Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 1, pp. 9–18, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00

Development of a radioimmunoassay for RX77368 (*p*Glu-His-3,3-dimethyl proline amide) — a stable analogue of thyrotrophin releasing hormone (TRH)

ANDREW G. LYNN*† and GEOFFREY W. BENNETT‡

†*Analysis Department, Reckitt and Colman plc, Pharmaceutical Division, Hull HU8 7DS, UK*
‡*Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK*

Abstract: The recent interest in RX77368 for the treatment of Motor Neurone Disease (MND) has led to the requirement for an assay (RIA) capable of detecting the peptide at low levels in plasma. Several drug conjugates were prepared in which RX77368 was covalently linked to larger proteins, e.g. bovine serum albumin, keyhole limpet haemocyanin or bovine thyroglobulin, the best yield being obtained with the bis-diazotized benzidine reaction (BDB) linking RX77368 to KLH. The latter conjugate was injected into sheep and ultimately produced an antibody of sufficiently high titre to be used. This combined with an iodinated radiolabel formed the basis of the radioimmunoassay.

Cross-reactivity studies using similar analogues and RX77368 metabolites showed that the antibody was specific for RX77368. The greatest cross-reactivity was exhibited by the *p*Glu-His-monomethylProNH$_2$ peptide (RX74355), but, not being a natural metabolite, this did not interfere with the assay. The RIA was used to measure RX77368 in MND patients in a recent clinical study, where RX77368 was administered both by the intravenous and oral routes. High plasma concentrations of RX77368 were found in the patients given intravenous drug by infusion. The oral route exhibited much lower levels, but had a sustained duration of action of up to 12 h.

Keywords: *TRH; TRH analogues; RX77368; RIA.*

Introduction

The tripeptide RX77368 (*p*Glu-His-dmProNH$_2$), a potent, stable analogue of thyrotrophin releasing hormone (TRH), has always been in demand by investigators in peptide research since it was first synthesized. Primary interest in TRH analogues as pharmacological tools reached a peak in the mid–late 1970s when several TRH peptides were tested as antidepressants in patients, with limited success. Recently, there has been a resurgence of interest in other therapeutic areas, associated neurodegenerative diseases, including motor neurone disease (MND). Experimental evidence suggests that RX77368 may be of benefit in the treatment of a wide range of disorders associated with ageing, for example, MND, Alzheimer's and Parkinson's disease, as well as spinal shock and stroke.

RX77368, a stabilized analogue of TRH, is of interest because it possesses a greater degree of biological stability than the parent TRH peptide and other analogues (see Fig. 1). The peptide resists degradation by plasma enzymes and brain tissue, resulting in half-lives in man of 1080 and 168 min, respectively [1]. The peptide displays a range of CNS potencies from 14 to 200 times that of TRH with little or no change in the endocrine profile. The effect of RX77368 is currently being studied in MND patients [2, 3].

The interest in RX77368 and its drug profile in plasma from clinical studies has meant that an assay for the peptide was required. As TRH itself is assayed successfully by several groups using a radioimmunoassay, RX77368 was initially tested and confirmed not to cross-react in a TRH RIA [4].

The present study describes the development of an RIA for RX77368, from the preparation of the immunogen to the assay of plasma samples in various clinical studies.

Materials

All TRH and related RX77368 peptides were obtained from Reckitt and Colman (Hull, UK) or as follows: TRH, Sigma (Poole, Dorset, UK); the peptides MK-771 (MSD),

* Author to whom correspondence should be addressed.

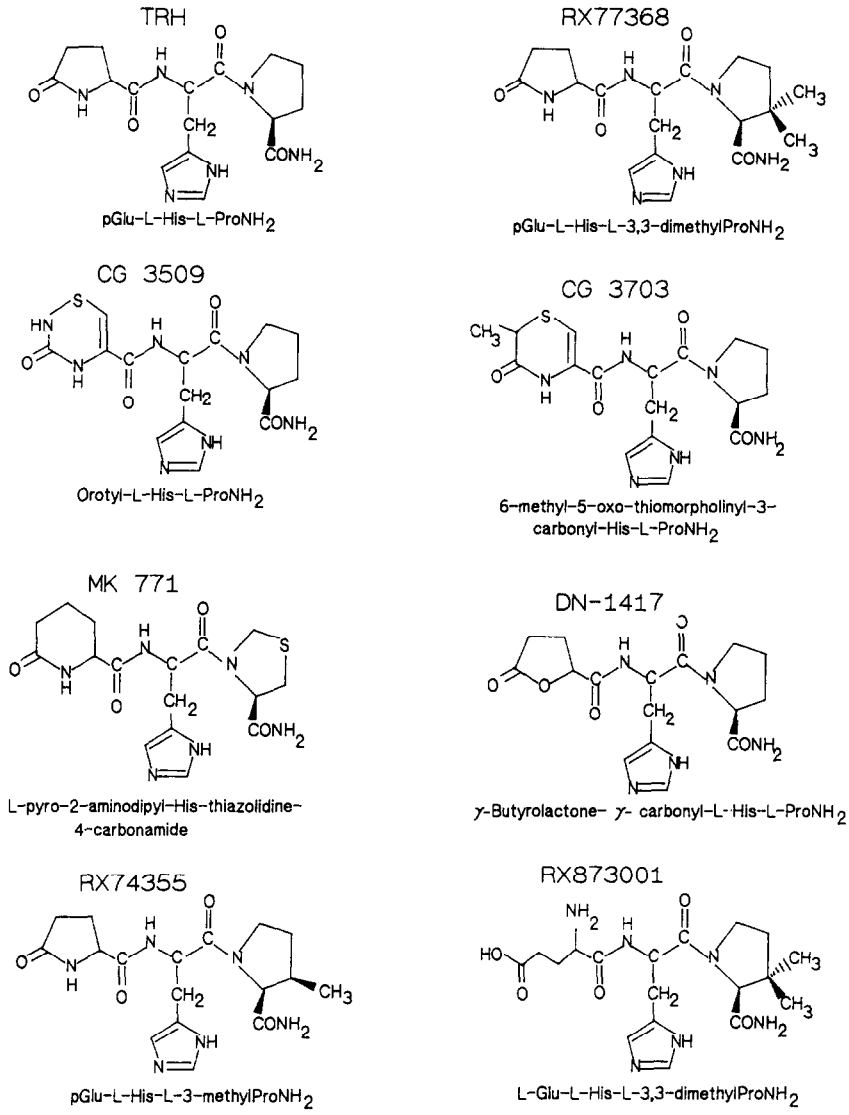

Figure 1
TRH, RX77368 and some closely related analogues used in the cross-reactivity studies.

CG3509 and CG3703, Grunenthal GBDH; reagents for the conjugation reactions were also obtained from Sigma; the ^{3}H-TRH and ^{3}H-RX77368 radiolabels were obtained from Dupont (UK) Ltd (Herts, UK) and Cambridge Research Biochemicals (Gadbrook Park, Northwich, Cheshire, UK), respectively.

Dialysis was carried out in 8/32″ and 24/32″ visking tubing (cut-off 12–14,000 mol. wt) obtained from Medicell International (London, UK).

Freund's (incomplete and complete) adjuvant was obtained from Sigma (Poole, UK). The rabbit immunizations were carried out with a non-ulcerative adjuvant (Morris) obtained from Guildhay Antisera Ltd (Surrey, UK) with the complete version prepared using a BCG vaccine [BP, BNF (intradermal), Glaxo] obtained from Evans Medical (Horsham, UK).

The iodination procedure was carried out by the iodo-gen method of Fraker and Speck [5]. The NaI^{125} being obtained from Amersham International (Bucks, UK).

Rabbits — New Zealand White, Full Barrier Bred were obtained from Interfauna UK Ltd. Sheep — the ewes (second year lambs) used in the study were supplied by the School of Agriculture (Sutton Bonington, Nottingham University, Nottingham, UK).

SAC-CEL second antibody was obtained from IDS (Washington, Tyne and Wear, UK).

Methods

Preparation of RX77368 immunogen

Various methods were used to attempt to link RX77368 and/or TRH to proteins. The proteins used in the study were bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH) and bovine thyroglobulin (BTG).

(a) *Glutaraldehyde.* This conjugation was carried out according to the method of Reichlin *et al.* [6], where ACTH was coupled to BSA. Both RX77368 and TRH were used, as well as the preparation of a non-protein control for incorporation determination.

(b) *Water soluble carbodiimides.* Two methods were used to link the proteins and peptides using the reagent 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (Ethyl-CD1).

The first method was based on that of Goodfriend *et al.* (1966) [7], where rabbit serum albumin (RSA) was coupled to angiotensin. Essentially, the three reagents were mixed in a small volume and incubated for 1–12 h, or until a colloidal precipitate indicated the end point.

The second method was essentially the same as Orth (1979) [8], where, again, ACTH was coupled to BSA. The RX77368 and TRH peptides were linked to all three proteins.

(c) *Difluorodinitrobenzene (DFDNB).* This procedure was based on that of Tager [9] and modified by Visser *et al.* [10] using the bifunctional reagent 1,5-difluoro-2,4-dinitrobenzene (DFDNB). The method is carried out in essentially two stages with firstly, the preparation of the active peptide, and secondly, the coupling of the peptide–FDNP complex to the protein.

(d) *Bis-diazotized benzidine (BDB).* The method used was essentially that of Bassiri and Utiger [11] as modified slightly by Sharp [12]. The modification employed a reduction in the volume of BDB reagent added to the protein (BSA) and peptide mixture. RX77368 and TRH were linked to BSA, KLH and BTG, using this procedure. Again, this method was carried out in two stages, with firstly, the preparation of the benzidine, and secondly, the linkage of the protein and peptide under alkaline conditions.

All products from the above conjugation procedures were freeze-dried and stored at −20°C.

In addition, the rate of incorporation in all reaction schemes was similarly tested. The TRH and RX77368 reactions were followed by addition of the appropriate tritiated ligand to the reaction mixture, and examination of the dialysate for activity, following dialysis for 24 h. Details of the incorporation rates are shown in Table 1.

Immunization of RX77368 conjugates

Sheep (2-year-old ewes). Each protein–RX77368 conjugate was resuspended in 2 ml of double distilled water, but due to solubility problems with the RX77368–KLH from both the DFDNB and BDB procedures, the mixtures were sonicated into a fine suspension using a soniprobe. For each conjugate, aliquots were prepared providing a 1-ml aliquot for the primary injection, and 6 × 0.4 ml aliquots for subsequent booster injections. The booster aliquots were stored at −80°C.

The primary injection consisted of 1 ml of protein–RX77368 conjugate plus 3 ml complete Freund's adjuvant, which was briefly sonicated to a stable emulsion. Each sheep received 1 ml aliquots of adjuvant intramuscu-

Table 1
Summary of conjugation procedures

Conjugation method	RX77368			TRH		
	BSA	KLH	BTG	BSA	KLH	BTG
Glutaraldehyde [6]	3.7	—	—	9.3	—	—
Water soluble [7]	15.8	—	—	1.9	—	—
Carbodiimides [8]	3.0	0	1.2	11.5	5.0	—
Difluorodinitrobenzene [9, 10]	33.1	33.6	51.6	34.3	33.0	—
BDB [11, 12]	16.0	19.6	12.8	5.3	17.2	0

Proteins used in the conjugation studies were bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH) and bovine thyroglobulin (BTG). Dialysates were analysed by liquid scintillation (^{3}H) after 24 h. Incorporations were expressed as a percentage of the tracer retained compared with a non-protein control.

larly and subcutaneously over three to five sites. Subsequent booster injections were given approximately monthly (1–8) and consisted of 0.4 ml RX77368 conjugate in 2 ml incomplete Freund's adjuvant, sonicated to a stable emulsion. Injections were administered subcutaneously with approximately 1.2 ml over three to five sites. Blood samples were obtained (20 ml) and allowed to clot overnight at 2–8°C. The blood was then centrifuged and the sera stored at 2–8°C with a preservative (sodium azide, 0.1 mg/100 ml) added prior to testing.

Rabbits. For immunization in rabbits the adjuvant was prepared according to the method of Herbert [13] using a non-ulcerative version (Morris) of Freund's adjuvant. Six rabbits were immunized, with 500 μg of conjugate (1 ml adjuvant) injected intradermally over eight to ten sites.

Booster injections were carried out at monthly intervals. Blood samples (2 ml) were obtained from the ear vein for determination of anti-RX77368 activity.

Analysis of test bleeds for anti-RX77368 activity

Test sera from sheep were analysed using various radiolabels, i.e. ^{125}I-RX77368, ^{3}H-RX77368 and ^{3}H-TRH.

Dilutions of the test sera were prepared in phosphate buffer (0.1 M, pH 7.0 with 0.1% BSA), from 1:20 through to 1:10,240 in the early samples, and 1:320 to 1:81,920 for the later test bleeds. Samples of sera (100 μl) were incubated with radiolabel (100 μl, 20,000 cpm) and buffer (100 μl) for 24 h at 2–8°C. When the titres began to increase, as with later sera, the incubations were set up at room temperature for 2 h. A comparison of both incubation procedures showed that there were no differences in amounts of anti-RX77368 activity between the methods, suggesting that the equilibrium state had been reached after the shorter incubation time. Therefore, all subsequent assays (dilution curves and cross-reactivity studies) were carried out at room temperature for 2 h. Antibody-bound and free peptide were separated using an anti-sheep or anti-rabbit solid phase second antibody (SAC-CEL), according to the protocol of the supplier.

Radioiodination of RX77368

The iodination procedure was carried out according to the iodo-gen method. Iodo-gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril), which is virtually insoluble in aqueous media, forms a solid phase on the surface of the tube when added via a suitable solvent, and is then evaporated off. The RX77368 peptide, sodium iodide (^{125}I, 0.3 mCi) and buffer were added quickly to the previously coated (iodo-gen) tube and the reaction allowed to proceed at room temperature for 60 min. The reaction was terminated by removal of the reaction mixture and adding to a C18 Sep-pak cartridge. The column was washed with Analar water and eluted with a mixture (2 ml) of acetonitrile–water (70:30, v/v) and 0.08% trifluoroacetic acid. The resultant fraction was diluted accordingly to give approximately 20,000 cpm/100 μl in phosphate buffer (0.1 M, pH 7.2, with 0.1% Triton X-100 and 0.01% preservative) for use in the assay.

RX77368 assay development

Standards to RX77368 were first prepared in a buffer based system by dilution of a stock 1 mg ml^{-1} solution of the peptide to give a range of concentrations from 1 ng ml^{-1} to 100 μg ml^{-1}. This range was extended later in the assay development to include dilutions from 0.1 ng ml^{-1}. Aliquots (100 μl) of antiserum, radiolabel (20,000 cpm/100 μl) and standard or sample (100 μl) were vortex mixed and incubated at room temperature for 2 h, and the bound and free radiolabel separated as described above.

The assay was modified such that the RX77368 series of standards was prepared in a human control plasma matrix, again with a 2 h room temperature incubation. The human control plasma was separated from whole blood within 18 h of donation as supplied by the Yorkshire Blood Transfusion Service (Leeds). The plasma was suitable for laboratory purposes only.

Cross-reactivity studies were carried out with a series of related TRH analogues and some major metabolites of RX77368. Dilutions were prepared in plasma over the range 1 ng ml^{-1} to 100 μg ml^{-1}, and assayed in a similar way to the RX77368 standards previously described in the text. Calibration curves were plotted and compared with the RX77368 data.

Assay of clinical samples

Plasma samples were obtained from the intravenous and oral phases of a recent clinical

study. The intravenous pilot study was carried out on two patients using a low dose (0.2 and 0.25 mg kg^{-1}) over a 2 h infusion. The oral study was carried out in a larger group of MND patients with increasing doses of the peptide administered at intervals of at least 48 h. Assays were carried out on samples following 0.7, 1.4 and 3.0 mg kg^{-1} RX77368. Some samples were diluted over the range 1:2 to 1:100 in control plasma to ascertain the relationship between concentration and dilution. The samples and dilutions were assayed in the standard RIA described above.

Assay sensitivity

The assay sensitivity was determined from multiple replicates of the B_o dose. The data was subjected to analysis by the Packard (United Technologies) PC-DAAS curve fit program, where the mean B_o + 2 SD cpm level was interpolated into a peptide concentration, using the assay standard curve. This procedure was carried out for each individual assay and typically was less than 100 pg ml^{-1}.

The highest standard was considered to be 333.3 ng ml^{-1} since greater peptide concentrations exhibited % B/B_o data similar to or less than the non-specific binding (NSB, which was in the region of 1.5%). The proposed working range of the assay was thus 0.1 ng ml^{-1} (2.55×10^{-10} M) to 333.3 ng ml^{-1} (0.85×10^{-6} M).

Results

Immunogen preparation

The combined results for the conjugation reactions carried out are shown in Table 1. The major reaction schemes, and the percentage incorporations of peptide, are shown. Consistent incorporation was obtained with the DFDNB reactions for each of the proteins used. Incorporation rates of 33.6 and 51.6% were obtained for RX77368–KLH and RX77368–BTG respectively. Both these conjugates were used for immunization of sheep. A third conjugate used exhibited an incorporation of 19.6% using the BDB reaction and this proved to be the most successful conjugate with respect to the production of antibodies and went on to provide the basis for the radioimmunoassay.

Antiserum dilution curves

Sheep. Two sheep were immunized with each of the RX77368–KLH (benzidine bridge), RX77368–KLH and RX77368–BTG (dinitrophenol bridge) conjugates. Bleeds were primarily evaluated over the range 1:20 to 1:10,240 (serial dilutions of the sera in phosphate buffer, pH 7.0, 0.1 M). Later bleeds were evaluated over an extended range reflecting their increased antibody activity.

Titres were obtained from an approximate ED_{50} cpm level derived from each individual curve, and interpolated into dilutions. Sera were harvested and tested throughout the period of 9 monthly bleeds. The programmes for all but the RX77368–BDB–KLH immunized sheep were terminated early due to the lack of antibody production. The later test sera, after booster numbers 7–9, of both sheep injected with the latter conjugate, showed a high titre response in the region of 1:6000 to 1:12,000 (final antibody concentration).

Rabbits. The conjugate (BTG–Glu-His-3,3-dmProNH_2) was prepared using the carbodiimide synthesis of Orth [8].

Rabbits were immunized with the BTG–Glu-His-3,3-dmProNH_2 and with the RX77368-dnp–KLH conjugates. Analyses were carried out in a similar way to the sheep antisera, anti-rabbit SAC–CEL being used in this case for effecting the separation. After the final boost the % B/T values were high for two out of the three rabbits immunized with the BTG conjugate. One rabbit (R.0745) appeared to have a better dilution profile and showed a final antibody titre in the region of 1:4000.

Cross-reactivity studies

Using sera obtained from sheep 1, a 1:2000 dilution was prepared in 0.01 M phosphate buffer pH 7.0 (final antibody concentration of 1:6000). Radiolabelled ^{125}I-RX77368 was prepared to give approximately 20,000 cpm/100 µl. The assay was set up as described previously for a 2 h incubation at room temperature. The majority of cross-reactivity assays were performed using a human control serum as the carrier matrix (described earlier), in which the analogues and metabolites were prepared.

The cross-reactivity data were analysed in a similar way to that described by Franchimont *et al.* [14].

Standard curves for RX77368 in the presence of control plasma are shown in Fig. 2, where they are compared with the analogues

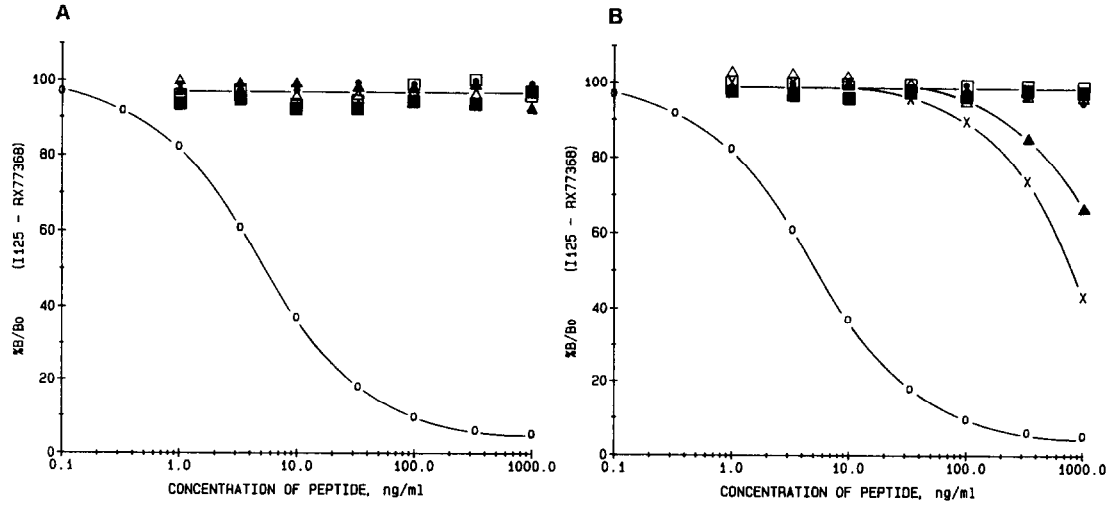

Figure 2
Cross-reactivity studies with metabolites of RX77368 (A) and TRH analogues (B) using sheep 1 antibody, compared with RX77368 (○). (A) RX77368-OH (△). *p*Glu-(D)-His-3,3-dmProNH$_2$ (▲), L-dmProNH$_2$ (□), His-dmProNH$_2$ (●), His-dmPro (DKP,■). (B) RX74355 (X), MK-771 (●), TRH (□), Glu-His-3,3-dmProNH$_2$ (▲), CG-3509 (△), CG-3703 (■). All assays were carried out using a 1:6000 dilution of antibody. Incubations were established at room temperature for 2 h.

and metabolites used in the study. The analogues and metabolites were studied over the range 1 ng ml^{-1} to 100 μg ml^{-1}. The standard curves for RX77368 are expressed as the mean from five or seven assays (respectively in A and B). The % B/B_0 values are very similar and showed very good correlation with all the range of concentrations used in the assay. The mean relative standard deviation (RSD) for all the concentrations is about 10%. The standard curve used in the assay has a working range of 0.1–333.3 ng ml^{-1}, giving % B/B_0 values from 97.3 to 6.4% for the above range. The NSB is in the region of 1.5%. The curve fits derived from the Packard crystal gamma counter are fitted according to a spline function and typically one to two iterations are obtained (one to three iterations demonstrates a good curve fit, whereas four to six iterations yield a poor curve fit).

The structures of RX77368, and some of the TRH analogues used in these studies, are shown in Fig. 1. The results show that the cross-reactivities of the analogues are negligible compared with the profile of RX77368. The only peptide to show any appreciable cross-reactivity was RX74355, *p*Glu-His-monomethyl proline amide, which is the nearest structural compound to RX77368. The cross-reactivity data compared with RX77368 are summarized in Table 2. The values of the 50% binding level have been interpolated into

Table 2
Cross-reactivity studies

	Concentrations at ED_{50} % Binding (ng)	Comparison with RX77368 (%)
RX77368	5.2	100
RX74355	800	<0.01
Glu-His-dmProNH$_2$	2350	<0.01
TRH	11,000	<0.001
MK-771	18,000	<0.001
CG-3509	>100,000	<0.001
CG-3703	>100,000	<0.001
L-dmProNH$_2$	>100,000	<0.001
His-dmProNH$_2$	>100,000	<0.001
*p*Glu-His-3,3-dmPro-OH	>100,000	<0.001
*p*Glu-(D)His-3,3-dmProNH$_2$	26,000	<0.001
His-dmPro (DKP)	>100,000	<0.001

Analogues and metabolites were assayed in the standard RIA (sheep antibody 1, incubated at room temperature for 2 h) over the range 1 ng ml^{-1} to 100 μg ml^{-1}. All compounds were prepared in human control plasma. Cross-reactivity was determined as described in the text.

concentrations from the individual graphs. The amount of analogue (ng) required to displace the RX77368 at this 50% level and the relative cross-reactivity compared with RX77368 (100%) are shown.

The curves displayed in Fig. 2A showed that the metabolites do not cross-react with the sheep 1–RX77368 antibody. Over the range of the RX77368 standard curve the metabolites all show zero cross-reactivity.

Similarly, all the peptides, some of which are presently being studied in the clinic, show very little cross-reactivity until concentrations well in excess of 1000 ng ml^{-1} are reached (see Fig. 2B).

Effect of first incubation time on the RX77368 assay

RX77368 standard curves were established in plasma over the range 0.1–333.3 ng ml^{-1}, and incubated for various times prior to the separation stage. This first incubation was carried out for 30, 60 and 120 min. All other treatments were essentially the same.

Somewhat surprisingly, the B/B_0 standard curves were almost superimposable (Fig. 3A). The greatest effects were observed with the 2 h incubation, with the largest differences occurring between the 0.1–10 ng ml^{-1} concentrations.

Effect of dilution of antibody on the standard curve to RX77368

Various dilutions of sheep 1 antibody were set up in RX77368 standard curves from 0.1 to 333.3 ng ml^{-2}. Dilutions of the antibody used were 1:6000 (standard solution), 1:8000, 1:10,000 and 1:12,000 final antibody concentrations, and incubation at room temperature for 2 h.

As expected, the amount of label bound was reduced with dilution of the antibody, as shown in Fig. 3B. However, the % B/B_0 data do not appear to be affected a great deal by the antibody dilution, with the standard curves for each of the dilutions being virtually superimposable.

Assay of plasma samples from a recent clinical study

Once a reproducible assay had been established the next stage was to carry out RX77368 determinations in clinical samples. Samples of plasma were obtained from a recent RX77368 clinical study at Westminster Hospital (London) under the direction of R. Guiloff [3] to examine the efficacy of the treatment of MND patients with RX77368.

The samples were assayed according to the previously described protocol. Samples were obtained from an intravenous study (0.2 and

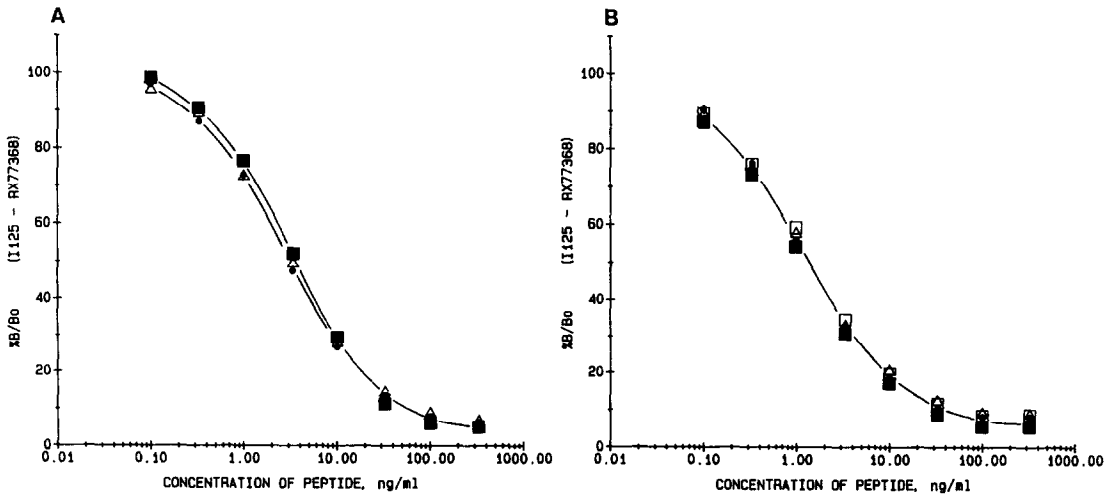

Figure 3
(A) Effect of incubation time on the RX77368 standard curve (% B/B_0). Assays were carried out for 30 (●), 60 (△) and 120 (■) min using a 1:6000 dilution of sheep 1 antibody. (B) Effect of dilution of anti-RX77368, sheep 1 antibody on the RX77368 standard curve (% B/B_0). Dilutions were prepared in phosphate buffer at 1:6000 (●), 1:8000 (△), 1:10,000 (■) and 1:12,000 (□). Incubations were at room temperature for 2 h.

0.25 mg kg^{-1}, a two patient pilot study) and from an oral multidose study (0.7, 1.4 and 3.0 mg kg^{-1}, seven patients).

Intravenous study

The intravenous study was a preliminary study and samples were only obtained from two patients. The intravenous RX77368 was administered by infusion over a 2 h period, with samples being collected up to +48 h. The results are shown in Fig. 4A. The detection limit for this assay was calculated by applying a 2 × SD limit on the B_0 data, as calculated according to the Packard Securia Plus, PC-DAAS curve fit package. The maximum concentrations observed were in the region of 250 ng ml^{-1}, declining to the basal levels by + 24 h.

Oral multidose study

The assay was carried out as previously described, and included three treatment groups, i.e. 0.7, 1.4 and 3.0 mg kg^{-1}, p.o.

All three dose levels showed similar profiles (see Fig. 4B) with the high, 3 mg kg^{-1} dose, showing a longer duration of action of up to 12 h post-administration. The three dose levels exhibited a dose-related effect. Plasma levels reached basal levels by +24 h.

The spline curve exhibited a detection limit in the region of 4–10 pg/tube, calculated as described earlier. The B_0 replicates were increased to six in the later assays, allowing a more precise detection limit to be calculated.

Effect of dilution of sample on the concentration of RX77368 in plasma

A sample of plasma was selected from the previously assayed intravenous study. The t = 1.5 h sample was used from which dilutions (1:2 to 1:100) were prepared in human control plasma. Quadruplicate determinations were carried out using the standard RIA. The RX77368 plasma concentrations were plotted against the reciprocal of the sample dilution, the resultant plot indicating that the effect of dilution is uniform and linear over the range studied (r = 0.997).

Discussion

A great deal of time was spent during the early phase of the study with the production of various RX77368–protein conjugates suitable for the development of specific antisera.

The best method in the literature previously described for TRH was the BDB method of Bassiri and Utiger [11], and this produced good incorporation of peptide in conjugation with KLH and BSA (Table 1).

The method of Visser *et al.* [10] showed higher levels of incorporation with all proteins, but did not raise antibodies to the same titre as the BDB conjugate. Antibodies were not

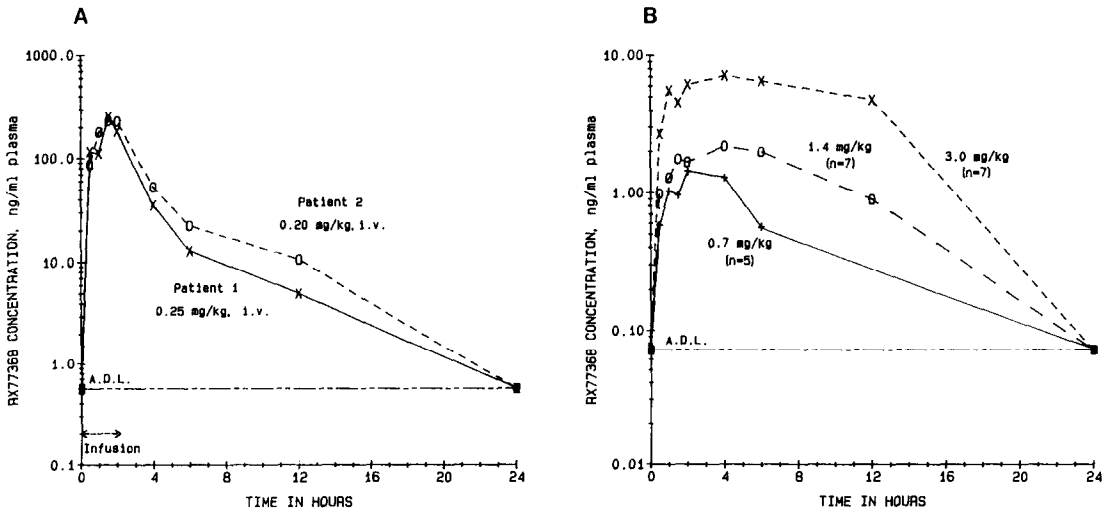

Figure 4
Intravenous (A) and oral (B) plasma profiles of RX77368 levels in plasma from patients with MND. (A) Intravenous (2 h infusion) administration of RX77368. (B) Effect of oral administration (0.7, 1.4 and 3.0 mg kg^{-1}) of RX77368. Assays were carried out as described in the text. The assay detection limit (ADL) was calculated using a 2 SD difference from the B_0 (see text).

obtained from other conjugates, with the exception of the BTG–Glu-His-3,3-dmProNH$_2$, prepared according to the method of Orth [8]. The antibodies raised in rabbits were not specific and recognized both RX77368 and the Glu-His-dmProNH$_2$ peptide equally. As the conjugate was linked through the C-terminus only the N-terminus was available for antigenicity. In contrast, the sheep antibody prepared with the BDB reagent and linked via the histidyl residue provided both C- and N-termini of RX77368 for antigenicity, giving a much more specific antibody as demonstrated previously for TRH [4].

During booster immunizations the sheep antibody titres rose consistently with each boost injection to a maximum at 4 months. After a delay in the immunization programme there then followed a period when the titre fell slightly, but after two further booster injections the titre reached the previous high level and a decision was made to terminate the immunization programme.

The final two bleeds (8 and 9) showed further increases in the titres over bleed 7 with the maximum titre obtained from sheep 1, bleed 8. Using these antisera, an RIA for RX77368 was established, incorporating a standard curve prepared in a plasma matrix.

Cross-reactivity studies (see Fig. 2 and Table 2) showed that the antibody was specific to RX-77368, and only recognized some analogues and metabolites in excess of 1000 ng ml^{-1}. The only peptide to exhibit any appreciable cross-reactivity, RX74355, is not a natural metabolite of RX77368. It will not be present in the clinical samples and, therefore, will not cause a problem in the assay. The working range of the assay is 0.1–333.3 ng ml^{-1} and is completed within 4 h.

The spline curve fit routine employed by the crystal gamma counter was adequate for the data, and the curve was normally fitted with one or two iterations. The % B/B_0 data was very reproducible and typically was within close limits. Intra- and inter-assay precision was estimated from the RSD(%) of the replicates of the standard curves. Essentially, the intra-assay variation was better than 5% for all assays used in the estimation. As expected, the inter-assay variation was higher, with a maximum % RSD of 16.5 at the highest concentration (mean over range was 10.1%). It is expected that samples showing high concentrations would be diluted and reassayed, with results interpolated from a more precise part of the standard curve.

Antibody-bound and free peptide were separated quickly by the use of the solid-phase anti-sheep or anti-rabbit second antibody (SAC–CEL). The SAC–CEL is available in amounts of 200 ml sufficient for 2000 tests, and provides a convenient means of separation at nominal cost.

The effect of incubation time (30 min to 2 h) on the binding reaction did not appear to affect the assay. There was an even greater similarity of the % B/T data and, again, the % B/B_0 curves were superimposable (Fig. 3A). The 2 h incubation data appeared to give the best curve profile.

Similarly, the effect of antibody dilution (over the range 1:6K–12K) on the RX77368 assay was almost negligible (see Fig. 3B). Although there were slight reductions in the % B/T values with dilution, the % B/B_0 data displayed superimposable standard curves.

Using the above assay system, clinical samples from an MND patient study at the Westminster Hospital (London) were analysed and the data presented. The intravenous samples were found to contain high levels (250 ng ml^{-1}) of RX77368. A second (oral) study showed reduced levels, but the highest dose (3.0 mg kg^{-1}) displayed levels which were sustained for up to 12 h (see Fig. 4). The plasma profile and drug concentrations were consistent with the endocrine changes observed during the same studies [3].

One of the most frequent problems involved with assaying clinical samples is that of dilution on the concentration of analyte present in the sample. The data in these assays, however, confirmed that there was a linear relationship with dilution, over the range studied. If dilution of plasma samples is unavoidable, the results obtained can be used with confidence.

Acknowledgements — We would like to thank Dr I.F. Tulloch for his helpful comments and constructive criticism during the preparation of the manuscript. We are also grateful to Mrs G.A. Gray for her expert typing.

References

[1] G. Metcalf, in *Thyrotropin Releasing Hormone* (E.C. Griffiths and G.W. Bennett, Eds), pp. 315–326. Raven Press, New York (1983).

[2] R.J. Guiloff, *J. Neurol. Neurosurg. Psychiat.* **50**, 1359–1370 (1987).

[3] H. Modarres-Sadhegi and R.J. Guiloff, *J. Neurol. Neurosurg. Psychiat.* In press.

[4] C. Lighton, C.A. Marsden and G.W. Bennett, *Neuropharmacology* **23**, 55–60 (1984).
[5] P.J. Fraker and J.C. Speck, *Biochem. Biophys. Res. Commun.* **80**, 849–857 (1978).
[6] M. Reichlin, J.J. Schnure and V.K. Vance, *Proc. Soc. Exp. Biol. Med.* **128**, 347–350 (1968).
[7] T. Goodfriend, G. Fasman, D. Kemp and L. Levine, *Immunochemistry* **3**, 223–231 (1966).
[8] D.N. Orth, in *Methods of Hormone Radioimmunoassay* (Jaffe and Behrman, Eds), 2nd edn. Academic Press, London (1979).
[9] H.S. Tager, *Anal. Biochem.* **71**, 367–375 (1976).
[10] T.J. Visser, W. Klootwijk, R. Doctor and G. Hennemann, *FEBS Lett.* **83**, 37–40 (1977).
[11] R.M. Bassiri and R.D. Utiger, *Endocrinology* **90**, 722–727 (1972).
[12] T. Sharp, Thesis, Dept. Phys. and Pharm., Medical School, QMC, Nottingham University (1983).
[13] W.J. Herbert, in *Handbook of Experimental Immunology* (Weir, Ed.), 2nd edn, Appendix A2.1. Blackwell Scientific, Oxford (1973).
[14] P. Franchimont, J.-C. Hendrick and A.-M. Reuter, in *Principles of Competitive Protein Binding Assays* (Odell and Franchimont, Eds), 2nd edn, Chap. 3. Wiley, London (1983).

[Received for review 14 December 1989; revised manuscript received 28 August 1990]