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A radioimmunoassay combined with solid-phase extraction for the determination of a novel anti-obesity agent, ARL 15849XX, in dog plasma

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Abstract

A radioimmunoassay has been developed for the determination of ARL 15849XX, a cholecystokinin-8 (CCK-8) analogue, in dog plasma. The method incorporates solid-phase sample extraction and is suitable for the determination of the analyte at picogram per millilitre concentrations. The antiserum was raised in Suffolk-cross sheep following primary and booster immunisations with an immunogen prepared by conjugating ARL 16935XX, an analogue of ARL 15849KF, to bovine serum albumin. The radioligand was prepared by the no-carrier-added ^{125}I iodination of a non-sulphated derivative, ARL 15745XX. The solid-phase extraction procedure, carried out using ion-exchange aminopropyl and octadecyl sorbents sequentially, was introduced to remove matrix interferences in the plasma and to enhance the method sensitivity. The calibration range is 20–1000 pg ml^{-1}, using a 1 ml sample of undiluted dog plasma.

Keywords: ARL 15849KF; Anti-obesity drug; Plasma; Solid-phase extraction; Radioimmunoassay

1. Introduction

ARL 15849KF (Fig. 1) is a novel and highly potent peptide anorectic CCK-A receptor agonist, currently under development for the treatment of obesity. Obesity is acknowledged to be a major risk factor for morbidity and mortality. Studies indicate that 25% of coronary artery heart disease and 35% of congestive heart failure and stroke could be attributable to obesity alone [1]. Mortality [2] is 25% above average at a body mass index (BMI) of 30 kg m^{-2} and 175% higher at a BMI of 40 kg m^{-2}. The prevalence of obesity (BMI above 30 kg m^{-2}) is 12% in the USA, 7% in

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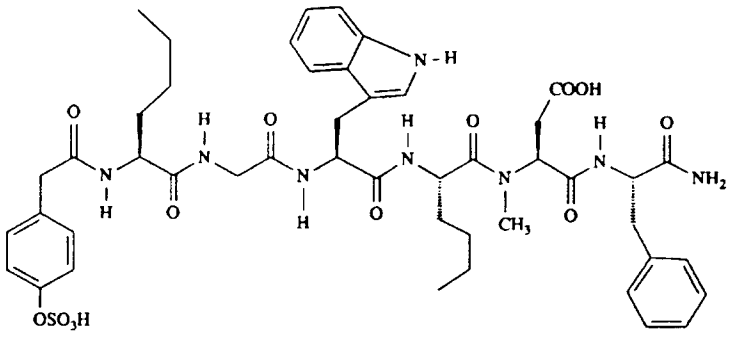

ARL 15849XX

$HpaSO_3H$-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2

Fig. 1. Chemical structure of ARL 15849XX and related compounds: ARL 15849XX, free acid ($R = -SO_3H$); ARL 15849KF, ammonium salt ($R = -SO_3^- NH_4^+$); ARL 15745XX ($R = -H$); ARL 16935XX ($R = -CH_2NH_2$); ARL 16935–BSA conjugate ($R = -CH_2NH-(C_6H_2N_2O_4)-BSA$).

Europe, and 5% in Japan, equating approximately 60000000 people worldwide. Many of the drugs that are currently used are amphetamine-like CNS stimulants with significant side-effects and/or abuse potential. There is, therefore, a clear medical and economic need for effective and safe anti-obesity drug treatment. ARL 15849KF acts peripherally following systemic absorption (limiting its potential for CNS side-effects and abuse) and, by imitating some of the effects of the naturally occurring peptide hormone cholecystokinin (CCK), triggers a feeling of satiety.

A validated analytical method is available for the determination of ARL 15849XX in dose formulations which employs fluorescence HPLC. However, this method is unsuitable for use with plasma samples. A radioimmunoassay (RIA) combined with solid-phase extraction (SPE) has therefore been developed to enable the determination of pg ml^{-1} concentrations of ARL 15849XX present in samples of dog plasma taken during exploratory toxicology and pharmacokinetic studies.

This paper describes the development and validation of an RIA for the specific determination of ARL 15849XX, employing solid-phase extraction for the removal of plasma interferences.

2. Experimental

2.1. Chemicals

Chemicals were obtained from several sources. Buffer salts (analytical grade) were purchased from Sigma UK and Fisons Laboratory Supplies, and methanol (HPLC grade) was supplied by Fisons Laboratory Supplies. ARL 15849KF was provided by the Department of Chemistry, Astra Research, Rochester, New York, USA. ARL 16395XX–bovine serum albumin (BSA) conjugate and radiolabelled ARL 15745XX were synthesised in the Chemical Development Department, Astra Charnwood, Loughborough, Leics. Potential cross-reacting peptide fragments of ARL 15849XX used for the specificity studies were also obtained from the same laboratories. No-carrier-added sodium ^{125}I-iodide was supplied by Amersham International plc. Purified endogenous gut peptide hormones were purchased from Sigma UK. BSA (RIA grade) and Freunds incomplete adjuvant were purchased from Sigma Immunochemicals. BGC vaccine, BP was obtained from Evans, Ltd.

Solid-phase extraction cartridges supplied by International Separation Technology Ltd. were obtained from Jones Chromatography.

Deionised water (more than 18 MΩ cm^{-1}) was obtained using a Milli Q system.

2.2. Preparation of radioimmunoassay working solutions

The RIA diluent consisted of 0.1 M sodium phosphate buffer (pH 6.0) containing 0.05% (w/v) BSA. This solution was stored for up to 1 month at a nominal 4°C.

Working solutions of the ARL 15745XX radioligand and antiserum (described below) were prepared on each day of analysis. Radioligand working solution was prepared by adding approximately 100 μl of the stock solution to 10 ml of assay diluent; 100 μl of this solution contained approximately 15000 dpm. Antiserum working solution was prepared by diluting the neat antiserum 300000 times in assay diluent. Primary aqueous stock standard solutions were prepared at intervals of approximately 3 months and stored at or below −20°C. Plasma calibration standards and quality control samples were stored at or below −20°C for up to 3 months.

Phase separation of antibody-bound and free (unbound) radioligand was performed by second antibody precipitation of the bound radioligand with diluted donkey anti-sheep IgG antiserum. This reagent (1:4 dilution of neat antiserum in assay diluent) was prepared and used on the day of assay.

2.3. Apparatus

Solid scintillation counting was performed by means of a Packard Cobra gamma counter with integral data reduction software. The four-parameter logistic (4PL) algorithm was employed to convert radioactivity measurements, expressed as counts per minute (cpm), into concentration values (pg ml^{-1}).

Solid-phase extraction was carried out using 3 ml IST cartridges containing 500 mg of aminopropyl adsorbent, followed by 3 ml IST cartridges containing 500 mg of octadecyl end-capped adsorbent, and performed using a 24-position vacuum box. The final sample extract was reduced to dryness under nitrogen in a Turbovap evaporator. The Turbovap evaporator was supplied by Zymark Inc., and Novopak and Resolve™ HPLC columns (150 mm × 3.9 mm; 5 μm spherical) were obtained from Waters.

2.4. Blood sample collection

Blood samples, for the preparation of a normal dog plasma pool (control plasma), and for the generation of individual plasma samples, were taken from healthy beagle dogs into tubes containing lithium heparin anticoagulant. The plasma was separated from the red cells by centrifugation.

All plasma samples were stored at or below −20°C and assayed within three freeze–thaw cycles. Under these conditions, the results of stability studies showed that ARL 15849XX is stable for up to 6 months.

2.5. Methods

2.5.1. Synthesis of ^{125}I-ARL 15745XX radioligand

ARL 15745XX was iodinated in the hydroxyphenylacetic acid (Hpa) residue (Fig. 1) via a direct electrophilic ^{125}I iodination utilising sodium ^{125}I-iodide as the ^{125}I source and chloramine-T as oxidant.

The radioiodination reaction was carried out within the microvial in which the sodium ^{125}I-iodide was supplied. Phosphate buffer (5 μl; pH 7.1; 1.0 M) was added followed by the stock solution of ARL 15745XX (10 μl), methanol (30 μl) and finally an aqueous solution of Chloramine-T solution (5 μl; 0.2 mM). The vial was mixed by agitation several times and incubated at room temperature for 30 min. An aqueous solution of sodium metabisulphite (5 μl; 0.4 mM) was added, mixed for 1 min and the product mixture was left to stand for another 2 min. The required monoiodinated product was isolated by collecting the appropriate fraction after separation by reversed-phase HPLC (Novapak C-18; detection at 210 nm; flow rate, 1 ml min^{-1}; solvent A, acetonitrile; solvent B, 0.2% trifluoroacetic acid in water; gradient (time, %B): 0, 65; 25, 65; 27, 5; 30, 65; 35, 65). The purity of the monoiodinated product was checked by reversed-phase HPLC (Resolve spheri-

cal C-18; solvent A, acetonitrile; solvent B, 0.2% trifluoroacetic acid; flow rate, 1 ml min^{-1}; gradient (time, %B): 0, 65; 25, 50; 27, 5; 33, 5; 35, 65).

2.5.2. Antiserum production

2.5.2.1. Preparation of the immunogen

In view of the relatively low molecular weight of this compound (994.12 for the ammonium salt), conjugation to a suitable protein was considered necessary to obtain a satisfactory antigenic response. Conjugation to BSA was carried out as follows.

To ARL 16935XX (26 mg) dissolved in acetonitrile–water (2:1, v/v; 3 ml) was added triethylamine (5 μl) and then difluorodinitrobenzene (9 mg ml^{-1}; 1 ml in acetonitrile). The reaction mixture was left for 1 h. The product was subjected to preparative TLC using chloroform–methanol–acetic acid (20:3:1, v/v) as mobile phase on Merck $20 \times 20 \times 1$ mm^3 plates with concentration zone, single plate only, yielding 28 mg of the product after stripping with methanol–chloroform (1:1, v/v). Three-quarters of the product was rotary evaporated to dryness and dissolved in methanol (0.5 ml), and the solution was added to BSA (RIA grade; 30 mg) in water (1 ml). Triethylamine was added in 5 μl amounts until a clear yellow solution resulted (pH 9.0). The reaction mixture was then allowed to stand for 1.5 h before being passed through a Sephadex G-25 (12 g) column. After elution with water, the protein fraction was subjected to TLC, which revealed traces of the fluorodinitrobenzene (FDNB) derivative of ARL 16935XX. The fractionation was repeated, the protein fraction revealing only a very faint trace of the FDNB derivative. The fractions were pooled (7.15 ml) and a portion (6.5 ml) was freeze-dried, yielding 27.9 mg of the conjugate. The remainder of the sample was used for determining the epitopic density of ARL 16935XX in the conjugate by UV-Vis spectrophotometry at 455 nm. The epitopic density was calculated to be 6.9 residues per mole of BSA.

2.5.2.2. Antiserum production in sheep

The immunogen (200 μg per animal) was emulsified with Freunds complete adjuvant and used to immunise three Suffolk-cross sheep by the intramuscular route. The animals were subsequently boosted, at 1, 2 and 3 months after the initial immunisation, with further 200 μg amounts of the conjugate emulsified in Freunds incomplete adjuvant. Test bleeds were taken approximately 10 days after each booster immunisation, and an acceptable titre (1:33000), corresponding to 50% maximum binding of the radioligand, was found in one sheep after the first boost. The serum from the third bleed from sheep 68 (titre approximately 1:800000) was judged to be the most suitable for development of an RIA method, and was stored at or below -20°C.

2.5.3. Solid-phase extraction-radioimmunoassay (SPE-RIA)

2.5.3.1. Extraction

Two solid-phase extraction steps were employed prior to the RIA; an initial ion-exchange extraction on aminopropyl sorbent was followed by a desalting and concentration step on octadecyl endcapped sorbent. The order of additions to the aminopropyl cartridges was as follows.

(1) Methanol (1×2 ml; 100%, v/v).

(2) Sodium acetate buffer (5 ml; pH 5.5; 1 M).

(3) Sodium acetate buffer (4 ml; pH 5.5; 50 mM).

(4) Diluted plasma sample (3 ml).

(5) Sodium acetate buffer (3 ml; pH 5.5; 50 mM).

(6) Sodium citrate buffer (3 ml; pH 6.0; 50 mM).

Eluates, containing ARL 15849KF, were collected during step 6 and applied directly to octadecyl endcapped cartridges equilibrated with 2 ml of 100% (v/v) methanol, followed by 2 ml of water. After a 2 ml wash with 10% (v/v) methanol, the compound was eluted with 3 ml of 100% methanol and the eluate was evaporated to dryness under nitrogen at 45°C. The extracts were reconstituted in 1 ml of assay diluent by vortex mixing.

2.5.3.2. RIA

Calibration standards were prepared over the range 10–2000 pg ml^{-1} ARL 15849XX in pooled

dog plasma. A zero plasma calibration standard, and plasma quality control samples at three levels were also analysed. All plasma samples were extracted as described previously. Analysis of the extracts was carried out in duplicate using 12 × 75 mm borosilicate glass tubes (Corning), for all calibration standards, quality controls and test samples. In each instance, the final RIA incubation contained 0.1 ml of a sample extract, 0.1 ml of the radioligand working solution and 0.1 ml of the antiserum working solution. Non-specific binding (NSB) and total radioactivity tubes were included with each batch of analyses.

The assay tubes were incubated overnight for at least 15 h at a nominal 4°C. After allowing the tubes to reach ambient temperature, phase separation was then carried out by the addition of 0.1 ml of donkey anti-IgG antiserum working solution (1:4 dilution) and 0.1 ml of non-immune sheep serum (1:50 dilution), each prepared in assay diluent. Each of the tubes was vortex mixed and incubated at ambient temperature for 30 min, then centrifuged (3500 rev min^{-1}; 20 min; 15°C). After transfer to a suitable holding rack, the supernatants were decanted to waste by inversion of the rack over a suitable sink. The tubes were then transferred to gamma counter racks and counted for at least 2 min.

The SPE-RIA procedure was used for method validation studies, and to analyse dog plasma samples from toxicology studies.

2.5.4. *Validation of the SPE-RIA method*

The SPE-RIA was validated in accordance with recommendations [3–6] which assess the performance of a method with regard to its specificity, sensitivity, accuracy and precision. Quality control samples were included throughout these validation studies, and in each test sample analysis batch. In addition, the stability of the compound in plasma under a variety of conditions was established.

2.5.4.1. *Specificity studies*

Cross-reactivity.

The specificity of the antiserum, with regard to potential cross-reacting peptide fragments of the compound and to a range of endogenous peptide hormones with related structures, was determined by analysing aliquots of control plasma which had been spiked with the compound at concentrations up to 5000 pg ml^{-1}. Cross-reactivity, where quantifiable, was calculated from the measured apparent concentration in the RIA as a percentage of the nominal concentration. The following compounds were tested.

ARL 17413XX (Hpa-Nle-Gly-Trp-Nle-MeAsp-Phe-OH)

ARL 16047XX (Hpa-SE-Nle-Gly-Trp-Nle-DMeAsp-Phe-NH_2)

ARL 15745XX (Hpa-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2)

ARL 17590XX (Hpa-Nle-Gly-Trp-Nle-MeAsp-OH)

(Trp-Nle-MeAsp-Phe-NH_2)

CCK-8 (Asp-TyrSO_3H-Met-Gly-Trp-Met-Asp-Phe-NH_2)

CCK-8 (desulph.)(Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2)

CCK-4 (Asp-TyrSO_3H-Met-Gly-NH_2)

VIP (Vasointestinal peptide)

Gastrin I (*p*-Glu-Gly-Pro-Trp-Leu-$(Glu)_5$-Ala-Tyr-Gly-Trp-NH_2)

Interference by endogenous plasma components.

Control plasma samples taken from six different animals were analysed by the SPE-RIA to assess any interference by endogenous material.

2.5.4.2. *Assay accuracy and precision*

The inter-assay and intra-assay accuracy and precision of the method were determined by analysis of aliquots of control plasma spiked with ARL 15849KF at each of the concentrations of the calibration standards. The inter-assay investigations were performed over five independent analysis batches using identical spiked samples, aliquots of which had been stored at −20°C prior to analysis. Intra-assay performance was assessed by six replicate determinations of samples from the same spiked control plasma in a single analysis batch.

2.5.4.3. *Stability*

Aliquots of control plasma spiked with ARL 15849KF were analysed immediately following

preparation, then after storage under the following conditions: 24 h at a nominal 4°C, at or below −20°C, and at ambient temperature; up to 4 months at or below −20°C. The stability during up to three freeze–thaw cycles was investigated, as was the stability of the sample extracts at or below −20°C. Each result represents the mean of two separate samples.

2.5.4.4. Analysis of diluted extracts

The agreement between results obtained by analysing extracts at different dilutions in assay diluent was investigated at dilutions up to twentyfold in order to extend the range of the method.

3. Results and discussion

3.1. Solid-phase extraction RIA (SPE-RIA)

Early investigations indicated that significant interferents with ligand antibody binding were present in unextracted dog plasma; this made use of a direct RIA method impracticable. It was therefore necessary to develop a suitable solid-phase extraction procedure for purification and concentration of the sample prior to RIA. The procedure described here effectively removes plasma interferents, and has the additional advantage of concentrating the extracted plasma, thereby increasing the sensitivity of the method.

3.2. Validation of the SPE-RIA method

3.2.1. Specificity studies

3.2.1.1. Cross-reactivity

Aliquots of blank pooled plasma samples spiked with the following compounds were analysed by SPE-RIA to assess their potential for cross-reaction if present in test plasma samples.

Cross-reactivity was detectable only for ARL 15745XX (0.75%; 37.3 pg ml^{-1} at a 5000 pg ml^{-1} nominal concentration) and for the fragment Trp-Nle-MeAsp-Phe-NH_2 (0.44%; 22 pg ml^{-1} at a 5000 pg ml^{-1} nominal concentration). There was no detectable cross-reactivity with any of the other potential breakdown products of ARL 15849KF or with any of the endogenous gut peptides tested, as listed in the experimental section, including CCK-8 and CCK-4. The combination of antiserum specificity and SPE extraction before RIA thus results in a highly specific assay for the parent compound.

Table 1
Concentrations of ARL 15849XX measured in individual dog plasma samples

Sample	Concentration determined in plasma blank (pg ml^{-1})	Blank B/B_0 value (%)
1	<20	105.1
2	<20	106.8
3	<20	103.9
4	<20	108.2
5	<20	105.3
6	<20	106.8
Mean	<20	106.0
RSD	—	1.5

3.2.1.2. Interference by endogenous plasma components

Plasma samples from six different dogs were analysed. The results are presented in Table 1, and show all plasmas to be free of any interfering components detectable above the limit of quantification of the method.

Table 2
Inter-assay precision and accuracy of validation samples ($n = 5$)

ARL 15849XX concentration in validation sample (pg ml^{-1})	Accuracy (%)	Precision, RSD (%)
10	93.0	66.0
20	102	12.5
50	90.8	23.5
100	85.4	12.2
200	106	10.8
500	88.2	5.4
1 000	109	19.5
2 000	218	130

Table 3
Intra-assay precision and accuracy of validation samples ($n = 6$)

ARL 15849XX concentration in validation sample (pg ml^{-1})	Accuracy (%)	Precision, RSD (%)
10	172	18.8
20	113	20.7
50	89.6	21.5
100	102	19.8
200	106	12.5
500	89.6	8.5
1 000	87.6	13.7
2 000	60.9	55.7

3.2.2. Method accuracy, precision, calibration range and sensitivity

Summary inter-assay and intra-assay accuracy and precision data for the determination of ARL 15849XX in dog plasma are presented in Tables 2 and 3. The accuracy and precision data were used to define the lower limit of quantification (LOQ) of the method. The LOQ was defined as 20 pg ml^{-1} on the basis of unacceptable inter-assay and intra-assay performance of the validation sample spiked at 10 pg ml^{-1}. The upper reporting limit for undiluted sample extracts was set at 1000 pg ml^{-1}.

3.2.3. Stability

Data obtained for the stability of ARL 15849XX in dog plasma in samples containing 200 pg ml^{-1} ARL 15849XX are presented in Table 4.

Table 4
Stability of ARL 15849XX[a] in dog plasma stored under different conditions ($n = 2$)

Storage conditions	Concentration (pg ml^{-1})	% Nominal concentration
Baseline	227	114
Freeze–thaw, three times	175	87.5
24 h at ambient temperature	224	112
24 h at 4 °C	206	103
24 h at −20 °C	203	102
24 h, extract	260	130
6 months at −20 °C	206	103

[a] Nominal added concentration, 200 pg ml^{-1}.

Table 5
Effect of sample extract dilution on measured ARL 15849XX concentration ($n = 2$)

Dilution factor	Concentration (pg ml^{-1})	% Nominal concentration
5.0	2060	103
10	1880	94.0
20	2160	108

The results indicate a generally minimal effect of storage on the measured concentration of the compound in plasma, apart from an apparent slight but significant loss after three freeze–thaw cycles. The apparent over-recovery of the extract stored for 24 h is likely to have been due to poor agreement between the duplicate analyses obtained for this data point, and does not reflect data obtained from repeat analyses of stored extracts after this validation was carried out (unpublished observations).

3.2.4. Analysis of diluted sample extracts

The effect of sample dilution on measured ARL 15849XX concentrations in extracts of a 2000 pg ml^{-1} validation sample analysed undiluted and diluted fivefold, tenfold and twentyfold with assay diluent are shown in Table 5. Agreement between dilutions up to twentyfold is good. This justifies the practice of analysing samples with measured concentrations above the working range of the calibration curve using diluted sample extracts.

4. Conclusions

A radioimmunoassay, incorporating a solid-phase sample extraction procedure, has been developed and fully evaluated for the determination of ARL 15849XX in dog plasma. The method is highly sensitive (LOQ, 20 pg ml^{-1}), highly specific, and has levels of precision and accuracy acceptable for early development. It has been used to demonstrate good stability of the compound in plasma, and it is expected to be sufficiently sensitive for the determination of ARL 15849XX in plasma samples from low-dose toxicokinetic stud-

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