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RESEARCH PAPER

Absorption of Thyrotropin-Releasing Hormone in Rats Using a Mucoadhesive Buccal Patch

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ABSTRACT

Mucoadhesive buccal patches were evaluated in vitro and in vivo using rats for release of thyrotropin-releasing hormone (TRH). TRH (10% w/w) was incorporated into mucoadhesive buccal patches that were custom coformulated with silicone and organic polymers (Dow Corning, Midland, MI) and its release profile was characterized in vitro using a modified Franz diffusion cell. TRH released into pH = 7.0 phosphate buffered saline at 37°C under sink conditions was detected using high-performance liquid chromatography (HPLC). Release of TRH in vitro from the buccal patches was rapid during the first 2 hr, with 51% of the total amount of TRH incorporated into the patches released after 24 hr. HPLC analysis indicated that TRH extracted from buccal patches thermally stressed at 40°, 55°, and 70°C showed negligible degradation after 6 months. In contrast, an aqueous TRH solution stored at 70°C showed degradation of TRH as soon as 10 days following incubation at this temperature. TRH patches placed on the buccal mucosa of anesthetized rats demonstrated rapid stimulation and release of thyroid-stimulating hormone (TSH) from the anterior pituitary. Thirty minutes after patch application, plasma concentrations of TSH fluctuated but remained approximately 4-

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to 7-fold greater than baseline (prior to patch application) TSH concentrations. Therefore, this preliminary study has demonstrated that physiologically active TRH was released from the TRH mucoadhesive buccal patches and systemically absorbed. Thus, the TRH mucoadhesive buccal patches may represent a convenient delivery system for therapeutic peptides.

INTRODUCTION

Due to recent advances in the biotechnological production of therapeutic proteins and peptides, there exists a need for successful delivery of these macromolecules by nonparenteral routes of administration. This stems from the significant limitations to delivery of these compounds by traditional routes of drug administration. As an example, the oral route of administration of protein drugs is limited due to poor absorption, the presence of lumenal and membrane proteolytic enzymes, and first-pass metabolism. Thus, other alternative routes of administration are currently being investigated. Some of these other routes of protein drug administration are buccal, sublingual, rectal, nasal, dermal, and vaginal.

Buccal administration of various proteins and peptides has previously been studied (1,2). While the surface area available for buccal absorption of drugs is limited, it offers other advantages. First, the buccal mucosa is readily accessible and a mucoadhesive patch may readily be applied and rapidly removed if desired. In addition, there exists the potential for modifying the local environment of the buccal mucosa by inclusion of protease inhibitors and penetration enhancers. Some examples of proteins and peptides that have been investigated for buccal administration include thyrotropin-releasing hormone, TRH (3), insulin (4), and interferon-α_2 (5), and calcitonin (6).

The present study was designed to evaluate an improved mucoadhesive buccal patch to deliver a physiologically active peptide in rats. The patch was fabricated from a custom mucoadhesive coformulation of silicone and organic polymers, and was supplied by Dow Corning. The peptide selected was TRH due to its well-characterized physicochemical properties and its exclusive use as a test compound for buccal administration. It has been reported that TRH is absorbed to a limited extent from the buccal mucosa of rats (7). TRH stimulates the release of prolactin and thyroid-stimulating hormone (TSH) from the anterior pituitary (3,8). Therefore, to assess whether physiologically active TRH is absorbed from an extravascular route of administration, plasma concentrations of prolactin or TSH are typically determined.

In addition, we also evaluated the release profile of TRH in vitro from the mucoadhesive buccal patches. Lastly, using liquid chromatography, we qualitatively assessed whether parent TRH extracted from buccal patches stored at 40°, 55°, and 70°C for up to 6 months was still present.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were used as received from Fisher Scientific (Chicago, IL) and all solutions were prepared using double-deionized (MilliQ) water. All TRH had a purity greater than 95% and was obtained from Sigma (St. Louis, MO). TRH (L-pyroglutamyl-L-histidyl-L-proline amide) had a molecular weight of 362, an apparent pK_a of 6.2 (9), and an octanol/water partition coefficient of 0.0376 at pH 7.4 (10). Cellulose acetate syringe filters (0.22 μm) were purchased from Fisher Scientific (Chicago, IL).

All high-performance liquid chromatography (HPLC) was conducted using a Waters 610 Fluid Unit pump, U6K injector, Nova-Pak C18 (3.9 × 150 mm) column, and a Model 486 tunable absorbence detector. The chromatographic peaks were automatically integrated and recorded via a Waters 746 data module (Waters, Milford, MA).

Fabrication of Mucoadhesive Buccal Patches

The buccal patches were fabricated by a solvent-casting technique. In brief, the mucoadhesive formulations were solvent cast onto a release liner as laminates. The laminates were allowed to devolatilize under ambient conditions for 30 min and then heated in an oven at 50°C for another 30 min. The drug–polymer matrix was sandwiched between the release liner and a backing membrane coated with a silicone adhesive layer. The laminates were then die cut to the approximate size and shape. Each buccal patch contained 10% w/w TRH. The schematic diagram of the patch is shown in Fig. 1.

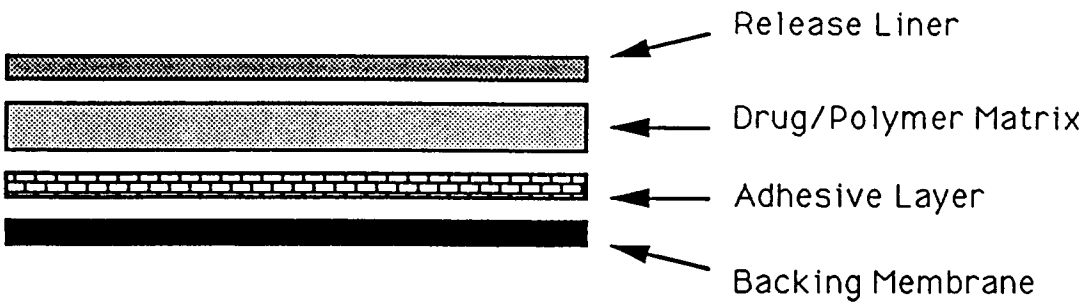

Figure 1. Schematic of the TRH mucoadhesive buccal patch.

HPLC Analysis of TRH

Reversed-phase high-performance liquid chromatography was used to quantitate TRH in aqueous solutions (11). The mobile phase consisted of 2% acetonitrile/0.01 M ammonium acetate with a flow rate of 1.5 ml/min. Absorbance detection was at 210 nm. All injection volumes were 10 µl.

A calibration curve was constructed following HPLC analysis of a series of TRH standard solutions. Concentrations of TRH in pH = 7.0 phosphate buffered saline (PBS) used for preparation of the calibration curve were 0.25, 0.50, and 0.75 mg/ml. These were assayed as described above. All TRH solution samples resulting from the in vitro release studies and assayed by HPLC employed a freshly prepared series of standard solutions (0.25, 0.50, and 0.75 mg/ml). The peak area for a given TRH solution sample was then compared to the calibration curve of peak area versus TRH concentration to determine the concentration of TRH in the solution sample. For elevated-temperature studies, only the values of the retention time for TRH and the presence of additional peaks were used to qualitatively assess whether parent TRH was still present and intact. This approach was selected with the elevated-temperature studies because it was not possible to extract all of the TRH initially incorporated into the polymer matrix once the system had cured. Peak area would only indicate the amount of TRH extracted from an individual buccal patch, which would be expected to vary.

In Vitro Release of TRH

A nine-station, thermostatted Franz cell system was used for assessment of the TRH released from all buccal patches. Five TRH buccal patches (surface area available for diffusion = 1.11 ± 0.03 cm^2) were placed on top of the receiving chamber of five separate cells. The mean weight (± standard deviation) of the five buccal patches were 178 ± 14.6 mg. The mean weight of the patches included the weight of the impermeable backing (≈30 mg/patch). The receiving chamber of each cell was filled with pH = 7.0 PBS and maintained at 37°C using a circulating water bath. Sink conditions were maintained in the receiving chamber during the release study. A 0.2-ml sample of the receptor solution contained in the receiving chambers was obtained through the sampling side arm at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hr following placement of the patches on the apparatus. Each sample was replaced by an equal volume of PBS at all time points. All collected samples were assayed by HPLC as described above.

The cumulative amount of TRH released at time t was calculated using the following equation:

$$M_n = C_n \cdot V_t + \sum_{i=1}^{n-1} C_i \cdot V_s$$

where M_n is the amount of TRH released at the nth sampling point, C_n is the concentration of TRH in the solution sample as determined by HPLC, V_t is the total volume of receptor phase, C_i is the concentration of the sample at the ith sampling time point, and V_s is the sampling volume at each time point. The amount of TRH released per unit area was then calculated at each time point.

Elevated-Temperature Studies

These studies were conducted to determine qualitatively whether parent TRH incorporated in the polymer matrix was still present when incubated at elevated temperatures. Each of nine buccal patches was initially cut into four even-sized pieces. The TRH patch sections were individually placed inside 36 15-ml glass vials.

Nine vials representing three different TRH buccal patches were then placed in a 40°C incubation cabinet, a second set of nine vials were placed in a 55°C incubation cabinet, and a third set of 15 vials were placed in an incubation cabinet maintained at 70°C. The remaining three sections of three different patches were used to obtain an overall chromatogram for TRH at time $t = 0$. At predetermined time points up to 6 months, three vials were removed from the 70°C incubation cabinet to assess whether significant changes had occurred in the overall appearance of the chromatogram relative to both the chromatogram obtained at time $t = 0$ and chromatograms observed with fresh TRH solutions prepared and analyzed that same day. For comparative purposes, we also conducted an experiment in which TRH was dissolved in PBS and stored at 70°C for varying time periods.

Extraction of TRH from buccal patches incubated at elevated temperatures was performed by adding 7.0 ml of PBS to each vial after removal from the incubator. Vials were then placed on an orbit shaker and rotated at 150 rpm for 24 hr. Each solution was then filtered through a 0.22-μm filter and the filtrate analyzed for TRH using HPLC as described above. Previous studies had shown that TRH was not lost when filtered through the 0.22μm filters. In addition, previous pilot studies had demonstrated no disappearance of TRH dissolved in PBS when stored at room temperature for 72 hr.

Evaluation of TRH Release In Vivo

This study was conducted to determine whether TRH administered to anesthetized rats using the buccal patches still retained biological activity. Rats (male, Sprague–Dawley, 200–250 g) were obtained from Harlan Laboratories (Indianapolis, IN). The animals were housed individually under controlled conditions for at least 2 weeks prior to experimentation. During this time the animal room was maintained at a temperature between 21° and 23°C. Rats were exposed to a standard 12-hr light–dark cycle (light on 0700–1900). Animals were provided unlimited access to a diet of Purina chow and water. At the time of the experiment, the mean weight of the animals was 284 ± 14.6 g.

To assess whether the buccal patches would deliver physiologically active TRH, we determined the plasma concentrations of TSH in anesthetized rats following application to the buccal mucosa. Each buccal patch was cut to a diameter of approximately 0.6 cm to obtain a target dose of 1 mg of TRH per patch. Prior to patch application, each rat was anesthetized with sodium pentathol (Abbott Laboratories, North Chicago, IL) by intraperitoneal injection of 0.24 ml of a 50 mg/ml solution. Five minutes following induction of anesthesia and prior to patch application, the terminal portion of the tail of each rat was clipped to obtain a 1.0-ml blood sample. The zero-time blood samples represented the normal levels of TSH expected for an anesthetized rat. Application of the TRH patches was performed by first moistening the buccal mucosa with a cotton swab application and then placing the patch on the moistened mucosa for approximately 1 min. All patches were in contact with the buccal mucosa throughout the 4-hr study. Once the patch had adhered to the mucosa, blood samples were obtained at 0.5, 1.0, 1.5, 2.5, and 4.0 hr. All blood samples collected were then centrifuged, the plasma retrieved, and the plasma frozen at -70°C until the time of analysis. The plasma samples were not assayed for TRH because the estimated plasma of TRH concentrations based on literature values for the bioavailability of TRH following buccal administration would have been below the detection limit of our assay (3,8).

Rats were injected with sodium pentobarbital as needed throughout the 4-hr study to maintain a light plane of anesthesia. No attempt was made to determine the amount of TRH remaining in each buccal patch following the in vivo study since our primary objective in this initial feasibility study was to determine whether any TRH still retained biological activity following system absorption. To eliminate the possibility that released TRH was absorbed from the gastrointestinal tract, a small pledget of cotton was placed towards the back of the oral cavity of each rat during the study. The cotton pledget served to absorb any secretions which could potentially contain TRH. Analysis of TSH in rat plasma was performed by radioimmunoassay (Hazelton Washington, Inc., Vienna, VA). Plasma concentrations of TSH determined at each time point for all rats were averaged and the data expressed as the mean ± SE plasma TSH concentration versus time.

RESULTS

In Vitro Release of TRH

The release of TRH from the buccal patches in vitro is illustrated in Fig. 2. Release of TRH proceeded very rapidly during the first 2 hr. At later time points, the release rate decreased, with the total percent of TRH released at 24 hr being 51% of the initial amount of TRH incorporated into the buccal patches.

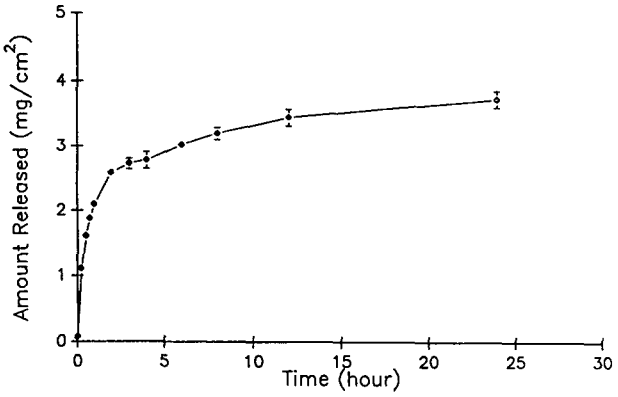

Figure 2. The amount of TRH released in vitro from TRH-containing mucoadhesive buccal patches normalized for the surface area in contact with the receptor phase. All values are the mean ± SEM of five patches.

Thermal Stress of TRH Buccal Patches

The qualitative changes in the chromatograph of a TRH solution incubated at 70°C for 3 months is shown in Fig. 3. It can be noted in Fig. 3 that the retention time for TRH increases from approximately 5.4 min at time t = 0 months to 6.0 min at 3 months. Additionally, a new peak can be noted in the resulting chromatograms at approximately 3.4–3.6 min which was observed to increase in height (area) from 0.33 3 to 3 months. In contrast, TRH which had been incorporated and subsequently extracted from the TRH buccal patches stored at 70°C for up to 6 months showed no change in either the retention time for TRH or the overall appearance of the chromatogram (data not shown).

TRH Absorption In Vivo

Figure 4 demonstrates that physiologically active TRH was systematically absorbed following application of the TRH patches to the buccal mucosa of anesthetized rats. A greater than 3-fold increase in the plasma concentration of TSH was noted as soon as 0.5 hr following application of the mucoadhesive TRH patches. At later times, plasma concentrations of TSH fluctuated but remained approximately 4- to 7-fold greater than the baseline TSH plasma concentration of 3.02 ± 0.57 ng/ml determined from blood samples collected prior to patch application. It should be noted that placebo patches (no TRH) applied to the buccal mucosa throughout the 4-hr study resulted in plasma TSH concentrations within 1 standard deviation of the mean plasma TSH concentration determined prior to patch application (data not shown).

DISCUSSION

We have demonstrated in the present study that TRH incorporated into the mucoadhesive buccal patches is still biologically active following application to the buccal mucosa of anesthetized rats. Previous studies using TRH as a model tripeptide for buccal delivery have demonstrated poor overall bioavailability in humans and rodents. In humans, the availability following buccal administration of TRH is approximately 1–5% compared to TRH administered intravenously (3). In rats, the bioavailability is approximately 1% or less for TRH administered buccally (12,13). However, our overall objective in this investigation was to determine if any of the TRH initially incorporated into the patches was still biologically active following buccal administration in the rat. Our results would suggest that physiologically active TRH was absorbed into the systemic circulation of anesthetized rats.

Buccal absorption of biologically active TRH from the mucoadhesive patches would suggest the potential of these patches for delivery of other peptides known to be absorbed through buccal mucosa. While no attempt was made in the present study to elucidate the mechanism by which TRH was absorbed following its release from the patches, the tripeptide may have been absorbed by simple diffusion. Dowty et al. (13), using excised rabbit mucosa, demonstrated that TRH apparently traversed buccal mucosa by simple diffusion with a steady-state permeability of approximately 10^{-7} cm/sec, and that the permeability was independent of pH. In addition, these same authors suggested that the primary pathway of TRH absorption across buccal mucosa was the intercellular space in the rate-limiting barrier, which for rabbit mucosa was the upper 50 μm of the epithelium (13). While there are distinct differences in rabbit and rat buccal mucosa, particularly the fact that rat buccal mucosa is keratinized, the mechanism of TRH transport may still be similar. Selection of the anesthetized rat model for evaluation of our TRH buccal patches was based on convenience, cost, and comparative purposes, since others have evaluated buccal absorption of TRH using this model (14).

The present study would also suggest that TRH extracted from patches incubated at elevated temperatures for up to 6 months did not undergo significant alterations in chemical structure. If thermal stress had in-

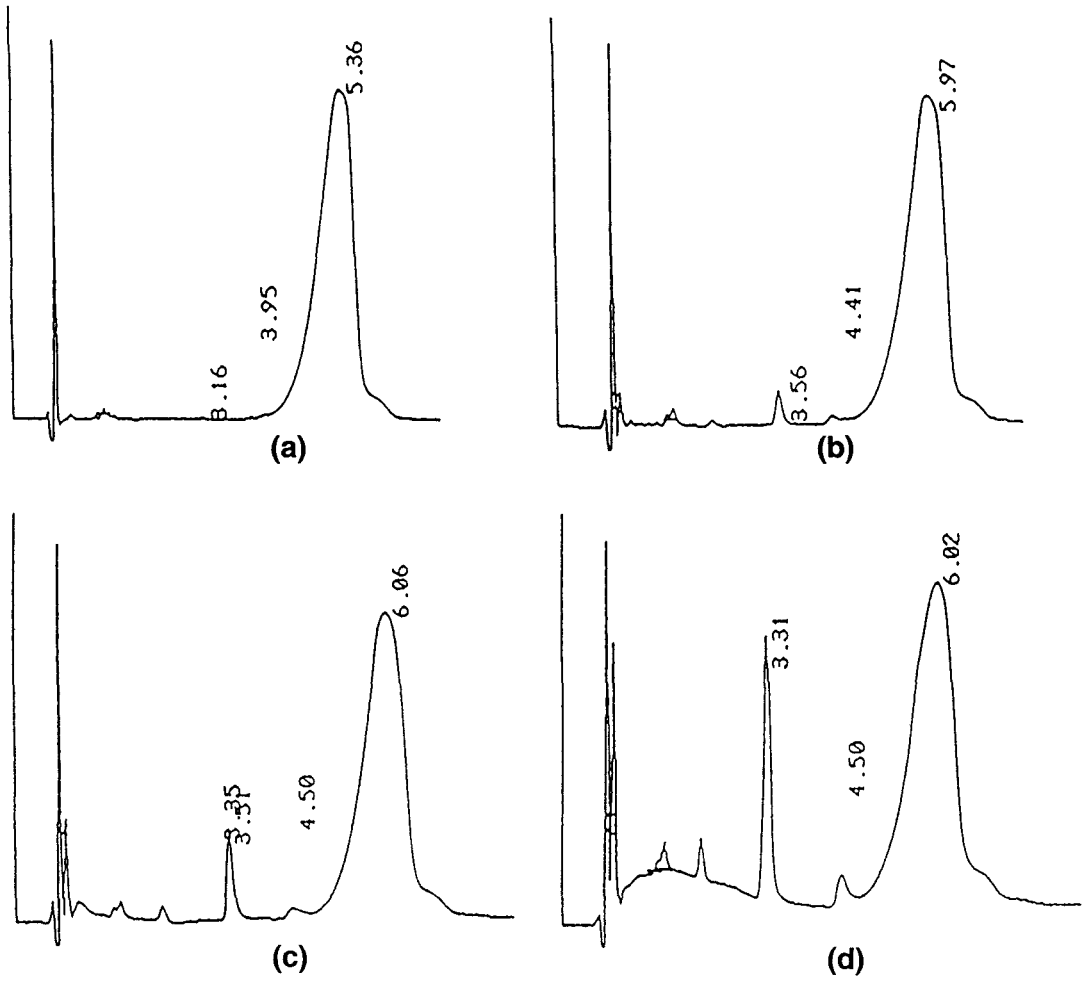

Figure 3. Qualitative changes observed in the chromatograms for a TRH solution incubated at 70°C. (a) 0.0 months, (b) 0.33 months, (c) 1 month, and (d) 3 months.

duced fragmentation of the tripeptide, we would have expected to have detected additional peaks representing smaller molecular weight species. Smaller molecular weight fragments such as pyroglutamic acid, histidine, or prolinamide resides are typically observed when bonds are cleaved in the parent molecule. In contrast, TRH in solution demonstrated an additional peak in the chromatogram as soon as 10 days following incubation at 70°C. However, it should be noted that others have demonstrated that incubation of a TRH solution (1 mg/ml in water, pH = 6) for 20 hr at 80°C did not result in any fragments detectable by HPLC assay (15). Presumably, chromatograms for TRH solutions obtained after extraction of TRH from the buccal patches may have resembled the chromatograms for the TRH solution had an aqueous environment been present within the patch matrix. It has been reported that fragmentation of TRH in solution involves lactam ring (pyroglutamic acid) opening, peptide bond hydrolysis, oxidation of the histidine segment, racemization of any three asymmetric carbon atoms, and/or any combination of the above (15). These same authors (15) have shown that the primary degradation products of thermally 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). In situ, degradation of TRH occurs primarily

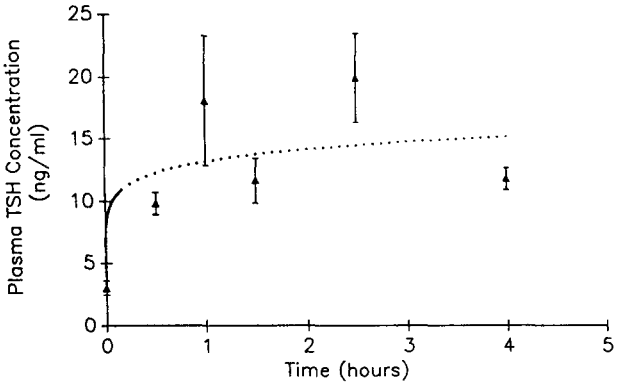

Figure 4. Plasma thyroid-stimulating hormone (TSH) concentrations following application of TRH mucoadhesive buccal patches in anesthetized rats. Time $t = 0$ hr represents baseline (prior to patch application) TSH concentrations in rats. All values represent the mean ± SEM of five rats. The dotted line represents a mathematical fit of the data using a standard power function of the form $TSH_{conc} = a(\text{time})^b$, where a and b are constants.

by deamidase activity, which is followed by, to a lesser degree, carboxypeptidase metabolism (13).

Merkle et al. (7), using buccal patches containing TSH demonstrated that plasma levels of TRH were elevated in rats 15 min after a 30-min patch application period and remained elevated for the duration of their 90-min study. We observed elevations in plasma levels of TSH in approximately 15 to 30 min following application, with elevated plasma concentrations of TSH remaining throughout the 4-hr application period. The fluctuations in plasma TSH concentrations in our study might have been due to stress, anesthesia, or endogenous fluctuations in TRH and/or TSH. It has been reported that pretreatment of rats with tri-iodothyronine leads to a strong reduction of endogenous TRH and TSH (7). However, we did not pretreat our rats with tri-iodothyronine. Nevertheless, it is apparent from our experiments that physiologically active TRH was released and absorbed into the systemic circulation of anesthetized rats. Future studies in our laboratory will be directed at determining whether any gross damage or histological changes occur in the buccal mucosa of rats and other species following application and removal of the mucoadhesive buccal patches.

In conclusion, we have demonstrated that release of TRH in vitro from the mucoadhesive buccal patches was rapid (≈37% at 2 hr) and continued for the duration of the release period (≈51% at 24 hr). Chromatograms for TRH solutions obtained by extracting TRH from buccal patches incubated at elevated temperatures for up to 6 months were not qualitatively different than chromatograms obtained from freshly prepared TRH solutions. Lastly, physiologically active TRH was released and absorbed systemically from TRH buccal patches which had been applied to the buccal mucosa of anesthetized rats. Therefore, the current biocompatible buccal patch may represent an alternative nonparenteral route of administration for other peptides known to diffuse across buccal epithelium. In addition, pharmaceutically acceptable enhancers and protease inhibitors could potentially be incorporated with a particular peptide to enhance buccal transport of the intact peptide.

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