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High-pressure liquid chromatographic assay for the determination of thyrotropin-releasing hormone and its common metabolites in a physiological salt solution circulated through the isolated perfused rat lung

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Abstract

A specific and sensitive assay for TRH (L-pyroglutamyl-L-histidyl-L-proline amide), ^{3}H-TRH (L-proline 3,4-^{3}H(N),histidyl-3-^{3}H(N)), and four possible metabolites of TRH, present in the recirculated perfusate of an isolated perfused rat lung preparation, was developed. Unlike previous methods, the method developed does not require extraction of the analytes from the biological matrix. The crude sample was adjusted to a pH of 3.2 with concentrated trifluoroacetic acid and injected on to a PRP-1 (polystyrene divinylbenzene) column (10 μm, 25 cm × 4.6 mm i.d.). The mobile phase was 10% v/v acetonitrile and 90% v/v 0.75 g l^{-1} 1-hepantanesulfonic acid in 0.004 M trifluoroacetic acid, adjusted to a pH of 2.4 with concentrated NaOH. The flow rate was 0.5 ml min^{-1} and the analytes were detected by UV absorption at a wavelength of 26 nm and by radiochemical detection utilizing a liquid scintillation counter. The nominal retention times for L-PRO, L-PRO-NH_2, TRH, cyclo(HIS-PRO) and TRH-OH were 4.0 ± 0.9, 10.0 ± 0.2, 15.5 ± 0.4, 19.2 ± 0.5 and 25.3 ± 0.5 min respectively. The assay performs well in terms of precision and accuracy as indicated by linear regression and intra-assay variability analysis.

Keywords: Isolated perfused rat lung; Radiochemical detection; Reversed-phase liquid chromatography; Thyrotropin-releasing hormone

1. Introduction

Existing assays developed for the determination of thyrotropin-releasing hormone (TRH) and its common metabolites in plasma [1,2] and other biological matrices [3,4] contain complicated extraction steps and/or the necessity of gradient elution. The present assay is a simple isocratic method which is capable of resolving TRH and its metabolites (Fig. 1) in Kreb's/Henseleit physiological salt solution [5], before and after passage

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of the solution through the vasculature of an isolated perfused rat lung, without the need for sample clean-up. The assay is performed following UV and radiochemical detection; it is presently being used with radiochemical detection in the determination of TRH and its metabolites following circulation of ^{3}H-TRH through the pulmonary vasculature of the isolated perfused rat lung.

2. Materials and methods

2.1. Chemicals

pGLU-HIS-PRO-NH_2(TRH), pGLU-HIS-PRO(TRH-OH), cyclo(HIS-PRO), L-PRO and L-PRO-NH_2 were obtained from Sigma Chemical Co. (St. Louis, MO). The peptides were reported to be >98% pure and were used as supplied. Titrated TRH [L-proline3,4-^{3}H(N)], histidyl-3-^{3}H(N)] was obtained from NEN DuPont Research Products (Boston, MA); radiochemical purity was reported to be 99% and it was used as supplied. Trifluoroacetic acid (TFA) was also obtained from Sigma. HPLC-grade 1-heptanesulfonic acid, acetonitrile and ACS-grade NaCl, NaOH, NaH_2CO_3, $CaCl_2$, dextrose, $MgSO_4$, KCl and KH_2PO_4 were obtained from Fisher Scientific (Fair Lawn, NJ).

2.2. Chromatographic system

The system consisted of a variable-wavelength Gilson Holochrome UV detector (Le Bel, France), an Altex Model 110A solvent pump (Chicago, IL), a Rheodyne 7125 manual injector (Cotati, CA) fitted with a 50 μl loop and a linear dual-channel chart recorder (Fisher Recordall Series 5000). A Hamilton PRP-1 RP 10 μm column (250 mm × 4.1 mm i.d.) (Reno, NV) was used, protected by a Hamilton PRP-1 RP 10 μm (25 mm × 1.3 mm i.d.) pre-column. When radiochemical detection was employed, eluate samples were collected by Pharmacia Frac-100 fraction collector (Uppsala, Sweden) and total disintegrations per minute (dpms) were counted on a Beckman LS 1801 scintillation counter (Schaumburg, IL).

The mobile phase was a mixture of acetonitrile and 0.75 g l^{-1} 1-heptanesulfonic acid in 0.004 M aqueous TFA (10:90, v/v), adjusted before use to a pH of 2.4 with concentrated NaOH. 50 μl injections were made in a variety of solutions after adjusting these to a pH of 3.2 ± 0.2 (ambient temperature) with concentrated TFA. The flow rate was 0.5 ml min^{-1}, and UV detection was performed at 206 nm. The separation of the analytes was conducted at ambient temperature.

2.3. Preparation of standard stock solutions

TRH (0.50 mg ml^{-1}), TRH-OH (1.0 mg ml^{-1}), cyclo(HIS-PRO) (0.50 mg ml^{-1}), L-PRO-NH_2 (2.5 mg ml^{-1}) and L-PRO (10.0 mg ml^{-1}) were prepared as stock solution in reverse osmosis purified water in grade A volumetric flasks, by dissolving accurately weighed solids or known volumes of previously purchased stock solutions in fixed volumes of solvent. Stock solutions were prepared fresh every 4 days and stored below 5°C between analyses. These stock solutions were used in the preparation of successive dilutions to prepare the calibration curves as well as in the test solutions: T1 (analytes in freshly prepared Kreb's/Henseleit solution) and T2 (analytes in Kreb's/Henseleit previously circulated through the isolated perfused rat lung preparation).

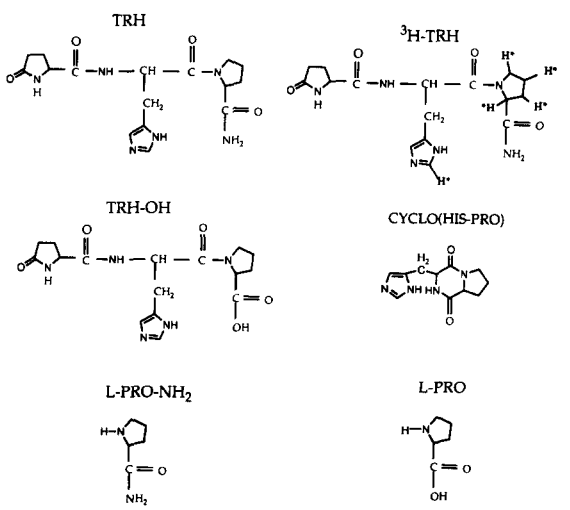

Fig. 1. Structure of TRH and its major metabolites.

Table 1
Regression analysis of the calibration data

Compound	Slope (mC ml μg^{-1})	Intercept (mV)	Correlation coefficient	n
TRH	0.150	0.137	0.997	5
TRH-OH	0.063	0.295	0.999	5
Cyclo(HIS-PRO)	0.116	0.070	1.000	5
L-PRO-NH_2	0.017	0.392	0.996	5
L-PRO	3.670[a]	0.028	0.993	5
^{3}H-TRH	4193.1[b]	28.299[c]	0.999	5

[a] Slope units for L-PRO are mV ml mg^{-1}.
[b] Slope units for ^{3}H-TRH are dpm ml ng^{-1}.
[c] Intercept units for ^{3}H-TRH are dpm.

2.4. Isolated perfused rat lung [6] and lung-circulated perfusate preparation

Adult, male Sprague–Dawley rats weighing 300–450 g were anesthetized intraperitoneally with sodium pentobarbitone (60 mg kg^{-1}, 50 mg ml^{-1}). The neck region was opened and a stainless-steel cannula (30 mm long, 1.94 mm i.d., 2.29 mm o.d.) was inserted 14 mm inside the trachea and tied in place. After opening the thorax at the diaphragm the lungs were ventilated via the cannula (33 respirations min^{-1}, 2 ml tidal volume). The rib cage was cut on both sies and the thoracic cavity revealed. Sodium heparin (0.1 ml; 1000 units ml^{-1}) was injected directly into the lumen of the right ventricle and one loose ligature was placed around the pulmonary artery (PA) and aorta. Bubble-free perfusate at 37°C (Kreb's/Henseleit buffer) was introduced through a blunt, 16G stainless-steel-tipped cannula (19 mm long), inserted into the PA (not to the point of the arterial bifurcation which hinders blood clearance) via an incision in the upper right ventricle. The cannula was clipped in place and the left ventricle and atrium served immediately to enable blood and perfusate to be pumped freely (15 ml min^{-1}).

Positive pressure ventilation was halted while the lungs remained partially inflated. The PA ligature was tightened and the clip removed. The lungs were excised, washed with perfusate held at 37°C and a steel rod was slipped through the esophagus, which was left attached to the isolated perfused rat lung preparation. The lungs were suspended horizontally from this rod in a jacketed glass thorax (1000 ml volume, 10.5 cm i.d.) at 37°C, with the tracheal exit cannula (78 mm long, 2.37 mm i.d., 2.82 mm o.d.) fitting snugly around the tracheal cannula; this joint was sealed with a short piece of silicone tubing. The whole procedure took less than 8 min. Residual blood components were washed from the preparation for a further 5–10 min after which the thorax was sealed and perfusate recirculated from a 200 ml heat-jacketed reservoir, Following recirculation for 30 min, the perfusate was collected and stored below 5°C. The lung isolation procedure was repeated three times and the collected perfusate for each of the three lungs was pooled and used to prepare test solutions containing analytes; hence analytical interferences due to the presence of extracelluar lung exudates were evaluated.

2.5. Preparation of test solutions

Kreb's/Henseleit solution consisted of dextrose (2.0 g l^{-1}), KCl (0.35 g l^{-1}), $MgSO_4$ (0.29 g l^{-1}), $NaHCO_3$ (2.10 g l^{-1}), $CaCl_2$ (0.27 g l^{-1}), NaCl (6.92 g l^{-1}) and KH_2PO_4 (0.16 g l^{-1}) completely dissolved in distilled water and adjusted to a pH of 7.4. A test solution (T1) of TRH (50.0 μg ml^{-1}), TRH-OH (100.0 μg ml^{-1}), cyclo(HIS-PRO) (50 μg ml^{-1}), L-PRO-NH_2 (250 μg ml^{-1}) and L-PRO (2.0 mg ml^{-1}) was prepared using aliquots of standard stock solutions and Kreb's/Henseleit solution as a diluent. This solution was

further diluted with Kreb's/Henseleit to produce the five different concentrations (Table 2) used to calculate the calibration data shown in Table 1 with the lowest concentration in each case equal to the apparent limit of detection determined earlier as three times the baseline. In addition, a test solution (T2), of identical concentrations as given above, of TRH and its metabolites was prepared and diluted as above, but with Kreb's/Henseleit solution which had been circulated through the lung. A radioactive solution of ^{3}H-TRH was perpared in Kreb's/Henseleit to produce a starting

Table 2
% Recovery of analytes

True concentration	Found concentration mean (SD), $n = 3$
TRH (μg ml^{-1})	
3.62	3.82 (0.66)
4.34	3.91 (0.57)
8.68	9.00 (0.34)
17.38	15.62 (0.78)
34.78	33.12 (0.75)
Cyclo(HIS-PRO) (μg ml^{-1})	
6.24	5.44 (0.33)
12.48	11.56 (0.64)
25.00	27.88 (1.75)
37.50	38.43 (1.28)
50.00	48.47 (0.86)
L-PRO (mg ml^{-1})	
0.250	0.299 (0.015)
0.500	0.490 (0.035)
1.00	0.925 (0.025)
1.50	1.410 (0.064)
2.00	1.800 (0.067)
TRH-OH (μg ml^{-1})	
12.5	11.91 (2.14)
15.0	23.30 (0.69)
50.0	52.55 (1.46)
81.76	79.98 (3.26)
99.80	98.74 (1.18)
L-PRO-NH$_2$ (μg ml^{-1})	
31.0	27.55 (2.25)
63.2	61.85 (6.19)
124.4	132.74 (8.20)
171.1	166.58 (10.66)
250.0	247.06 (7.75)

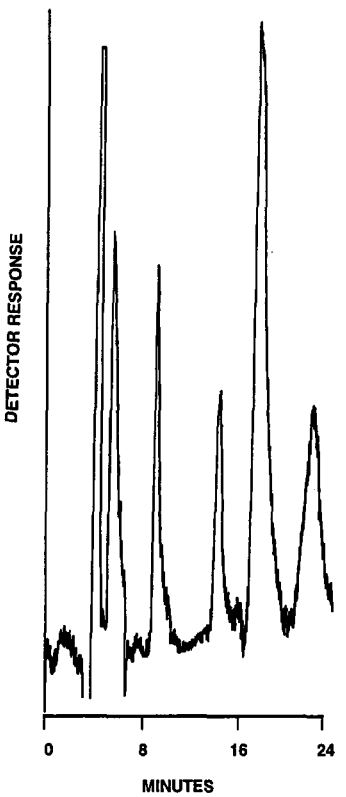

Fig. 2(a).

TRH concentration of 0.520 pg m^{-1} with a specific activity of 70.0 Ci mmol^{-1}. Four successive dilutions of the stock solution were prepared, using Kreb's/Henseleit as a diluent. All solutions were prepared fresh daily and stored below 5°C between analyses. The pH of each of these dilutions was adjusted to 3.2 ± 0.2 (ambient temperature) using concentrated TFA, immediately before injection.

2.6. Assay characteristics

Chromatograms for all analytes were evaluated for peak height (trough-to-trough baseline), average retention time and signal-to-noise ratio following UV detection. ^{3}H-TRH chromatograms were constructed following radioanalysis of each 30 s sample and evaluated for peak height (measured in disintegrations per minute). Calibration curves of mean peak height ($n = 4$) versus concentration were constructed at five concentrations for

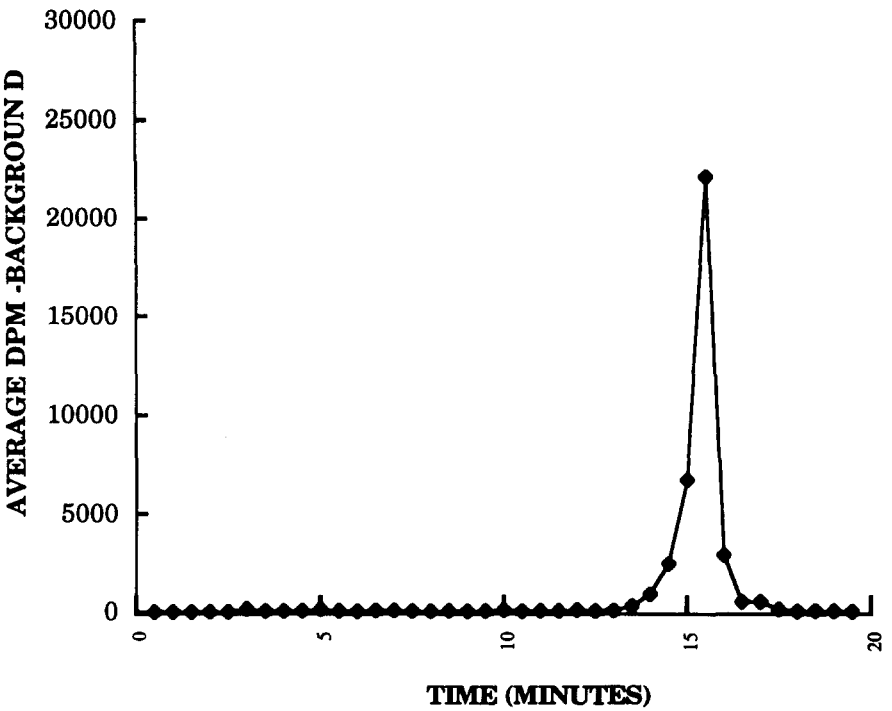

Fig. 2. (a) Chromatogram of test solution containing 1.5 mg ml^{-1} of L-PRO, 250 $\mu g\ ml^{-1}$ of L-PRO-NH_2, 50.0 $\mu m\ ml^{-1}$ or TRH, 50.0 $\mu g\ ml^{-1}$ of cyclo(HIS-PRO) and 100.0 $\mu g\ ml^{-1}$ of TRH-OH in Kreb's/Henseleit solution. (b) Radiochromatogram of test solution containing 0.4 $\mu Ci\ ml^{-1}$ ^{3}H-TRH (specific activity 70.0 Ci $mmol^{-1}$) in Kreb's/Henseleit.

each of the analytes (Table 1) and evaluated for linearity. Additional solutions (T1 series) were prepared utilizing previously prepared standard stock solutions and Kreb's/Henseleit as a diluent. Found concentrations of T1 series samples were compared to actual concentrations. In addition, T1 concentrations were compared to their T2 counterparts for two concentrations of each analyte using Student's t-test. The probability with which average apparent concentrations ($n = 3$) could be ascribed to different values between T1 and T2 was calculated in each case.

3. Results and discussion

The structures of TRH and its reported major metabolites are shown in Fig. 1. The retention times (measured from the time of injection) of these reference compounds, prepared in both Kreb's/Henseleit before circulation and after circulation through the isolated perfused rat lung, were found to be 4.9 ± 0.9, 10.0 ± 0.2, 15.5 ± 0.4, 19.2 ± 0.5 and 25.3 ± 0.5 min ($n = 12$) for L-PRO, L-PRO-NH_2, TRH, cyclo(HIS-PRO) and TRH-OH respectively. The solvent front appeared at 3.5 min. Peak shape and typical chromatograms are shown following UV (all solutes) and radiochemical detection (^{3}H-TRH only) in Fig. 2. Regression analyses ($n = 5$) of calibration data showed a linear relationship between concentration and peak height for each of the five analytes and the tritiated TRH (Table 1). The minimum detectable limits of the assay, determined by a minimum signal equal to three times the baseline noise, were 3.62, 12.5, 6.24, 31.0 $\mu g\ ml^{-1}$, 0.250 $mg\ ml^{-1}$ and 13.0 $ng\ ml^{-1}$ for TRH, TRH-OH, cyclo(HIS-PRO), L-PRO-NH_2, L-PRO and ^{3}H-TRH respectively.

The "found concentration" data in Table 2 show results from repeat injections ($n = 3$) of TRH, TRH-OH, cyclo(HIS-PRO), L-PRO-NH_2, and L-PRO solutions in pure Kreb's/Henseleit solution (T1) as if they were unknown. The results

Table 3
Comparison of % recovery of analytes in Kreb's/Henseleit and in Kreb's/Henseleit circulated through the isolated perfused rat lung
Cyclo(HIS-PRO) True concentration T1 Found concentration, T2 Found concentration, p value[a]

Compound	True concentration (μg ml^{-1})	T1 Found concentration, mean(SD), $n = 3$	T2 Found concentration, mean(SD), $n = 3$	p value[a]
TRH	4.34	3.91 (0.57)	4.04 (0.26)	0.010
	17.38	15.62 (0.78)	16.62 (0.85)	0.020
TRH-OH	25.0	23.30 (0.69)	26.35 (0.26)	0.039
	81.76	79.98 (3.26)	82.36 (0.69)	0.009
Cyclo(HIS-PRO)	12.48	11.56 (0.64)	13.25 (0.69)	0.043
	37.50	38.43 (1.28)	36.52 (0.96)	0.016
L-PRO-NH_2	63.2	61.85 (6.19)	64.58 (2.13)	0.014
	171.1	166.58 (10.66)	172.36 (5.69)	0.011
L-PRO[b]	0.500	0.490 (0.035)	0.492 (0.062)	0.001
	1.50	1.410 (0.064)	1.62 (0.089)	0.044

[a] Probability of a difference existing between T1 and T2.
[b] Units for L-PRO concentrations are mg ml^{-1}.

show the reproducibility and reliability of the assay at the concentrations investgated. In addition, when the assay was utilized to determine the presence of TRH and its metabolites added to Kreb's solution which had been circulated through the isolated perfused rat lung (T2) for 30 min, apparent concentrations determined using the same calibration curves (Table 3) were found to be statistically equivalent ($p < 0.05$) to analytes in Kreb's which did not contain extracellular lung exudates. This method appears to be sensitive, reproducible, unaffected by lung exudates and much less complicated than those previously published. The assay appears to rely dually upon the existence of a relatively high concentration of heptanesulfonic acid, thus giving the column a net negative charge, and upon the ionic nature of the analytes at the adjusted sample pH of 3.2. The elution order shows initially the rapid exit of the small molecular weight, L-PRO, followed by the elution of the L-PRO-NH_2 (zwitterionic and cationic respectively, under these conditions). The final three larger molecular weight analytes, TRH, cyclo(HIS-PRO) and TRH-OH, are cationic, cationic and zwitterionic respectively, both at their adjusted sample pH of 3.2 and at the mobile phase pH of 2.4. It is believed that the successful separation of these three structurally similar analytes is due both to their differential interaction with the ion-pairing characteristics of TFA, added pre-injection, and their encounter with a mobile phase environment including an additional ion-pairing agent, 1-heptanesulfonic acid, at a decreased pH along with additional polar interactions of a non-ionic nature. Coupled with radiochemical detection, it is believed that the assay will be useful to study the metabolism of TRH and the appearance of metabolites in the circulating perfusate of the isolated perfusated rat lung.

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