Preface

The endothelins are a remarkable family of signaling peptides: molecular biology predicted the existence of their receptors and synthetic enzymes prior to both the identification of the encoded proteins and the synthesis of antagonists and inhibitors for use as pharmacological tools. Although considerable advances have been made, culminating in the design of endothelin antagonists with therapeutic potential in cardiovascular disease, much remains to be discovered.

Tantalizingly, new research frontiers are emerging. To support further progress, *Peptide Research Protocols: Endothelin* encompasses experimental protocols that interrogate all facets of an endogenous mammalian peptide system, from peptide and receptor expression through synthetic pathway to peptide function and potential role in human disease.

Chapters describe the use of molecular techniques to quantify the expression of mRNA for both endothelin receptors and the endothelin-converting enzymes. Peptides, precursors, receptors, and synthetic enzymes may be localized and quantified in plasma, culture supernatants, tissue homogenates, and tissue sections using antibodies, while additional information on receptor characterization may be obtained using radioligand binding techniques. Several protocols cover in vitro assays that determine the function of the endothelin peptides in isolated preparations, that characterize new endothelin receptor ligands, or provide information on the tissue-specific processing of endothelin precursor peptides. Finally, in vivo protocols illustrate the role of the endothelin peptides in healthy human individuals, describe animal models that reveal the alteration of the endothelin system in cardiovascular disease, and therefore predict the therapeutic potential of drugs that manipulate endothelin synthesis or function. A particular strength of *Peptide Research Protocols: Endothelin* is that, although each protocol may be used independently, many of the techniques are written to be complementary. The sequencing of the human genome presents new challenges in understanding the role in human physiology and pathophysiology of novel encoded proteins and peptides. The protocols described in this book have proved successful in endothelin research and the experimental strategies described have a wider relevance for determining the functional importance of the emerging orphan receptors and their cognate peptidic ligands.

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Analysis of Endothelins by Enzyme-Linked Immunosorbent Assay and Radioimmunoassay

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1. Introduction

This chapter describes procedures for the measurement of endothelin peptides by antibodies, focusing on the two main immunoassay techniques that are widely used. In a two-site "sandwich" enzyme-linked immunosorbent assay (ELISA) one antibody is immobilized to a solid phase and captures the endothelin (ET) peptide(s), which is quantified by the binding to this complex of a second, enzyme-labeled antibody in the liquid phase. In a radioimmunoassay (RIA), the ET peptide(s) to be measured competes for the binding of a fixed concentration of radiolabeled peptide to a fixed concentration of antibody in the liquid phase. In contrast to the ELISA, the immune complex measured in a RIA does not contain the analyte and therefore inverse (falling) standard curves of peptide concentration vs bound labeled peptide are produced. For a more detailed discussion of the two techniques, **refs. *1*** and ***2*** are recommended.

Both immunoassay techniques are characterized by limited purification of the analyte and may therefore be susceptible to interference by unrelated molecules or crossreact with structurally-related peptides. Where the precise identification of the ET peptides present in the tissue samples is required, these immunoassays can be preceded by chromatographic separation (*see* Chapter 3). ELISAs can also be used to test the specificity of antisera used to visualize, but not to quantify, ET peptides in tissue sections by immunocytochemistry (*see* Chapter 1).

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1.1. ET-1 and Big ET-1

ET-1 is the principal isoform in the human cardiovascular system and remains the most potent constrictor of human vessels discovered. ET-1 is unusual amongst the mammalian bioactive peptides in being released from a dual secretory pathway ***(3,4)***. The peptide is continuously released from vascular endothelial cells by the constitutive pathway, producing intense constriction of the underlying smooth muscle and contributing to the maintenance of endogenous vascular tone ***(5)***. The peptide is also released from endothelial cell-specific storage granules (Weibel-Palade bodies) in response to external physiological, or perhaps pathophysiological, stimuli producing further vasoconstriction ***(3,4)***. Thus, ET-1 functions as a localy released, rather than circulating, hormone and concentrations are comparatively low in plasma and other tissues.

In pathophysiological conditions, tissue levels of ET-1, and its precursor big ET-1, are significantly increased, e.g., within the wall of human vessels containing atherosclerotic lesions ***(6)***. Increases in the plasma levels of immunoreactive ET have been measured in number of pathophysiological conditions including coronary vasospasm and congestive heart failure ***(7,8)***. Raised plasma levels of big ET-1 appear to be particularly predictive of disease severity and prognosis ***(9)***.

1.2. ET-2 and Big ET-2

ET-2 has been less extensively studied than other ET peptides. We detected ET-2 mRNA ***(10)*** and mature peptide ***(11)*** in human cardiovascular tissues and ET-2 was as potent a vasoconstrictor as ET-1 in human arteries and veins ***(12)***. Big ET-2 has been detected in the cytoplasm of endothelial cells ***(13)*** and, surprisingly, in normal human plasma, big ET-2 levels are higher than big ET-1 ***(14)***. ET-2 has also been identified in failing hearts from humans ***(15)***. A specific ELISA has been developed for big ET-2, giving plasma levels of 0.85 ± 0.03 pmol/L, $n = 42$ (unpublished observations). However, the physiological or pathophysiological role of this isoform remains to be discovered.

1.3. ET-3 and Big ET-3

Endothelial cells do not synthesize ET-3, but the mature peptide and big ET-3 are detectable in plasma ***(14,16,17)*** and other tissues including heart ***(11)*** and brain ***(18)***. ET-3 is unique in that it is the only endogenous isoform that distinguishes between the two endothelin receptors. It has the same affinity at the ET_B receptor as ET-1 but, at physiological concentrations, has little or no affinity for the ET_A sub-type. In humans, ET_A receptors predominate in the human vasculature and the low density of ET_B receptors ($<15\%$) present on the smooth muscle of the vasculature contribute little to vasoconstriction ***(12)***. ET_B receptors are the principal sub-type in the kidney, localizing to nonvascular tissues. Evidence is emerging that the ET_B sub-type functions as a clearing

receptor to remove ET from the circulation. Blockade of the ET_B receptor results in a rise in circulating immunoreactive ET. Blockade of the ET_B receptor by receptor antagonists results in a corresponding rise in circulating levels of ET-3 ***(17)***. A selective ET-3 ELISA can be used to measure the extent of ETB receptor blockade ***(17)***.

ET-3 levels are also altered in disease ***(16)***. ET-3 may play a beneficial role in human disease by activating ET_B receptors to release opposing vasodilators, thus limiting unwanted vasoconstriction.

1.4. Species Differences

The majority of published immunoassays have been developed to measure ET in human tissue and plasma. However, there are no reported differences in the predicted amino-acid sequences in other mammalian species where the ET-1 (dog, bovine, pig, guinea pig, rat, mouse, rabbit) or ET-3 (mouse, rat) genes have been sequenced. Vasoactive intestinal contractor, the rodent (mouse and rat) equivalent of human ET-2, differs by only one amino acid. The assays described below would be expected to detect the mature peptides in most, if not all, mammalian species. In other vertebrates (e.g., fish), the N-terminus is reported to vary from the human ET-1 sequence but the C-terminus is conserved; selecting antisera directed to the C-terminus should therefore ensure 100% crossreactivity. The C-terminus of big ET precursors can also vary between species.

1.5. Two-Site "Sandwich" ELISA or Radioimmunoassay?

Quantification of endogenous levels of ET peptides in plasma, other tissues, in cell cultures and following the fate of exogenously applied peptides in vitro or in vivo represents a challenge because of the potential for crossreactivity between the three isoforms and their precursors. Although ET-1 is the predominant isoform in many tissues, the term immunoreactive endothelin (IR-ET) is used to reflect that some assays may detect and therefore measure ET-2 and/or ET-3 together with precursors. It is not surprising that there is variation in the absolute amounts of "immunoreactive ET" measured, e.g., in human plasma, reflecting variation in recovery following the method of solid-phase extraction, the type of immunoassay employed, and the specificity of the antisera. This limitation must be considered in the design of experiments to ensure appropriate control to allow measurement of relative changes rather than absolute levels of immunoreactivity.

Sandwich ELISAs are available from a number of commercial sources including the Biotrak™ endothelin-1 ELISA from Amersham Pharmacia Biotech ***(19)***. This kit, one of the first to be developed, is recommended because of excellent intra- and inter-assay coefficients of variation and good

recovery following solid phase extraction ***(19)***. In this assay, samples are incubated in microtiter wells that are precoated with an antibody against the C-terminus of ET to which ET isoforms in the sample bind specifically. Unrelated molecules are removed during a subsequent washing step. Bound ET is measured using a peroxidase-labeled Fab' fragment of an anti-N-terminal ET-1 antibody conjugate (creating the sandwich), which catalyzes the conversion of a chromogenic substrate to a visible reaction product. This assay has been well characterized for crossreactivity against ET peptides and their precursors and the lack of crossreactivity with pharmacological tools used in ET research, such as receptor antagonists, has been demonstrated. The main advantages of the sandwich ELISAs are speed, they avoid the use of radioactivity, and they can be more specific than the more commonly used RIAs, as two antibody binding sites are required on the same molecule for a signal to be obtained. Sandwich ELISAs are the method of choice for most applications. The sensitivity of an ELISA can be comparable to a RIA when making direct measurements of comparatively small volumes of conditioned tissue culture media or physiological saline. However, owing to potential interference in the ELISA, it is not yet possible to measure plasma or tissue ET levels without solid-phase extraction, as it is with RIA.

Radioimmunoassay kits are also widely available from several sources; those from Amersham Pharmacia Biotech have been well characterized ***(20)***. For large studies, it may be more economical to purchase antisera suitable for RIA separately or to generate antisera “in house” and then combine these with commercially available tracers.

1.6. RIA for ET-1/ET-2 & Precursors (N-Terminus)

Two kits are available using antisera directed to the N-terminus of ET-1 and ET-2 (RPA535 and the high sensitivity assay, RPA545) that have virtually no crossreactivity with ET-3. As expected these antisera crossreact with, and would therefore detect, big ET-1 and big ET-2. These kits will detect peptides (ET-1 and ET-2) selective for the ET_A receptor but cannot be used to monitor the conversion of big ET-1 to ET-1.

1.7. RIA for ET-1/ET-2/ET-3 (C-Terminus)

Antisera directed to the common C-terminus of the ET isopeptides (e.g., RPA555) display the expected crossreactivity for ET-1, ET-2 and ET-3 with little crossreactivity for the precursor peptides ***(20)***. This assay is recommended to quantify all the mature, and therefore biologically active, peptides and can also be used to measure the conversion of big ET to ET-1.

1.8. RIA for the Separate Measurement of ET-1, Big ET-1, and CTF

More than 90% of ET-1 synthesized by endothelial cells is thought to be released towards the smooth muscle cells, where it immediately binds to

its receptors. Only a small fraction escapes into the circulation and plasma levels may not accurately reflect potential increases in ET-1 as a result of disease or following treatment with ET receptor antagonists. Synthesis of ET-1 from big ET-1 also results in the formation of the biologically inactive C-terminal fragment (CTF) in an equimolar ratio. Since this peptide does not bind to ET receptors, it can provide a better measure of the conversion of big ET-1 ***(21–23)***. In the solid phase extraction method described below, the CTF fragment elutes differentially from the biologically active ET-1 and big ET-1 peptides, allowing all three components of the ET-1 synthetic pathway to be measured.

2. Materials

2.1. Preparation of Biological Samples for Solid Phase Extraction

2.1.1. Preparation of Solid Tissues

1. Homogenizer (Polytron or similar).
2. 0.5 *M* Acetic acid containing 0.1% Triton X-100 solution.
3. Boiling water bath.
4. Polypropylene centrifuge tubes.
5. Centrifuge.

2.1.2. Preparation of Plasma for Solid-Phase Extraction

1. Blood collection tubes containing EDTA at a final concentration of 1.2–2 mg/mL of blood. These can be either commercial evacuated tubes (Monovette—Sarstedt, Leicester, UK; Vacutainer—Becton Dickinson, London, UK) or polypropylene screw-capped tubes (K10pp—LIP, Keighley, UK).
2. Polypropylene tubes (e.g., Sarstedt, Leicester, UK).
3. 2 *M* Hydrochloric acid.

2.2. Solid Phase Extraction of Biological Samples

1. Silica mini-columns, e.g., Amprep 500 mg C2 or C18 columns (Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK) or C8 SPE-ED (Applied Separations, Allentown, PA).
2. Hydrochloric acid, trifluoroacetic acid, methanol, deionized water.
3. Vacuum manifold and centrifugal sample concentrator, e.g., Savant Speedvac, Thermoquest Life Sciences Ltd., Basingstoke, Herts, UK.

2.3. Detection of ET Peptides Using ELISA Kits

1. ELISA kit, e.g., Biotrak endothelin-1 ELISA (Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK).
2. Pipets (ideally multichannel) and tips to dispense between 50–200 µL vol.
3. Polypropylene tubes.
4. 37°C Incubator.

5. Microtiter plate shaker (e.g., Wellmixx 2, ThermoDenley, Basingstoke, Hants., UK).
6. Microtiter plate washer (e.g., Biotrak Microtiter plate washer, Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK).
7. Microtiter plate reader (e.g., Biotrak Microtiter plate reader, Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK).
8. Deionized water.
9. 1.0 *M* Sulphuric acid.

2.4. Detection of ET Peptides Using RIA Kits

1. RPA535 or high sensitivity assay, RPA545 for ET-1/ET-2 and precursors. RPA555 for ET-1/ET-2/ET-3 (Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK).
2. Polypropylene assay tubes (12 × 75 mm, 55.526, Sarstedt, Leicester, UK).
3. Magnetic separation racks (e.g., Amerlex-M Separator, Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK).

2.5. RIA for the Separate Measurement of ET-1, Big ET-1 and CTF

Materials as for **Subheading 2.4.** with the following additions/modifications.

1. [^{125}I]-ET-1 and [^{125}I]-big ET-1 (2200 Ci/mmol), (Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK).
2. ET-1(1-21, human) and big ET-1(1-38, human) peptides, (Peptide Institute, Osaka, Japan).
3. Assay buffer (per liter): 5.74 g Na_2HPO_4, 1.14 g NaH_2PO_4 2.5 g BSA, 0.5 g sodium azide and 100 µL Tween-20, at pH 7.2–7.4.
4. Antisera. The primary antisera against the endothelin peptides were all designed and produced “in house”. Commercial antisera are also available (e.g., Bachem, St. Helens, UK or Peptide Institute, Osaka, Japan).

3. Methods

3.1. Preparation of Biological Samples for Solid Phase Extraction

Cell culture media and physiological saline can be assayed directly in both the ELISA and RIA. However, solid tissues and plasma require solid phase extraction.

3.1.1. Homogenization of Solid Tissues

1. Cut tissue (typically 1–3 g wet weight) into small pieces, transfer to polypropylene tubes and homogenize with 10 vol of ice-cold 0.5 *M* acetic acid/0.1% Triton X-100, for 30 s on the maximum setting.
2. Heat the homogenates in a water bath at 100°C for 15 min, cool and clarify by centrifugation at 48,000*g* for 20 min at 4°C, prior to solid phase extraction (*see* **Subheading 3.2.**).

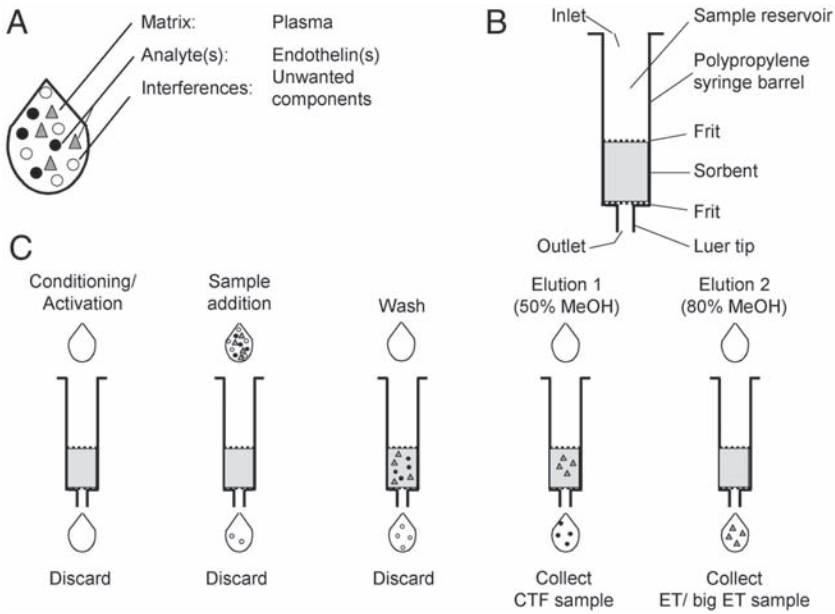

Fig. 1. Schematic diagram illustrating the differential solid phase extraction of IR-ET, big ET-1, and CTF.

3. Retain a portion of each homogenate for the measurement of protein concentration if the results are to be expressed as the amount of IR-ET/mg protein (*see* **Note 1**).

3.1.2. Collection and Preparation of Plasma

1. Collect whole blood into EDTA tubes, mix well, (but do not shake) and centrifuge at 2000*g* for 10 min at 4°C. Remove the plasma into polypropylene tubes. If necessary, samples of plasma may be stored at −80°C (*see* **Note 2**).
2. If samples are to be processed immediately, measure 5 mL of plasma into fresh polypropylene tubes and acidify by adding 1.25 mL of 2 *M* HCl (*see* **Note 3**). Cap the tube, mix by inversion, and then centrifuge for 10 min at 2000*g* to remove any precipitate.

3.2. Solid Phase Extraction of Plasma or Tissue Homogenates

3.2.1. Extraction of Tissue for Measurement by ELISA and RIA Kits

1. Attach the silica mini-columns (appropriately labeled with sample details) to the vacuum manifold and precondition by adding 2 mL of methanol. Allow this to pass completely onto the column before adding 2 mL of deionized water. It is important not to allow the columns to dry out before addition of the sample. If they do dry out, repeat the preconditioning step (*see* **Fig. 1**, **Note 4**).

2. Add the prepared plasma or homogenate samples (typically 2–5 mL) to the conditioned columns and allow these to pass through the column under gravity (do not use vacuum). If necessary, top the column up as the sample passes through until all of the sample is on the column. At this stage apply the vacuum carefully until no more sample drips through.
3. Wash the column through to waste (i.e., do not collect the eluate) with 2 × 2.8 mL vol of 0.1% TFA in water using vacuum to produce a flow-through rate of approx 1 mL/min. Continue to apply vacuum until no more wash drips through the column (*see* **Note 5**).
4. Place labeled tubes into the manifold to collect eluate.
5. Add 2 × 2 mL of 80% MeOH/0.1% TFA in deionized water. Allow both 2 mL aliquots to pass onto the column under gravity. Only then apply vacuum until no more eluate drips into the collection tubes.
6. Dry all tubes overnight in an evacuated centrifuge.
7. Cap tubes and store at −80°C, or process immediately.
8. Extracted samples, tissue culture media and physiological saline can be stored at −80°C until studies are complete.

3.2.2. Extraction of Samples for Detection of ET-1, Big ET-1, and CTF by RIA

1. Use C8 mini-columns for this extraction and carry out **steps 1–4** as described in **Subheading 3.2.1.** Continue as follows.
2. Elute the CTF fragment by adding 2 × 2 mL 50% MeOH/0.1% TFA in deionized water. Allow the solution to pass through, under gravity, until all of the solution is on the column. Only then apply vacuum until no more eluate drips into the collection tubes.
3. Place a new set of tubes into the manifold to collect the mature ET/big ET-1 fraction.
4. To elute the mature ET and big ET-1 fraction add 2 × 2 mL 80% MeOH/0.1% TFA, in deionized water, and proceed as described in **steps 5–8** under **Subheading 3.2.1.**

3.3. ELISA Protocol

1. Reconstitute and dilute kit reagents (buffers, standards, and antibody) as instructed.
2. Prepare a standards curve over the concentration range of 1–32 fmol/mL ET-1 by serial dilution in assay buffer.
3. Reconstitute the lyophilized samples by vortexing them in assay buffer (typically 250 µL) to allow duplicate aliquots of 100 µL to be used in the assay. It is crucial that the sample is thoroughly dissolved.
4. The recommended format of the 96-well plate precoated with anti-ET-$1_{(15–21)}$ comprises the first two wells for the blank, the second two for the nonspecific binding (NSB), 12 for the standards curve leaving 80 wells for the determination of 40 unknown samples in duplicate.

5. Pipet assay buffer (100 µL) into each NSB well and then the standards (2 × 100 µL aliquots from six standards of 1, 2, 4, 8, 16, and 32 fmol ET-1/well) and samples (2 × 100 µL of reconstituted sample or culture media used directly). Use new pipet tips for each duplicate.
6. Cover and incubate the microtiter plates for 16–24 h at 4°C.
7. To remove unbound material, wash and aspirate all wells 4× with 400 µL wash buffer using a 96-well microtiter plate washer. Blot the plate on tissue paper to remove any remaining liquid.
8. Detect bound ET-1 by pipeting 100 µL of the detection reagent (horseradish peroxidase [EC. 1.11.1.7] conjugated FAb' fragment of anti-ET-1 antibody reconstituted in assay buffer) into all wells, except the blank. Incubate for exactly 30 min at 37°C in a humidified container.
9. Repeat the washing and blotting step (**step 6** above).
10. Immediately add 100 µL of the chromogenic substrate (TMB, 3,3',5,5'-tetramethylbenzidine) into all wells. Cover and mix on a plate shaker at ambient temperature for exactly 40 min.
11. Add of 100 µL of 1.0 *M* sulfuric acid to each well to stop the development of the blue color reaction and produce a yellow color, which is more optically dense. Immediately mix, and measure the absorbance at 450 nm (within 30 min to avoid fading) using a 96-well microtiter plate reader.
12. Calculate the average absorbance for duplicate wells and subtract the mean NSB. Plot, mean absorbance (*y*-axis) vs ET-1 standard per well (*x*-axis) and interpolate unknown values from the standards curve. Alternatively, use the quadratic curve fitting programs that are supplied with plate readers that interpolate sample values from the standard curves automatically (*see* **Note 7** and **Fig. 2**).

3.4. RIA Kit Protocol

1. Reconstitute lyophilized samples by vortexing in assay buffer (typically 250 µL) to allow duplicate aliquots of 100 µL to be used in the assay. It is crucial that the sample is thoroughly dissolved and this may require several hours with sonication and/or trituration (*see* **Note 6**).
2. Prepare kit reagents as instructed (assay buffer, standards, antisera, and tracer).
3. Prepare the working standards by serial dilution of the stock solution provided to produce a standards curve for ET-1 over the concentration range 0.5–256 fmol/tube ET-1.
4. Aliquot samples, standards and blanks into labeled tubes according to the format suggested in the kit instructions. Briefly, use the first two tubes for the total count (Total, T), the second two for the nonspecific binding (Blank, NSB—add 200 µL assay buffer), the next two for the Zero standard (B_0—add 100 µL assay buffer). The next 16 tubes are for the standards curve (8 standards in duplicate, 100 µL of standard/tube, starting with the most dilute) and up to 40 samples in duplicate (80 tubes, 100 µL sample/tube). Use a new pipet tip for each duplicate.
5. Pipet 100 µL of the diluted primary antisera into all tubes except the Totals and Blank tubes. Vortex to mix thoroughly.

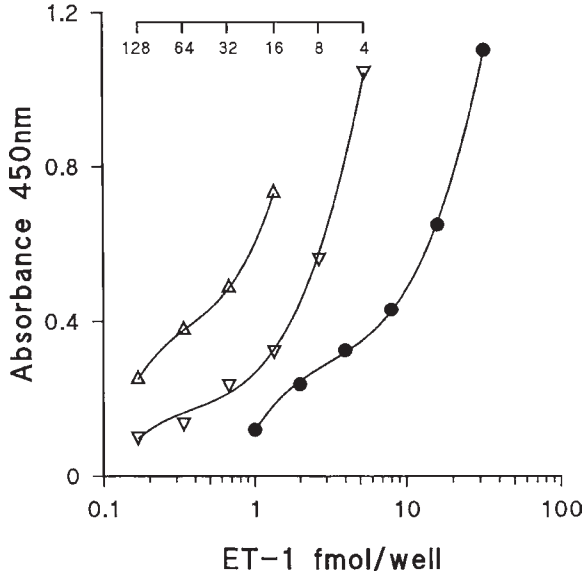

Fig. 2. An example of a standards curve for an ET-1 ELISA (•). The concentration of IR ET-1 in conditioned medium (Δ,∇) from human umbilical vein endothelial cells has been diluted as shown in the scale above the curves to illustrate parallelism with authentic standards.

6. Cover tubes with foil to prevent evaporation and incubate at 4°C for 16–24 h (i.e., overnight).
7. Prepare the radioligand tracer, [^{125}I]-ET-1 as instructed (*see* **Note 8**). Pipet 100 µL into all tubes, mix well, seal the Total tubes with caps, recover with foil and incubate for a further 16–24 h at 4°C.
8. Bring the Amerlex M separation reagent (donkey anti-rabbit antisera linked to magnetizable beads) to room temperature and aliquot 250 µL into all tubes (except the Total tubes). Vortex to mix and then incubate at room temperature for 10 min.
9. Separate the antibody bound fraction by transferring the tubes (except Totals) to the magnetic separation racks. Make sure that all tube bases are in contact with the magnetic base plate and that the racks fit the magnetic bases and are not loose.
10. Incubate the tubes for 15 min at room temperature. After this time the reagent should change from an opaque green mixture to a clear blue solution covering a small brown pellet that contains the bound counts. Invert the magnetic racks sharply over a sink (make sure it is suitable for the disposal of radioactive material), tipping the blue solution and unbound counts to waste (*see* **Note 8**). Do not reinvert the racks, leave inverted and place immediately onto absorbent paper, over foil, to drain for 5–30 min. Tap the bases of racks once or twice during this time. If magnetic racks are not available then the tubes can be

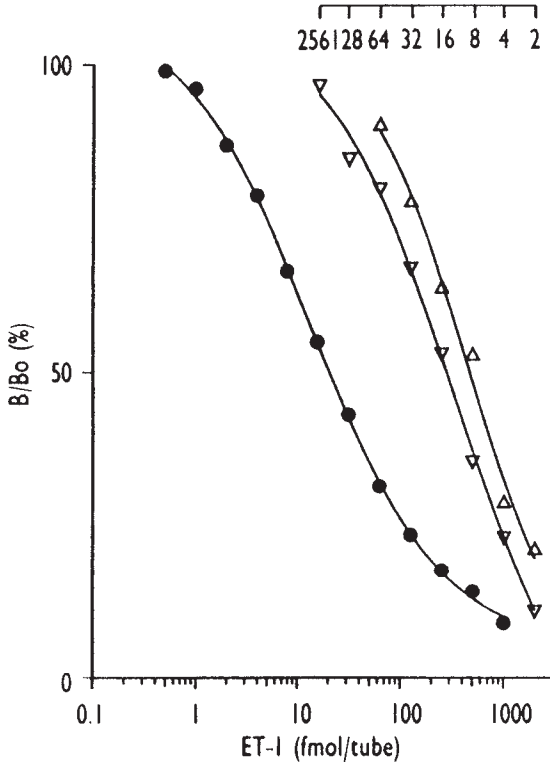

Fig. 3. An example of a standards curve for an ET RIA (•). The concentration of IR ET in extracts from human heart (∇,Δ) has been diluted as shown in the scale above the curves to illustrate parallelism with authentic standards.

centrifuged at 4°C for 10 min at 1500*g* or higher and the solution decanted to leave a pellet.

11. Transfer all tubes to gamma counter racks and measure radioactivity in each tube, including the Totals. Count each tube for at least 60 s.
12. Calculate the average CPM for each duplicate and subtract the background. Calculate $\%B_0/T$:

$$\%B/\text{Total} = (B_0 \text{ cpm} - \text{NSB cpm})/(\text{Total cpm} - \text{NSB cpm}) \times 100$$

13. Calculate %B/B0 for each standard and sample:

$$\%B/B_0 = (\text{Standard or sample cpm} - \text{NSB cpm})/(B_0 \text{ cpm} - \text{NSB cpm}) \times 100$$

Plot mean $\%B/B_0$ (*y*-axis) vs log ET-1 standard fmol/tube (*x*-axis) and interpolate unknown values from the standards curve (*see* **Fig. 3**). Alternatively quadratic curve fitting programs supplied with gamma counters can be used and sample values interpolated from the standard curves automatically.

3.5. RIA for the Separate Measurement of ET-1, Big ET-1, and CTF

The RIA is carried out as described in **Subheading 3.4.** with the following modifications/additions to each step.

1. Reconstitute samples by the addition of 250 µL of RIA buffer for the 50% MeOH, CTF fraction and 500 µL for the 80% MeOH, mature ET/ big ET fraction (*see* **Note 6**).
2. Prepare standard curves for both ET-1 and big ET-1 from the stocks of each peptide. Dilute the ET-1 stock solution (10^{-4} *M*) in RIA buffer to give 2 mL of a 10^{-8} *M* solution. This is the top concentration of the standard curve. From this, perform serial dilutions of 1 mL + 1 mL eleven times, each in RIA buffer, to give a total of 12 concentrations/standard curve (*see* **Note 9**).
3. Aliquot samples, standards and blanks into labeled tubes according to the format given in **Table 1** (*see* **Note 10**).
4. Dilute the appropriate primary antibody for each assay in RIA buffer to the previously empirically determined dilution. Aliquot 100 µL into each tube (*see* **Table 1**), vortex to mix, cover the tubes with foil to prevent evaporation, and incubate at 4°C overnight.
5. Prepare the radioligand tracer (*see* **Note 8**) for each assay, [^{125}I]-ET-1 for the mature assay and [^{125}I]-big ET-1 for the big ET-1/CTF assay, by dilution into RIA buffer to achieve 10–15,000 cpm/100 µL. Aliquot 100 µL into all tubes, mix well, cap the Total tubes, recover with foil and incubate overnight at 4°C.
6. Continue **steps 6–12** as described in **Subheading 3.4.**

4. Notes

1. There are a variety of methods for protein determination commercially available. When measuring protein levels in sections of tissue we solubilize the proteins in 0.5 *M* NaOH/1% SDS (heated to 80°C for 30 min and centrifuged to remove any precipitate). Therefore, our protein assay of choice is the Bio-Rad DC assay (500-0116, Bio-Rad, Hemel Hempstead, Herts, UK), which is specifically designed to be compatible with detergents.
2. The tubes containing whole EDTA blood can be spun at speeds equating to 1200–2000*g*, for 6–10 min, (i.e., less *g*—more time). Excessive *g*-force will result in hemolysis, which is undesirable. Too low a speed results in poor volume recovery and low plasma ET estimation. Polypropylene tubes are recommended for storage of plasma samples as they exhibit low peptide binding and are structurally competent at −80°C.
3. The ratio of plasma to 2 *M* HCl is 4:1 v/v, smaller volumes of plasma may be extracted but the final sensitivity of the assay will be lower. With a 5 mL plasma volume, reconstituted after extraction to 250 µL and assayed (2 × 100 µL) for a single analyte, each assay tube will contain the equivalent of 2 mL of plasma and the detection limit of plasma will be approx 0.5 pmol/L. Where the extracted

Table 1
Format for Radioimmunoassay

Tubes	Standard/sample (µL)		Buffer (µL)	1° Antibody (µL)	Label (µL)	Amerlex (µL)
1–2	TOTAL	–	–	–	100	–
3–4	BLANK/NSB	–	200	–	100	250
5–6	REF/B0	–	100	100	100	250
7–8	0.488*	100	–	100	100	250
9–10	0.977	100	–	100	100	250
11–12	1.95	100	–	100	100	250
13–14	3.91	100	–	100	100	250
15–16	7.81	100	–	100	100	250
17–18	15.63	100	–	100	100	250
19–20	31.25	100	–	100	100	250
21–22	62.5	100	–	100	100	250
23–24	125	100	–	100	100	250
25–26	250	100	–	100	100	250
27–28	500	100	–	100	100	250
29–30	1000	100	–	100	100	250
31–32	sample 1	100	–	100	100	250
33–200	samples 2–85	100	–	100	100	250

*Tubes 7–30 are standard curve dilutions (pmol/L) in duplicate.

sample is reconstituted in 500 µL and assayed in duplicate, e.g., for mature ET (2 × 100 µL) and big ET-1 (2 × 100 µL), each assay tube will contain the equivalent of 1 mL of plasma, making the lowest plasma concentration of ET-like immunoreactivity detectable to be 1 pmol/L. This assumes an assay detection limit (sensitivity) of 1.0 fmol/tube (100 µL).

4. Vacuum can be used to speed up the various stages of solid-phase extraction, however, it is essential that the column does not dry out during the conditioning or sample application stages. During sample application, only apply vacuum to the columns such that the flow rate through the column does not exceed 1 mL/min.
5. The columns in routine use in our laboratory have a volume above the sorbent bed of 2.8 mL. We therefore normally fill the columns to the brim twice in the washing stages, as this is both quicker, and ensures that all traces of sample are washed through the column. If robotic systems are being used, 5 mL of 0.1% TFA is a sufficient wash.
6. It is essential that the lyophilized, solid-phase extracted samples are allowed to fully dissolve in the ELISA/RIA buffer. The inclusion of Tween-20 (0.1%) in the RIA buffer facilitates this. Careful choice of the conditions of vacuum centrifugation (minimized time and applied heat) will assist this. Sometimes the samples will need to be left overnight at 4°C to redissolve completely.
7. Validation of the two-site ELISA: The sensitivities of detection for this assay (defined as two standard deviations above mean zero dose absorbance) are 0.5 fmol/well with ED_{50} values of 13 fmol/well. Intra- and interassay are $<$6.6% and $<$20.3%. The ELISA crossreacted with ET-1 (defined as 100%) and ET-2 ($>$100%). Crossreactivity with big ET-1 was very low (0.07%), and was undetectable with the C-terminal fragment of big ET-1, ET-3, big ET-2 and big ET-3 at the highest concentrations tested. There was no detectable crossreactivity with unrelated vasoactive peptides such as atrial natriuretic factor and porcine brain natriuretic peptide. Importantly, the ET_A selective antagonist FR139317 and the ET_B selective agonist BQ3020 did not crossreact, indicating that this assay can be used to measure IR ET in the presence of these compounds. Dilution curves of the conditioned medium from human umbilical vein endothelial cell cultures, known to secrete mature ET peptide, paralleled those of the standard ET-1 (*see* **Fig. 1**). This confirmed that the immunoreactivity detected in the tissue culture supernatants was the expected endogenous ET-1.
8. Follow local rules for the use of ionizing radiation in your laboratory. Dispose of aqueous radioactive waste in a designated sink.
9. We have found that 200 tubes are the optimum number that can be processed in one batch at the precipitating antibody stage. Each batch of 200 tubes must contain a full standard curve, which occupies 30 tubes, leaving 170 tubes, which is sufficient for the assay of 85 samples in duplicate. It may therefore be necessary to increase volumes of standards produced, as there should be at least one standard curve included per 200 tubes (i.e., per 85 samples in duplicate).
10. The big ET-1 RIA will have twice as many tubes as the mature ET assay as the 50% MeOH fractions (containing CTF) are assayed in this RIA.

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