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# RAPID SEPARATION OF TRITIATED THYROTROPIN-RELEASING HORMONE AND ITS CATABOLIC PRODUCTS FROM MOUSE AND HUMAN CENTRAL NERVOUS SYSTEM TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH RADIOACTIVE FLOW DETECTION

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## SUMMARY

Reversed-phase high-performance liquid chromatography with radioactive flow detection was utilized to investigate the catabolism of thyrotropin-releasing hormone (TRH) in central nervous system (CNS) tissues. Two different column/gradient solvent systems were tested: (1) octadecylsilane (ODS) with an acetic acid–acetonitrile gradient and (2) poly(styrenedivinylbenzene) (PRP-1) with a trifluoroacetic acid–acetonitrile gradient. Both systems used 1-hexanesulfonic acid as the second ion-pairing reagent and yielded excellent separation of TRH and its catabolic products, TRH acid, cyclo(histidyl-proline), histidyl-proline, proline, and prolinamide, produced in CNS tissue homogenates. The PRP-1 column with a trifluoroacetic acid–acetonitrile solvent system produced a better and more reproducible separation of TRH catabolic products than the ODS column with the acetic acid–acetonitrile solvent system. This PRP-1 technique was utilized to demonstrate different rates and products of TRH catabolism in mouse and human spinal cord compared with cerebral cortex.

## INTRODUCTION

The products formed from the catabolic metabolism of thyrotropin-releasing hormone (TRH) in central nervous system (CNS) tissues have been examined by high-performance liquid chromatography (HPLC) [1–14], thin-layer chromatography (TLC) [15–30], paper chromatography [31–33], electrophoresis [30,32–34] and radioimmunoassay (RIA) [5,8,10,11,14,35–40]. HPLC has been

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more commonly employed for purification of TRH and its metabolites before quantitation by TLC or RIA [5,8,10,11,14]. An HPLC method for the study, in one sample elution, of all the labeled metabolites derived from the catabolism of [$^3$H-Pro]TRH in CNS tissue homogenates was previously introduced by Schwartz et al. [12]. In this paper we present an evaluation of two column systems for reversed-phase HPLC with continuous radioactivity detection of [$^3$H-Pro]TRH and its metabolites in the study of spinal cord TRH catabolism. The advantages of this methodology are: (1) specific and simultaneous detection of all the metabolic products, (2) simplified biological sample preparation that reduces sample loss, and (3) increased accuracy of quantitation and minimization of artifacts [41]. We investigated the usefulness, in the separation of TRH and its catabolites, of the ion-pairing reagent 1-hexanesulfonic acid [13] and acetic acid (AA) or trifluoroacetic acid (TFA) [42] in octadecylsilane (ODS) or poly(styrenedivinylbenzene) (PRP-1) columns. The PRP-1 column with TFA–acetonitrile (TFA-AN) gradient provided improved and more reproducible separation of TRH catabolic products in homogenates from different regions of mouse and human CNS.

## EXPERIMENTAL

### *Materials*

Unlabeled standard peptides pyroglutamyl-histidyl-prolinamide (TRH), histidyl-proline diketopiperazine (cyclo(histidyl-proline), cyclo(His-Pro)), pyroglutamyl-histidyl-proline (TRH acid, TRH-OH), prolinamide (Pro-$NH_2$), and proline (Pro) were obtained from Sigma (St. Louis, MO, U.S.A.) and histidyl-proline (His-Pro) from Accurate Chemical (Westbury, NY, U.S.A.). Labeled standard peptides [L-proline-2,3,4,5-$^3$H]TRH ([$^3$H-Pro]TRH), [L-proline-3,4-$^3$H]cyclo(His-Pro) ([$^3$H-Pro]cyclo(His-Pro)), and [2,3,4,5-$^3$H]L-proline ([$^3$H]Pro) were obtained from New England Nuclear (Boston, MA, U.S.A.).

### *Extraction procedure*

Mouse or human CNS tissues were obtained immediately following necropsy[a]. Whole tissue homogenates of 0.1 g tissue per ml buffer were prepared with a buffer consisting of 100 m*M* potassium phosphate [19]. This buffer was prepared by the addition of a 100 m*M* $KH_2PO_4$ solution to a 100 m*M* $K_2HPO_4$ solution until a pH of 7.5 at 25°C was attained. Homogenates were prepared using an immersion sonicator (Ultrasonics, Farmingdale, NY, U.S.A.) with fifteen 500-ms bursts at 20 kHz.

### *TRH catabolism*

Each homogenate (0.5 ml) was incubated with 1 $\mu$Ci (9.38 pmol) of [$^3$H-Pro]TRH at 37°C for 0, 10, 20, 30, and 60 min, respectively. Reactions were stopped by precipitation with 10 volumes of methanol, capped, and left at room temperature overnight to allow the cyclization of histidyl-prolinamide (His-Pro-

[a]Approved AALAC procedures followed for all animal euthanasia.

$NH_2$) to cyclo(His-Pro). Samples were then centrifuged at 27 000 *g* for 15 min. The supernatants were removed and lyophilized for 12–16 h until dry in a Speed Vac concentrator (Savant Instruments, Hicksville, NY, U.S.A.). The residue was reconstituted in 500 μl of HPLC solvent (A–B, 80:20) and the pH was adjusted to 3.2 with 3 *M* acetic acid or 2 *M* TFA. The solution was filtered through a 0.45-μm nylon membrane micro-filter (MSI/Fisher, Itasca, IL, U.S.A.), and 100 μl were injected into the HPLC system.

*High-performance liquid chromatography*

Chromatography was performed on a Gilson (Middleton, WI, U.S.A.) computer-integrated gradient System 45 high-performance liquid chromatograph with two means of post-column detection. Post-column detection was accomplished using a Gilson variable-wavelength ultraviolet detector at 206 nm (40-μl flow cell) and a Radioanalytic Model BD radioactive flow detector (100-μl flow cell). The outputs from both detectors were fed directly into the Gilson Data Master unit and integrated. Channel A of the Gilson HPLC system was connected to the radioactive flow detector set for tritium and channel B to the UV detector (206 nm) in order that unlabeled standards could be chromatographed simultaneously with the labeled metabolites and standards [$^3$H-Pro]TRH, [$^3$H-Pro]cyclo(His-Pro), and [$^3$H]Pro to identify the labeled metabolites of [$^3$H-Pro]TRH.

Two different column/solvent systems were studied: (1) Zorbax ODS 80 mm×6.2 mm, 5 μm particle size, analytical column (DuPont, Boston, MA, U.S.A.) used in series with an Altex (Beckman, Fullerton, CA, U.S.A.) Ultrasphere ODS 150 mm×4.6 mm, 5 μm particle size, analytical column and an ODS guard column. (2) Two poly(styrenedivinylbenzene) (PRP-1) 250 mm×4.1 mm columns with 10 μm particle size (Hamilton, Reno, NV, U.S.A.) used in series with a PRP-1 guard column. The use of two Zorbax columns in series or two Altex columns did not produce as good a separation as the Zorbax–Altex column system. Solvents optimized in the ODS column system were solvent A [5% AN (Fisher, Itasca, IL, U.S.A.) and 1.5 g/l 1-hexanesulfonic acid (Fisher) in 0.02 *M* AA, pH 2.4] and solvent B [30% AN and 1.5 g/l 1-hexanesulfonic acid in 0.02 *M* AA, pH 2.4]. Solvents A and B in the ODS system composed a linear gradient from 20% to 76% solvent B with an elution time of 18 min and a flow-rate of 1.2 ml/min. Solvents optimized in the PRP-1 column system were solvent A [5% AN and 1.5 g/l 1-hexanesulfonic acid in 0.004 *M* TFA, pH 2.4] and solvent B [30% AN and 1.5 g/l 1-hexanesulfonic acid in 0.004 *M* TFA, pH 2.4]. Solvents A and B in the PRP-1 system composed a linear gradient from 21% to 43% solvent B with an elution time of 20 min and a flow-rate of 1.0 ml/min. In order that the columns would be equilibrated at their starting solvent composition for the next run the gradients were changed to their respective starting % B midrun (ODS 18 min and PRP 12 min). Therefore the next sample could be injected immediately after each chromatographic elution was over without added delay for excess column equilibration. The injector and injection loop was flushed after each sample injection with methanol and HPLC-grade water. The mean and standard deviation of retention times for standards evaluated in the first daily chromatogram following overnight flushing evaluated over different days is not significantly dif-

ferent than the mean and standard deviation of retention times for standards evaluated following multiple chromatograms at the end of the day over different days under the conditions described.

*Thin-layer chromatography*

To identify by a separate technique the metabolic products formed, fractions were collected on some chromatographic elutions, spotted on thin-layer silica GF plates (Analtech, Newark, DE, U.S.A.) and developed with a solvent composed of chloroform–methanol–ammonium hydroxide (Fisher) (125:75:25) [18]. Unlabeled standards were spotted with the aliquots containing unknown metabolites and developed by ninhydrin for detection of the free amino acids (Pro and Pro-$NH_2$) or by Pauly reagent for detection of the histidine-containing peptides (TRH-OH, TRH, cyclo(His-Pro) and His-Pro). The spots were scraped, placed in scintillation cocktail (Scintiverse LC/Fisher), and counted to confirm that each metabolite and appropriate standard co-eluted under similar conditions.

*[$^3$H-Pro]His-Pro-$NH_2$ cyclization*

[$^3$H]His-Pro-$NH_2$ was produced by incubating [$^3$H-Pro]TRH with bovine liver pyroglutamate aminopeptidase (PGAP) (Sigma). The enzyme was prepared by dissolving 10 mg of PGAP in 1 ml of 100 m*M* potassium phosphate buffer, pH 7.4, 2 m*M* dithiothreitol (DTT), 2 m*M* ethylenediaminetetraacetic acid (EDTA) and 1 m*M* bacitracin with preincubation at 20°C for 30 min. Then 10 $\mu$Ci of [$^3$H-Pro]TRH in 40 $\mu$l of buffer solution were incubated with 70 $\mu$l of the PGAP solution at 37°C for 5 min. The reaction was terminated by the addition of 600 $\mu$l of ethanol and microcentrifuged at 4°C at 15 000 *g* for 15 min. The samples were then lyophilized until dry for 30–60 min, reconstituted in HPLC solvent (A–B, 20:80), and injected onto the HPLC system. Experiments were repeated as above except that after the reaction was terminated with ethanol, the samples were allowed to stand 24 h at room temperature (18–22°C) prior to HPLC to yield complete cyclization of His-Pro-$NH_2$ to cyclo(His-Pro).

*[$^3$H-Pro]His-Pro-$NH_2$ metabolism*

[$^3$H]His-Pro-$NH_2$ was produced by adding 10 $\mu$Ci of [$^3$H-Pro]TRH to 0.2 mg PGAP in 200 $\mu$l of the above buffer, incubating at 37°C for 5 min, then diluting enzyme mixture 1:5 to 1 ml in above buffer and adding 100 $\mu$l of this solution to 500 $\mu$l mouse cerebral cortex or spinal cord homogenates (0.1 g/ml). These samples were incubated at 37°C for 0, 10, 20, and 30 min and the reaction was stopped by the addition of 10 volumes of ethanol prior to sample processing as described above to determine if [$^3$H-Pro]His-Pro-$NH_2$ were metabolized to His-Pro.

*TRH-OH metabolism*

Using the same experimental procedure as above His-Pro was produced from unlabeled TRH-OH (Sigma) by adding 40 $\mu$g TRH-OH to 70-$\mu$l aliquots of PGAP solution (0.2 mg PGAP in 200 $\mu$l of 100 m*M* potassium phosphate buffer, pH 7.4, 2 m*M* DTT, 2 m*M* EDTA, 1 m*M* bacitracin). The reaction mixture was incubated at 37°C for 0, 10, 20, 60, and 120 min before the reaction was stopped, lyophilized,

reconstituted, and injected into the HPLC system as described above. His-Pro formation was identified by both HPLC and TLC.

*[$^3$H-Pro]Cyclo(His-Pro) metabolism*

The metabolism of cyclo(His-Pro) was investigated by incubating 1 $\mu$Ci (17.1 pmol) of [$^3$H-Pro]cyclo(His-Pro) in 500 $\mu$l mouse cortex and spinal cord homogenates (0.1 g in 1 ml of 100 m*M* potassium phosphate buffer, pH 7.5, containing 2 m*M* DTT and 2 m*M* EDTA). The reactions were run for 0, 30, 60, 120, and 180 min at 37 °C. Samples were extracted in methanol, processed, and chromatographed as above.

*[$^3$H-Pro]TRH catabolic products*

NIH:N mouse ($n=3$) or human ($n=6$) cerebral cortex and mouse ($n=3$) or human ($n=13$) spinal cord homogenates were incubated with [$^3$H-Pro]TRH for 0, 10, 20, 30, and 60 min. Catabolic products were analyzed by HPLC. Radioactive TRH or TRH catabolic products in femtomoles were determined at each time point in each homogenate (Fig. 2). Total protein was measured in parallel homogenates with Bradford reagent (Bio-Rad Labs., Richmond, CA, U.S.A.) [43]. Products formed were calculated as fmol/mg of protein at each time point and plotted. ODS product curves were integrated with a Houston Hipad and BIOQUANT statistical software (Nashville, TN, U.S.A.) and PRP-1 curve areas were calculated using a MicroSoft Excel macro written for Macintosh computer. The products formed in 60 min were represented as fmol/mg of protein (mean $\pm$ standard deviation) for each species and CNS region (see Table II).

## RESULTS

*Quantitative and qualitative HPLC analysis of peptide standards*

Standard chromatograms and retention times, which demonstrate the different elution patterns of the two column/solvent systems, are presented in Fig. 1. The catabolic product separation and reproducibility data from both the ODS and PRP-1 column systems are collected in Table I. The PRP-1/TFA–AN system had an improved separation compared to the ODS/AA–AN system. The smallest PRP-1/TFA–AN system peak-to-peak separation, between TRH and cyclo(His-Pro) ($1.47 \pm 0.03$ min), was significantly ($p < 0.001$) greater than the two smallest ODS/AA–AN system peak-to-peak separations between TRH and cyclo(His-Pro) ($0.78 \pm 0.04$ min) and between TRH and His-Pro ($0.79 \pm 0.20$ min).

In both column systems His-Pro-$NH_2$ overlapped the TRH peak necessitating overnight incubation to stoichiometrically cyclize His-Pro-$NH_2$ to cyclo(His-Pro) which could be quantitated separately from the TRH peak. In the ODS/AA–AN system the His-Pro peak overlapped the TRH peak due to peak tailing. In addition, even with an ODS pre-column the ODS/AA–AN column system could not withstand the acidic pH needed to chromatograph [$^3$H-Pro]TRH metabolites for extended periods of time [41]. Eventual hydrolysis of the silane–silica bond made it necessary to repack the column approximately every four weeks of use. Al-

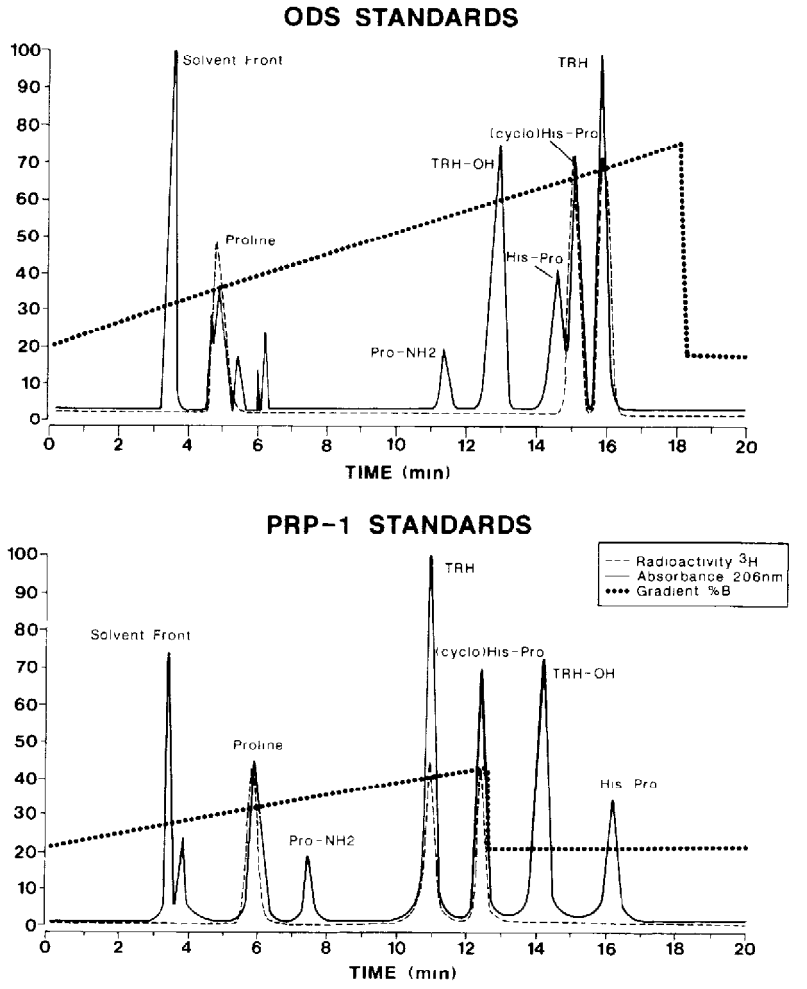


Fig. 1 Reversed-phase HPLC separation of unlabeled and [$^3$H]Pro-labeled TRH and metabolite standards. Standards were injected into a Gilson System 45 computer-integrated HPLC system and separated using two types of columns: (1) ODS/AA–AN system consisting of a Zorbax ODS and an Altex Ultrasphere ODS column eluted by a 20 to 76% B gradient (solvent A: 5% AN and 1.5 g/l 1-hexanesulfonic acid in 0.02 *M* AA; solvent B: 30% AN and 1.5 g/l 1-hexanesulfonic acid in 0.02 *M* AA). (2) PRP-1/TFA–AN system with two PRP-1 columns in series eluted by a 21 to 43% B gradient (solvent A: 5% AN and 1.5 g/l 1-hexanesulfonic acid in 0.004 *M* TFA; solvent B: 30% AN and 1.5 g/l 1-hexanesulfonic acid in 0.004 *M* TFA) Simultaneous UV output (206 nm) channel A and radioactive detector channel B were integrated and presented as area percent relative to TRH as highest peak.

though the relative metabolite elution order never changed, this loss of the column bonded phase and repackings caused reproducibility problems with peak shifts of as much as 3.9 min over the life of a column. Due to the ODS/AA–AN sensitivity to low pH we evaluated columns containing fully polymeric packing materials, which are resistant to the effects of acidic pH. The PRP-1 column, using a buffer containing TFA produced the best results. His-Pro overlapping

TABLE I

RETENTION TIMES

| Column system | $n$ | | Retention time (min) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | TRH | cyclo(His-Pro) | His-Pro | Proline | Pro-$NH_2$ | TRH-OH |
| ODS | 11 | Mean ± S D | 14.1 ± 1.0 | 14.9 ± 1.0 | 13.4 ± 1.2 | 5.0 ± 0.6 | 9.8 ± 1.1 | 11.3 ± 1.4 |
| | | Range | 13.1–16.3 | 13.9–17.1 | 12.3–15.6 | 4.3–5.6 | 8.9–11.7 | 10.0–13.9 |
| | | $\Delta$ | 3.2 | 3.2 | 3.3 | 1.3 | 2.8 | 3.9 |
| PRP-1[a] | 20 | Mean ± S D | 11.2 ± 0.2 | 12.6 ± 0.2 | 16.5 ± 0.2 | 6.0 ± 0.1 | 7.6 ± 0.2 | 14.5 ± 0.2 |
| | | Range | 10.9–11.6 | 12.4–13.1 | 16.2–16.9 | 5.8–6.2 | 7.4–7.9 | 14.2–15.0 |
| | | $\Delta$ | 0.7 | 0.7 | 0.7 | 0.4 | 0.5 | 0.8 |

[a]PRP-1 smallest peak separation (1.5 ± 0.1 min) was significantly greater ($p < 0.001$) than ODS smallest peak separation (0.8 ± 0.2 min).

with TRH was not observed in the PRP-1/TFA–AN system. In addition, this system was able to separate Pro from the solvent front whereas the ODS/AA–AN system could not. Both 5- and 10-$\mu$m particle sizes were used in PRP-1 columns. Although their separation was roughly equivalent, the 5-$\mu$m columns were found to be structurally weak within normal HPLC pressure limits and suffered several column collapses. The 10-$\mu$m columns exhibited greater mechanical stability than the 5-$\mu$m columns and produced excellent reproducibility with peak shifts no greater than 0.8 ± 0.2 min during the life of a column.

The use of continuous radioactive flow detection enabled [$^3$H-Pro]TRH and catabolic products to be specifically detected and integrated by the Gilson Data Master unit without decreased peak resolution inherent in fraction collection. Moreover, radioactive flow detection required minimal sample clean-up of the reaction mixture compared to that needed for UV detection. Therefore decreased sample loss occurred from tissue homogenates with increased experimental accuracy and fewer spurious peaks or artifacts.

*Thin-layer chromatography*

HPLC peak aliquots from reactions with CNS tissues were spotted on TLC plates with unlabeled standards to identify all the metabolites. All the labeled peaks were found to co-migrate with their corresponding standards. No undefined metabolites were derived from [$^3$H-Pro]TRH in CNS tissues.

*[$^3$H-Pro]His-Pro-$NH_2$*

We produced [$^3$H-Pro]His-Pro-$NH_2$ using bovine liver pyroglutamyl aminopeptidase (Sigma). When the extracted and lyophilized reaction mixture was injected onto the HPLC system within 60 min of formation a peak corresponding to [$^3$H-Pro]His-Pro-$NH_2$ was found which partially overlapped the TRH peak. When the extracted homogenate reaction mixture was allowed to incubate at room temperature for 24 h prior to lyophilization this peak disappeared and a peak with the same retention time as cyclo(His-Pro) appeared (data not shown). These experiments indicated that after ethanol extraction and 24-h incubation

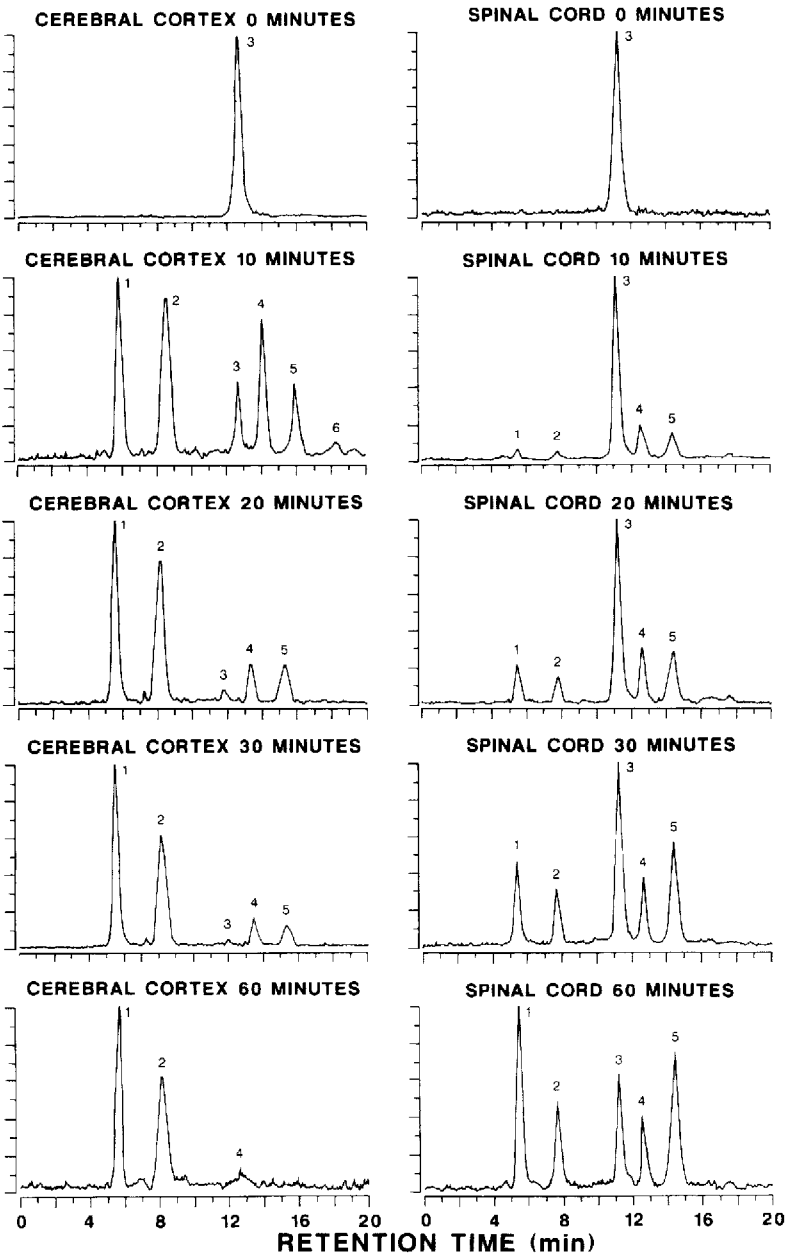


Fig. 2. Reversed-phase HPLC separation of [$^3$H-Pro]TRH metabolites. Mouse cerebral cortex and spinal cord homogenates of 0.1 g tissue in 1 ml of 100 m*M* potassium phosphate buffer, pH 7.5, were incubated with 1 $\mu$Ci (9.38 pmol) of [$^3$H-Pro]TRH at 37 °C for 0, 10, 20, 30, and 60 min, respectively. Reactions were stopped by precipitation with 10 volumes of methanol, capped, and left at room temperature overnight to allow the cyclization of His-Pro-$NH_2$ into cyclo(His-Pro). Chromatography was performed as described in the legend to Fig. 1. Post-column detection was accomplished using a Radioanalytic Model BD radioactivity detector (100-$\mu$l flow cell). Peaks: 1 = proline (Pro); 2 = prolinamide (Pro-$NH_2$), 3 = pyroglutamyl-histidyl-prolinamide (TRH); 4 = histidyl-proline diketopiperazine (cyclo(His-Pro)); 5 = pyroglutamyl-histidyl-proline (TRH-OH).

all the His-Pro-$NH_2$ was cyclized to cyclo(His-Pro). Therefore cyclo(His-Pro) in our system corresponds stoichiometrically to His-Pro-$NH_2$ formation.

*[$^3$H-Pro]His-Pro-$NH_2$ metabolism*

When freshly produced [$^3$H-Pro]His-Pro-$NH_2$ was incubated with mouse cerebral cortex or spinal cord homogenates for 20 and 30 min, respectively, [$^3$H-Pro]His-Pro was formed and was further metabolized to [$^3$H]Pro. Therefore [$^3$H-Pro]His-Pro can be formed from the enzymatic breakdown of [$^3$H-Pro]His-Pro-$NH_2$. Migration of [$^3$H-Pro]His-Pro was similar to that of unlabeled standard on TLC.

*TRH-OH metabolism*

His-Pro was made from unlabeled TRH-OH standard using the method used to produce [$^3$H-Pro]His-Pro-$NH_2$. The His-Pro formed was found to have the same HPLC retention time as standard His-Pro with the same TLC $R_F$ value and appropriate color formation when developed with Pauly reagent and ninhydrin.

*Cyclo(His-Pro) metabolism*

For over 30 min no degradation of [$^3$H-Pro]cyclo(His-Pro) was noted in cerebral cortex or spinal cord homogenates. Incubation times of 60, 120, and 180 min before methanol extraction were required to show minimum degradation of cyclo(His-Pro) in cortex (3.4, 7.0, and 9.7%) and in spinal cord (1.8, 4.2, and 7.5%), respectively.

*TRH catabolic products*

In 100 m*M* potassium phosphate buffer, pH 7.5, TRH catabolism is faster in mouse and human cerebral cortex than in spinal cord (Table II). The catabolic rates are comparable in mouse and human cerebral cortex. However, TRH catabolism is two times faster in mouse spinal cord compared to human spinal cord. In

TABLE II

TRH DEGRADATION RATE AND PRODUCTS FORMED

Cerebral cortex and spinal cord homogenates (0.1 g/ml) in 100 m*M* potassium phosphate buffer, pH 7.5, were incubated with [$^3$H-Pro]TRH and processed as described in the legend to Fig. 2

| Tissue | Degradation rate (mean ± S.D.) (fmol/mg/min) | Concentration products formed in 60 min (mean ± S.D.) (fmol/mg of protein) | | | | |
|---|---|---|---|---|---|---|
| | | cyclo(His-Pro) | His-Pro | Pro | Pro-$NH_2$ | TRH-OH |
| *Mouse* | | | | | | |
| Cortex | 84.3 ± 6.1 | 65.7 ± 17.9 | 27.9 ± 21.9 | 574.5 ± 21.0 | 348.7 ± 63.8 | 111.4 ± 97.3 |
| Spinal cord | 25.8 ± 2.9[a] | 101.3 ± 20.5 | 0.0 ± 0.0 | 553.4 ± 69.0 | 107.0 ± 16.7[a] | 309.9 ± 26.7[a] |
| *Human* | | | | | | |
| Cortex | 83.6 ± 18.2 | 362.5 ± 131.5 | 12.2 ± 9.9 | 253.2 ± 75.4[b] | 531.0 ± 48.6[b] | 261.9 ± 53.5[b] |
| Spinal cord | 11.1 ± 11.6[c] | 169.8 ± 186.6 | 22.3 ± 34.0 | 90.2 ± 70.4[a,b] | 185.9 ± 120.1[a] | 170.8 ± 153.7 |

[a] $p < 0.005$ mouse or human cortex versus spinal cord

[b] $p < 0.002$ mouse cortex versus spinal cord or human cortex versus spinal cord

both mouse and human spinal cord Pro-$NH_2$ formation is significantly decreased compared to cerebral cortex (Fig. 2). However, in mouse spinal cord significantly more TRH-OH and Pro formation occurs than in human spinal cord.

## DISCUSSION

We previously described the HPLC analysis of [$^3$H-Pro]TRH catabolism in murine spinal cord homogenates with fraction collection [12]. In this report we describe enhanced HPLC analysis of TRH metabolism in CNS tissues by the use of post-column detection with a radioactive flow detector. Optimal separation of TRH and its metabolites is achieved with ion-pairing reagents at an acidic pH. Both the ODS and PRP-1 column systems produced excellent separation of the peptides studied. However, we confirmed that the silane–silica bond in the ODS column system is easily hydrolyzed at acidic pH, producing column deterioration and decreasing reproducibility [42]. The PRP-1 columns permitted operation at acidic pH without breakdown of the column stationary phase over time and gave better separation of the products of TRH catabolism. This methodology increased the accuracy of metabolic measurements by (1) insuring that the products formed (i.e. cyclo(His-Pro)) were derived from TRH and not from another source [39,41] and (2) monitoring the metabolism of TRH at different times

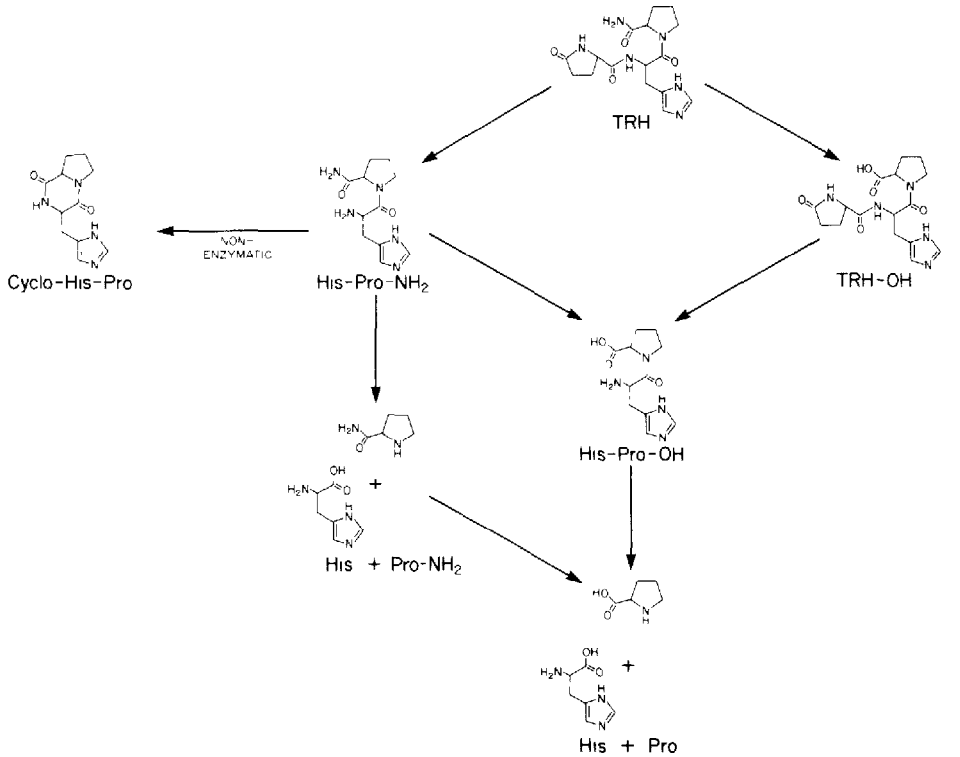


Fig. 3. TRH catabolism. Proposed catabolic pathways of TRH in cerebral cortex and spinal cord after Schwartz et al. [12] and Kreider et al. [27].

over a long incubation period when small product peaks, completely separated from the other produced peaks, could be identified as separate products.

We studied not only the production of TRH-OH and indirectly His-Pro-$NH_2$ but also the secondary metabolism of these compounds to His-Pro, Pro-$NH_2$, and proline. This secondary metabolism is important because of the possible bioactive functions of His-Pro [2,4,38] and cyclo(His-Pro) [17,37,40].

CNS TRH catabolism can involve many reactions due to enzymes located in different cellular compartments (membrane particulate, cytoplasmic soluble) which may have variable specificity for the pyroglutamyl-histidyl and His-Pro-$NH_2$ as well as the Pro-$NH_2$ bond (Fig. 3). The method decribed in this paper clearly delineated the differences in metabolism of exogenous TRH in spinal cord crude homogenates compared to cerebral cortex from mice and humans. Primary and secondary metabolic reactions were identified at each time point in one chromatographic run, permitting a means of determining regional differences in TRH catabolism. This method can be applied to the study of TRH catabolism in different buffer systems, different organ systems (cerebrum, cerebellum or spinal cord), different subcellular systems (membrane particulate or cytoplasmic soluble), or any combination of these aspects to more clearly define metabolic processing which may be optimized under different conditions.

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