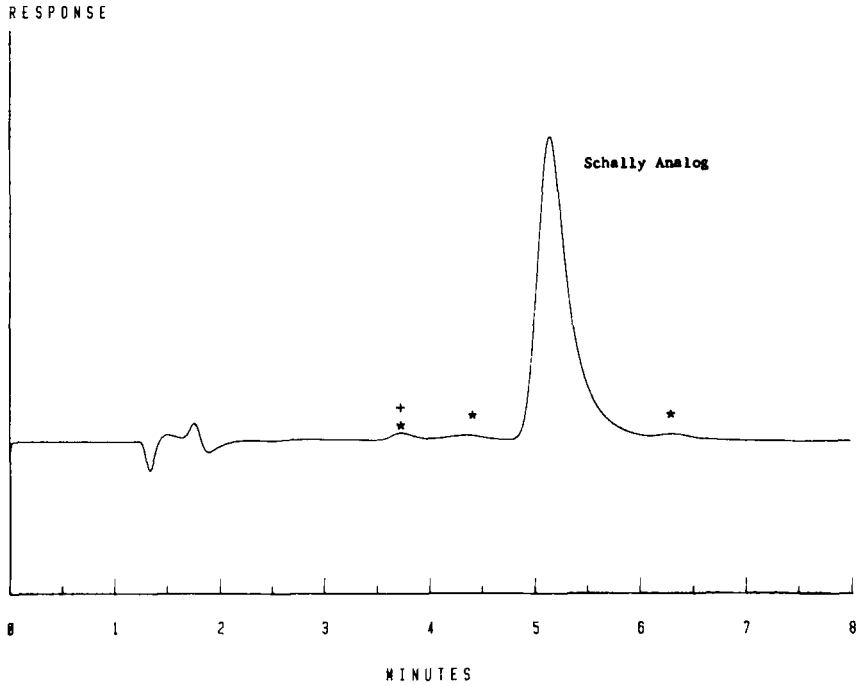

Figure 10: Solution Stability: 7 Days at pH = 2
Schally Analog - 200 μg/ml in 5% lactose/water
- dark, room temperature

* - Impurity in Raw Material
\+ - Solution Decomposition Product

Adsorption of Schally Analog on Glass

When distilled water was used to reconstitute the lyophilized vials, assays of those vials in the 5-25 μg/vial range gave low results based on claimed input. An apparent loss of 10-20% was seen.

An experiment was performed to see if there was adsorptive loss of Schally Analog on glass surfaces. At a peptide concentration of 5.4 μg/ml

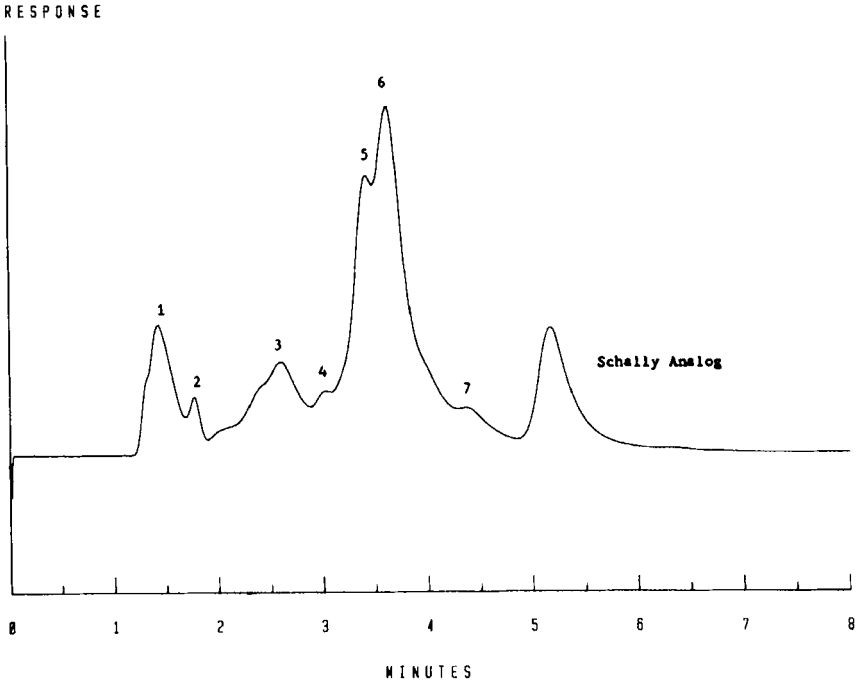

Figure 11: Solution Stability: 7 Days at pH = 12
Schally Analog - 200 μg/ml in 5% lactose/water
- room temperature, dark

Peaks #1-7 are Solution Decomposition Products

and a lactose content of 5%, portions of Schally Analog solutions with various solvents were contacted consecutively with a number of clean, dry lyophilization vials. Each solution was sampled for assay before contact with the vials and after 5 and 10 contacts with vials. The results are depicted in Figure 4. Three observations can be made: 1) pure water gives more adsorptive loss than 0.05M phosphate buffer; 2) adsorptive loss decreases as the pH of the buffer becomes more acidic and 3) use of mobile phase as the solvent virtually eliminates adsorptive loss of Schally Analog. As a result, the assay method uses mobile phase as the reconstitution solvent for the lyophilized vials to minimize adsorptive loss of the peptide.

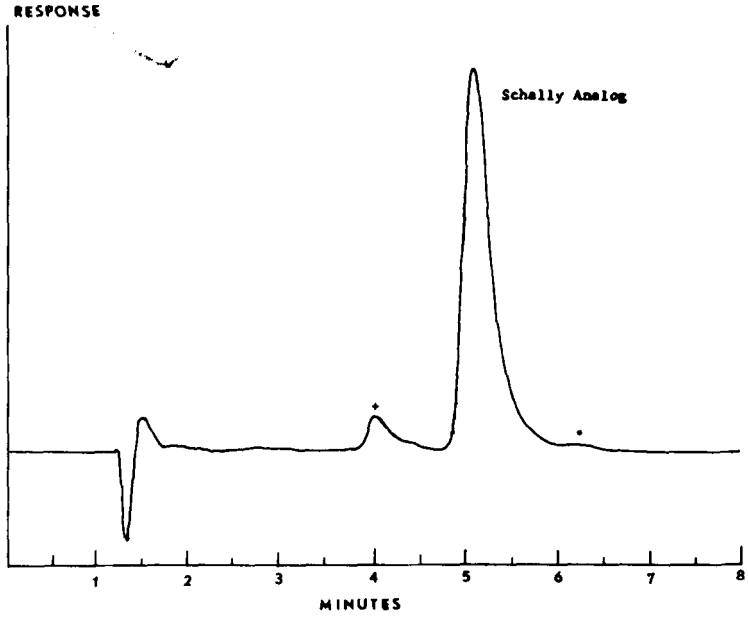

Figure 12: Lyophilized Vial Stability Sample

Schally Analog - 50 μg/vial * - Impurity in Raw Material

Lactose - 50 mg/vial + - Lyophilized Decomposition Product

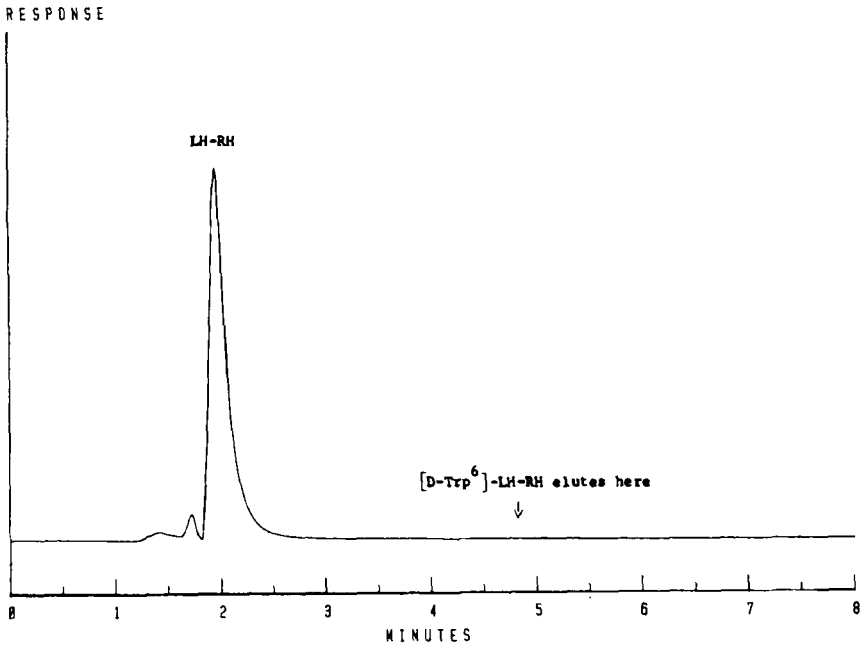

Figure 13: Column Specificity: LH-RH vs. [D-Trp^6]-LH-RH
Chromatogram of LH-RH using the chromatography for Schally Analog (Figure 1)

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Godwin Fong for his review and helpful discussion of the manuscript and Ayerst Laboratories (Montreal) for providing samples of Schally Analog reference standard and lyophilized vials.

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