Report

High-Performance Liquid Chromatographic Assay for Thyrotropin Releasing Hormone and Benzyl Alcohol in Injectable Formulation

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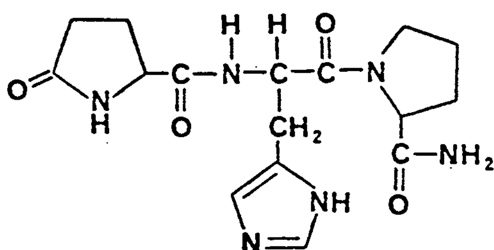
A stability-indicating, high-performance liquid chromatographic method is presented for the determination of thyrotropin releasing hormone (TRH) and benzyl alcohol in a multidose injectable formulation. Separation is achieved on a microparticulate octadecylsilica column, with pH 2.2 ion-pair reagent (octanesulfonic acid, sodium salt) solution/methanol/acetonitrile/triethylamine (92:4:4:0.01), at a flow rate of 1.5 ml/min and a detection wavelength of 215 nm. *p*-Aminobenzoic acid is used as an internal standard. The relative standard deviations of the method are ±0.6% for TRH and ±0.7% for benzyl alcohol. Recoveries from standard additions to placebos ranged from 99.0 to 101.3 and 99.7 to 101.8% for TRH and benzyl alcohol, respectively.

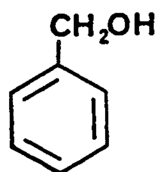
KEY WORDS: thyrotropin releasing hormone (TRH); benzyl alcohol; high-performance liquid chromatographic stability-indicating assay; multidose injection.

INTRODUCTION

Thyrotropin releasing hormone (TRH) (Scheme I) is currently used as an adjunctive agent in the diagnostic assessment of thyroid function. It is also being studied clinically for palliative use in the treatment of amyotrophic lateral sclerosis, commonly known as Lou Gehrig's disease. TRH is formulated at a concentration of 200 mg/ml with 0.9% (w/v) benzyl alcohol (Scheme II) in aqueous solution for injection for the latter use.



Scheme I. L-Pyroglutamyl-L-histidyl-L-prolinamide (TRH, protirelin).



Scheme II. Benzyl alcohol.

In recent years methods to assay TRH in biological fluids have been described (1–5). Among the assays are those based on radioimmunoassay (RIA) and high-performance liquid chromatography (HPLC) in conjunction with RIA and UV detection. Reversed-phase HPLC has been used to determine benzyl alcohol in combination with hydroxyzine (6), hydrocortisone (7), and glutaric acid and phenylephrine (8).

There are, as yet, no published methods for the determination of TRH in pharmaceutical preparations. A simple, precise, and specific assay method for TRH in a 0.9% benzyl alcohol matrix was needed for quality control purposes. For use in accelerated stability studies, potential degradation products as well as racemization products must be separated from the intact drug. A reversed-phase ion-pair HPLC method with UV detection was developed for the determination of TRH and benzyl alcohol in injectable formulation. The HPLC method is so designed that both TRH and benzyl alcohol can be analyzed concomitantly in a single chromatographic run, with only minor modification in the sample preparation. The method has been shown to be stability indicating for both TRH and benzyl alcohol.

EXPERIMENTAL

Instrumentation. The chromatographic system was equipped with a Waters Associates (Waters Associates, Milford, Mass.) Model 6000A pump and Model 710B WISP autoinjector and a Kratos (Kratos Analytical Instruments, Ramsey, N.J.) Spectroflow 757 variable wavelength detector. The separation was performed on a 15-cm × 4.6-mm-i.d. column containing microparticulate bonded (5-μm) octadecylsilane material (Nucleosil C_{18}, 5 μm, Alltech Asso-

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ciates Inc., Deerfield, Ill.). The chromatographic peaks were electronically integrated and recorded (SP-4100, Spectra-Physics, Santa Clara, Calif.).

Mobile Phase. A solution of 920 ml of 0.05% (w/v) octanesulfonic acid, sodium salt (Regis Chemical Co., Morton Grove, Ill.) (pH adjusted to 2.2 with 3 *M* phosphoric acid) was mixed with 40 ml of methanol (Analyzed Reagent, Baker, Phillipsburg, N.J.), 40 ml of acetonitrile (Analyzed Reagent, Baker, Phillipsburg, N.J.), and 0.1 ml of triethylamine (Aldrich Chemical Co., Milwaukee, Wis.). The solution was filtered, degassed under vacuum, and used as the mobile phase.

Internal Standard Solution. A solution of *p*-aminobenzoic acid (Analyzed Reagent, Baker, Phillipsburg, N.J.) (1 mg/ml) in water was used as the internal standard for TRH. A solution of *p*-aminobenzoic acid (0.06 mg/ml) (15 ml of 1 mg/ml solution above diluted to 250 ml with water) was used as the internal standard for benzyl alcohol.

Standard Preparation. A working standard solution of TRH was prepared by mixing 20 ml of a stock solution of TRH (Reference Standard, Abbott Laboratories, North Chicago, Ill.) (1 mg/ml in water) with 10 ml of the internal standard solution and diluting to 200 ml with water.

A working standard solution of benzyl alcohol was prepared by mixing 5 ml of a stock solution of benzyl alcohol (Reference Standard, Abbott Laboratories, North Chicago, Ill.) (0.36 mg/ml in water) with 15 ml of the internal standard solution and diluting to 50 ml with water.

Stressed TRH Solution. A stressed TRH solution was prepared by heating 5 ml of a TRH standard solution (0.1 mg/ml in water) with 75 μl of 1 *N* sodium hydroxide in an oven at 100°C for 1 hr, followed by cooling to room temperature and neutralizing with 40 μl of 1 *M* phosphoric acid (the isomerization products produced under the alkali-stress conditions are LDL-TRH and DLL-TRH).

System Suitability Check Solution. A system suitability check solution was prepared by mixing the stressed TRH solution above with 1 ml of the benzyl alcohol/internal standard solution mixture (2.0 ml of 0.36 mg/ml benzyl alcohol stock solution above mixed with 10.0 ml of 1 mg/ml internal standard solution and diluted to 100 ml with water).

Sample Preparation. A sample stock solution for TRH and benzyl alcohol was prepared by diluting a 4-ml aliquot of sample to 200 ml with water.

A working sample solution for TRH was prepared by mixing 5 ml of the stock solution with 10 ml of the internal standard solution and diluting to 200 ml with water.

A working sample solution for benzyl alcohol was prepared by mixing 10 ml of the stock solution with 15 ml of the internal standard solution and diluting to 50 ml with water.

Chromatographic Conditions. The conditions used were as follows: flow rate, 1.5 ml/min; detector, 215 nm; 0.2 AUFS; injection volume, 10 μl; and temperature, ambient.

System Suitability Test. The HPLC system suitability was established by injecting the system suitability check solution into the HPLC with the chart speed set at 1 cm/min (the elution order is the internal standard followed by benzyl alcohol, LDL-TRH, DLL-TRH, and TRH) and determining the resolution factors between the peaks for TRH and DLL-TRH, benzyl alcohol and LDL-TRH, and benzyl alcohol and the internal standard. The system was considered suitable for analysis, if the resolution factors were not less than 1.0, 1.5, and 2.5, respectively.

Analysis. The assay of TRH and benzyl alcohol was carried out by consecutively injecting in duplicate (RSD, ≤1.4%) standard and sample preparations for TRH, followed by standard and sample preparations for benzyl alcohol, and by measuring the respective peak areas electronically. The amount found for each component was calculated using peak area ratio with internal standard.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram from the analysis of TRH in the formulation. In the described HPLC method, benzyl alcohol is determined concomitantly with TRH in a single run by merely adjusting the concentrations of benzyl alcohol and internal standard of the sample and standard preparations to give large enough peak area responses for precise quantification. Figure 2 shows a typical chromatogram from the analysis of benzyl alcohol in the formulation.

Several possible modes of degradation/transformation of TRH in aqueous solution can be postulated. These include lactam ring (pyroglutamic acid) opening, peptide bond hydrolysis, proline amide bond hydrolysis, oxidation of the histidine segment, racemization of any three asymmetric carbon atoms, and/or any combination of the above. Table I lists the relative retention times for several postulated degradation/transformation products.

The primary degradation/transformation products observed in the HPLC chromatogram of mildly stressed (100°C; pH range, 3.0 through 10.6) aqueous solutions of TRH are L-pyroglutamyl-L-histidine (peptide bond hydrolysis), TRH free acid (proline amide bond hydrolysis), and LDL-TRH and DLL-TRH (isomerization products). The relative amounts of each of these four products varied depending on the pH. A chromatogram of a pH 6.5 heat-stressed sample of TRH in water is given in Fig. 3. All of these degradation/transformation products are separated from TRH, internal standard, and benzyl alcohol, making this analytical methodology sensitive to these changes.

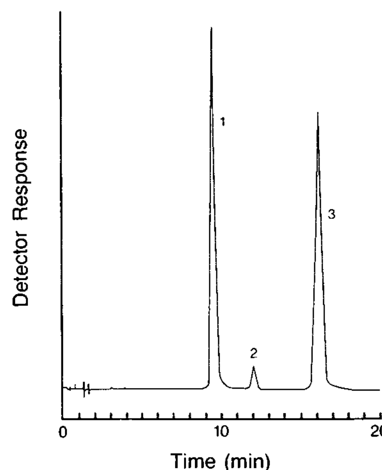

Fig. 1. Chromatogram typical of TRH analysis. 1, Internal standard; 2, benzyl alcohol; 3, TRH. HPLC conditions as stated in text.

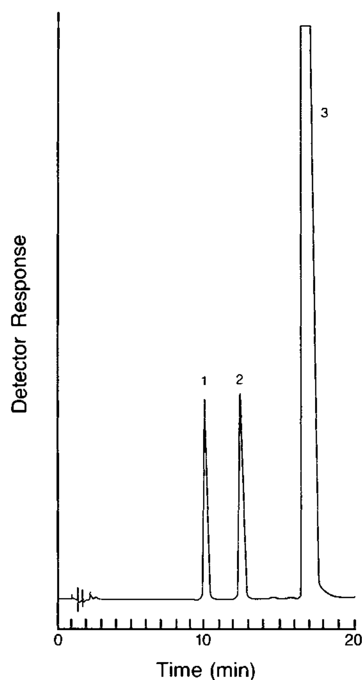

Fig. 2. Chromatogram typical of benzyl alcohol analysis. 1, Internal standard; 2, benzyl alcohol; 3, TRH. HPLC conditions as stated in text.

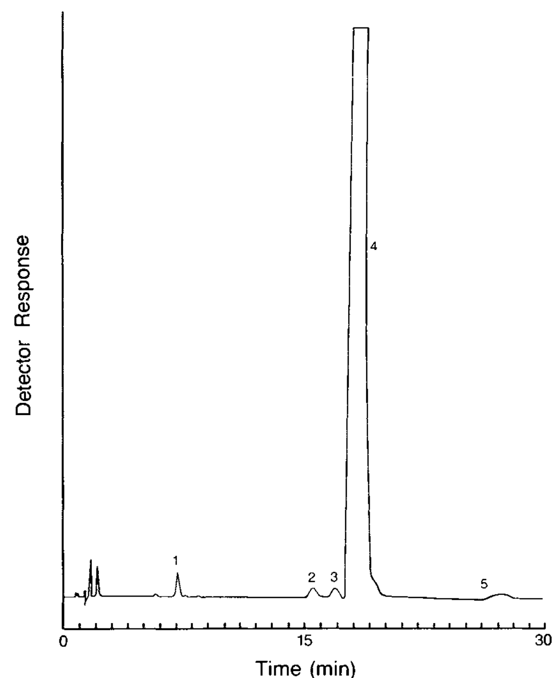

Fig. 3. Chromatogram of a stressed bulk drug solution. Stress conditions: aqueous solution, pH 6.5, heated for 24 hr at 100°C. 1, L-Pyroglutamyl-L-histidine; 2, LDL-TRH; 3, DLL-TRH; 4, TRH; 5, TRH free acid. HPLC conditions: pH of ion-pair reagent solution, 2.5; detection wavelength, 205 nm; concentration, 1 mg/ml; injection volume, 20 μl.

As confirmation of the method specificity for benzyl alcohol, the potential degradation products of benzyl alcohol (benzaldehyde and benzoic acid) were chromatographed (relative retention times: benzyl alcohol, 1.0; benzaldehyde, 2.2; benzoic acid, 2.4) and were found not to interfere in the analysis of either benzyl alcohol or TRH.

In order to test the applicability of the described HPLC method to detect degradation/transformation in the formulated product, samples of the injectable formulation and appropriate placebos were subjected to stress conditions shown in Table II. The stressed samples were then analyzed by the HPLC procedure. The results of the analyses are summarized in Table II. Only trace amounts of isomerization products of TRH were observed in the chromatograms of the mild alkaline (pH 10.3) stressed sample, indicating the stability of TRH in the formulated product. In the chromatograms of the stressed placebo samples no interfering peaks were observed to elute with the same retention volume as either TRH, benzyl alcohol, or the internal standard.

System suitability criteria based upon the separation of TRH and its isomerization products, LDL-TRH and DLL-TRH, benzyl alcohol, and the internal standard were used to

Table I. Relative Retention Times of Potential Degradation/Transformation Products of TRH

Potential degradation/transformation product	Relative retention time to TRH[a]
1. L-Pyroglutamic acid	0.09
2. Proline	0.20
3. L-Pyroglutamyl-L-histidine	0.38
4. Prolinamide	0.42
5. Histidine	0.72
6. LDL-TRH	0.83
7. DLL-TRH	0.92
8. TRH	1.00
9. γ-L-Glutamyl-L-histidine	1.20
10. L-Histidyl-L-prolinamide 2HBr	1.25
11. TRH (free acid)	1.47
12. [Glu[1]] TRH	—[b]

[a] All retention times were obtained using a Bio-Sil column.
[b] Does not elute.

Table II. HPLC Analysis of Stressed TRH Formulation Samples

Stress condition	% recovery	
	TRH	Benzyl alcohol
Initial[a] (unstressed)	100.0	100.0
pH adjusted 3.3, heated in oven (80°C) for 20 hr	98.6	97.8
pH adjusted 10.3, heated in oven (80°C) for 20 hr	98.1	100.1
Sample "as is" (pH 6.0), heated in oven (80°C) for 20 hr	100.5	98.5
Sample "as is" (pH 6.0), exposed to UV radiation (Hanovia lamp) for 20 hr	98.1	57.2

[a] Initial value normalized to 100% and the rest of the data calculated relative to the initial.

assure the quality of the HPLC separations run at any given time and place. A retention time of less than 10 min for TRH will generally result in unsatisfactory resolution. The optimum retention time of TRH for good resolution and sample analysis is between 13 and 18 min. The retention time of TRH is very sensitive to the aqueous/organic composition in the mobile phase.

The mobile phase of pH 2.2 ion-pair reagent solution/methanol/acetonitrile (88:6:6 to 90:5:5) can be used to give equivalent separation characteristics to the described mobile phase. However, an occasional abnormal (peak shouldering or splitting) chromatographic response of the internal standard is observed with that system. Trace amounts of triethylamine in the mobile phase eliminate this abnormal response, which is attributed to the interaction of the internal standard with free silanol groups in the column packing.

Resolution is also dependent on the column specificity. Three columns have been identified that give satisfactory resolution. These columns, in order of preference, are (i) Nucleosil C_{18}, 5 μm; (ii) Bio-Sil ODS-5S; and (iii) Burdick and Jackson OD5 Spherical C_{18}.

Linearity of detector response was established for TRH in the range of 20–250% levels in the sample preparation (correlation coefficient = 1.000; y intercept = -0.007) and for benzyl alcohol in the range of 40–300% levels in the sample preparation (correlation coefficient = 1.000; y intercept = 0.020). Standard addition recovery experiments performed in placebos at various TRH levels (160–240 mg/ml) and benzyl alcohol levels (6.75–11.25 mg/ml) showed recoveries of 99.0–101.3% for TRH ($N = 9$; RSD = ±0.7%) and 99.7–101.8% for benzyl alcohol ($N = 9$; RSD = ±0.6%).

The reproducibility of the method was demonstrated by performing replicate analyses by different analysts on a sample of the injectable formulation. The sample showed a mean assay value of 205.2 mg/ml for TRH ($N = 6$; RSD = ±0.6%) and 8.9 mg/ml for benzyl alcohol ($N = 6$; RSD = ±0.7%).

In conclusion, HPLC provides a simple and precise method for the quantitative determination of TRH and benzyl alcohol in the injectable formulation. The method is sensitive to the potential degradation that could occur in the formulated product and is stability indicating for both TRH and benzyl alcohol.

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