# Determination of endothelin-1 immunoreactivity in plasma, cerebrospinal fluid and urine

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**Abstract.** Endothelin-1 (ET-1) is a potent vasoconstrictor peptide apparently involved in a number of vascular diseases in man. Nonetheless, its determination in biological samples is difficult, and data on plasma or urine concentrations are controversial. We investigated different sample preparation procedures as well as different radioimmunoassays for their influence on ET-1 measurement. Recovery of ET-1 depended on the extraction procedure, the type and size of the extraction columns and on the biological matrix itself. Incomplete removal of matrix components by the extraction leads to the formation of particulate matter in the evaporated eluate. When dissolved in assay buffer, ET-1 was found to be absorbed to and only partly released from these particulates, so that it was not accessible for measurement in a radioimmunoassay. This was the case for all extraction procedures investigated except for that involving acetic acid. HPLC analysis of spiked samples revealed that ET-1 is in part degraded during extraction, most probably to Meth-sulphoxide ET-1. Dilution curves of synthetic pure ET-1 standards from different suppliers, prepared either in plasma with subsequent extraction or in assay buffer of the respective radioimmunoassay, resulted in considerable differences in the measured values for ET-1-immunoreactivity. Every radioimmunoassay tested had a specific pattern of recognizing different synthetic ET-1 standards. In conclusion, the measurement of ET-1-immunoreactivity is strongly influenced by the experimental conditions of sample preparation as well as by the radioimmunoassay employed.

**Key words:** Endothelin-1 – Radioimmunoassay – HPLC – Immunoreactivity – Extraction

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*Abbreviations:* ET-1, Endothelin-1; AA, acetic acid; TFA, trifluoroacetic acid; MeOH, methanol; EtOH, ethanol; RIA, radioimmunoassay; HPLC, high-pressure liquid chromatography

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## Introduction

Endothelin-1 (ET-1), which is the most potent vasoconstrictor peptide yet discovered, was originally isolated from culture supernatants of vascular endothelial cells [1]. Other mammalian endothelin isopeptides (endothelin-2 and -3) have also been identified, but only ET-1 is produced by endothelial cells and seems to occur in substantial amounts in human plasma. ET-1 is apparently involved in a number of vascular diseases in man. Elevated plasma levels of ET-1 immunoreactivity (ET-1 IR) have been detected in myocardial infarction [2], vasospastic angina [3], hypertension [4, 5], vasculitis [6], subarachnoid haemorrhage [7, 8], diabetes mellitus [9, 10], and HIV infection [11, 12].

In spite of these numerous studies, data on plasma endothelin concentrations are hardly comparable and in part controversial [9, 10, 13–16]. Although several radioimmunoassays (RIA) for the selective measurement of ET-1 are available, results from different laboratories vary considerably. There is no consensus technique for the determination of this peptide, nor is there a generally accepted normal range in human plasma [9, 13]. Moreover, the antisera used in the respective RIAs are sensitive to small modifications in the chemical structure of ET-1, as shown by the (often considerable) cross-reactivity of antisera between the three different but structurally closely related endothelins.

ET-1 cannot be measured directly in biological fluids, but must be purified and concentrated by an extraction step. The chemical structure of this 21-amino-acid peptide includes two intrachain disulphide bonds (between positions 1–15 and 3–11) as well as one methionine in position 7 [1]. Therefore, it is very susceptible to chemical modification; reduction with subsequent cleavage of the disulphide bonds as well as oxidation of the methionine may occur during storage or processing of samples, depending on the assay conditions. Very little is known of the influence of these degradation products on the binding affinity of ET-1 to the antibodies used in the respective radioimmunoassays. Moreover, matrix components like proteins, lipids, or salts not removed by the extraction procedure may influence the binding of the antibodies to ET-1 in a given radioimmunoassay. The purpose of this study, therefore, was to evaluate several extraction procedures described in terms of recovery and stability of ET-1, and to investigate several available RIAs regarding the immunoreactivity of different synthetic ET-1 preparations in order to establish a reliable method for the determination of ET-1 in biological fluids.

## Materials and methods

### *Reagents*

Synthetic purified ET-1 was obtained from several suppliers: Serva (Heidelberg, Germany), Sigma (Deisenhofen, Germany), Peninsula (Belmont, Calif.) and NEN/Dupont (Dreieich, Germany). Synthetic ET-2 and ET-3 were purchased from Serva and 3-[$^{125}$I] iodotyrosyl ET-1 with a specific activity of 74 TBq/mmol and a radiochemical purity of > 90% from Amersham (Braunschweig, Germany).

Extraction was performed on silica-C18 cartridges (Sepak C18 Vac, 500 mg, Waters/Millipore, Eschborn, Germany) unless otherwise indicated. Other extraction cartridges tested were Sepak C18 classic 360 mg (Waters/Millipore) and Bond-Elut Si-C18 500 mg, 200 mg, 100 mg (Analytichem International, obtained from ict, Frankfurt, Germany). Columns were used only once.

Five RIAs for the measurement of ET-1 that were commercially available at the time of the study were evaluated: Endothelin 1-21 specific [$^{125}$I]assay system (Amersham, Braunschweig, Germany); Endothelin-1, 2 RIA (Biomedica, Vienna, Austria); Endothelin-1, 2 [$^{125}$I] RIA Kit (NEN/Dupont, Dreieich, Germany); Endothelin RIA (Nichols Institute, Bad Nauheim, Germany); Endothelin-1 RIA (Peninsula Laboratories, Belmont, Calif.). Trifluoroacetic acid (TFA, 1-ml sealed ampoules) and siliconizing reagent (Sigmacote) were purchased from Sigma (Deisenhofen, Germany). Phosphate-buffered saline (PBS) contained 0.145 *M* NaCl, 0.018 *M* $Na_2HPO_4$ and 0.010 *M* $KaH_2PO_4$. All other chemicals used were of reagent grade or better and obtained from Merck (Darmstadt, Germany).

## *Sample collection*

For the collection of pooled human plasma, blood from healthy volunteers was drawn into EDTA-containing vacutainer tubes (Greiner, Frickenhausen, Germany) without other additives, placed immediately on ice, and centrifuged at 4°C. Plasma was pooled and stored until use at –20°C. Cerebrospinal fluid (3 ml from each) was collected after informed consent from patients undergoing spinal anaesthesia. Urine was collected from three volunteers in the morning, centrifuged at 4°C and stored at –20°C.

## *Extraction procedures*

Recovery of the respective extraction procedures was investigated by adding to the sample prior to extraction the $^{125}$I-labelled ET-1 at different concentrations (0.4–120 fmol/ml) with or without non-labelled ET-1 (400–40,000 fmol/ml). Radioactivity in the collected fractions was measured in a 12-well multi gamma counter (LB 2111, Berthold, Wildbad, Germany). In order to study the fate of ET-1 after extraction, the evaporated eluate was dissolved in 0.5 ml of RIA buffer by repeated vigourous vortexing and left on ice for 1 h. The residual particulate matter and the supernatant were separated by centrifugation. To the remaining pellet, another 0.5 ml of RIA buffer was added, and the procedure was repeated with additional sonification. Each step was monitored for labelled ET-1. All these experiments were done in triplicate.

Stability of ET-1 in the respective extraction procedures was determined by HPLC analysis. An aliquot of 10 µg of ET-1 (Serva, Heidelberg, Germany) was added to a 2.5-ml plasma or urine sample or to 0.2 ml of PBS and extracted as described. The eluate was dried under reduced pressure and redissolved in 100 µl PBS; 80 µl was injected onto the column.

The capacity of the respective extraction procedures to remove matrix components was judged by chemical analysis. The eluate was dissolved in 1 ml of distilled water, centrifuged and the supernatant investigated. Osmolarity was measured with a micro osmometer (3 MO, Advanced Instruments, obtained from Dinkelberg Labortechnik, Neu-Ulm, Germany), total protein with Coomassie Brilliant Blue G 250 according to the method of Bradford [17], Na and K on a flame photometer (Eppendorf, Hamburg, Germany) and uric acid, creatinine and urea on an automated clinical chemical analyser (Hitachi 717, Boehringer Mannheim, Germany). All these experiments were done in duplicate. Lipid content in the eluate was analysed by qualitative thin-layer chromatography on silica gel 60 plates (200 × 200 × 0.25 mm, Merck, Darmstadt, Germany), using a standard one-dimensional system for the separation of simple lipid classes with hexen/ether/glacial acetic acid (80/20/2, v/v/v) as solvent. Plates were sprayed with 0.001% 8-anilino-1-naphtalen sulphonate and lipids were visualized as fluorescent spots under UV light. Alternatively, plates were stained with iodine.

The following extraction procedures were tested:

1. Extraction with acetic acid (AA) [18]. Si-C18 cartridges were conditioned with one column reservoir volume (CV) of MeOH followed by one CV of water and one CV 10% AA, then 2.5 ml of plasma or urine was diluted with an equal volume of 20% AA, centrifuged (10 min, 2,600 *g*) and applied to the columns. The sample was passed through at a flow rate determined by gravity (1 × *g*). Subsequently, columns were washed with one CV 10% AA and excess AA was removed by applying reduced pressure. Columns were then washed with 2 CVs of ethyl acetate and dried under reduced pressure. ET-1 was eluted with 3 ml of MeOH/0.05 *M* ammoniumbicarbonate (80/20, v/v) and the eluate dried in a centrifugal evaporator (Speedvac, Bachhofer, Stuttgart, Germany).

2. Extraction with trifluoroacetic acid (TFA) [8]. Si-C18 cartridges were conditioned with 4 ml of 60% acetonitrile in 0.1% TFA followed by 20 ml of 0.1% TFA, then 2.5 ml of plasma or urine was diluted with an equal volume of 0.2% TFA, centrifuged (10 min, 2,600 *g*) and applied to the columns. The sample was passed through at a flow rate determined by gravity. Subsequently, columns were washed with 20 ml of 0.1% TFA and ET-1 was eluted with 3 ml of 60% acetonitrile in 0.1% TFA. Care was taken not to dry the columns during this procedure.

3. Extraction with ethanol (EtOH) [19]. Si-C18 cartridges were conditioned with 5 ml of MeOH, 5 ml of water and 5 ml of 4% AA, then 2.5 ml of plasma or urine was diluted with an equal volume of 4% AA, centrifuged, applied to the columns and passed through at a flow rate determined by gravity. Columns were washed with 3 ml of water and 3 ml of 25% EtOH in water. ET-1 was eluted with 3 ml of 4% AA in 86% EtOH.

4. Extraction with methanol (MeOH) [20]. Si-C18 cartridges were conditioned with 2 ml of MeOH followed by 3 ml of water, then 2.5 ml of plasma or urine was applied to the columns and passed through at a flow rate determined by gravity. Columns were washed with 4 ml of 10% MeOH in water and ET-1 was eluted with 4–6 ml of 85% MeOH in water.

### *Absorption of ET-1 on surfaces*

To investigate the adherence of ET-1 to various surfaces, 30 fmol $^{125}$I-ET-1 in 0.2 ml of PBS or in 0.2 ml of plasma was pipetted in triplicate into test tubes (5 ml) of borosilicate glass, polystyrol, polypropylene or siliconized polypropylene, or into 1.5-ml reaction vials (Eppendorf, Hamburg, Germany) or polypropylene-like HPLC micro vials (250 µl, Weidmann Plastik, Switzerland). After standing for 30 min at room temperature the tubes were vigorously vortexed, spun down and the contents completely transferred to a new tube of the same type. This procedure was repeated twice, and in each step transferable and adherent radioactivity was counted. In all other experiments with ET-1, siliconized polypropylene tubes were used.

### *Separation of ET-1, -2, -3 by HPLC*

The HPLC system consisted of a L 6200 gradient-controlled pump, an AS 2000 autosampler and a L 4000 variable-wavelength UV detector connected by a D 6000 interface to a D 6000 Chromatography Data Station (all Merck-Hitachi, Darmstadt, Germany). Analysis was performed on a C18 reversed-phase column (250 × 4.6 mm, Superspher RP 18 endcapped 4 µm, Merck, Darmstadt, Germany), using a linear gradient from 10% acetonitrile in 0.1% TFA to 60% acetonitrile in 0.1% TFA over 60 min, followed by a purge gradient over 15 min to 100% acetonitrile in 0.1% TFA and re-equilibration for 20 min. Flow rate was set to 1 ml/min, column temperature maintained at 40°C and column effluent monitored at 210 nm.

### *Comparison of concentration with immunoreactivity of ET-1*

Standard curves (1–50 fmol/ml) were prepared with different synthetic ET-1 standards (see above), either in pooled human plasma followed by extraction with AA, or directly in the assay buffer of the respective RIA. These samples, together with extracted blank plasma samples, were analysed in the five RIAs evaluated. The added amount of ET-1 was compared with the immunoreactivity measured. All these experiments were done in triplicate, and in at least two different RIAs from the same supplier. To investigate the influence of the different extraction procedures on the RIA performance, plasma and urine samples with and without 10 fmol/ml ET-1 (NEN) added ($n$ = 3 each) were extracted by the procedures described and analysed for immunoreactivity in the RIA obtained from NEN.

### *Statistical analysis*

Statistical calculations were performed using the SPSS software package (SPSS/PC 4.0, Chicago, USA). Tests for statistical significance were calculated with Student's $t$-test and the Chi-square test.

**Table 1.** Comparison of the different extraction procedures. Recovery of $^{125}$I-labelled ET-1 and residual components of the sample matrix contained in the eluate are shown for the different extraction procedures. Values are expressed as mean ± SD. CSF, cerebrospinal fluid

| | Recovery of $^{125}$I-ET-1 (% of total) | Matrix residues in the eluate/obtained from a 2.5 ml sample | | |
|---|---|---|---|---|
| | | Total protein (mg/l) | Osmolarity mosmol/l) | pH |
| Extraction from plasma with | | | | |
| AA | 88.4 ± 1.2 | 47.8 ± 4.7 | 11 ± 1 | 5.5 ± 0.1 |
| TFA | 87.0 ± 0.2 | 1205.0 ± 33.8 | 8 ± 1 | 2.0 ± 0.1 |
| EtOH | 84.9 ± 1.8 | 216.6 ± 17.5 | 0 ± 0 | 5.2 ± 0.1 |
| MeOH | 67.2 ± 2.0 | 164.9 ± 19.1 | 0 ± 0 | 5.1 ± 0.2 |
| Extraction from urine with | | | | |
| AA | 98.6 ± 0.4 | 41.9 ± 3.4 | 7 ± 1 | 5.2 ± 0.1 |
| TFA | 98.0 ± 0.3 | 33.1 ± 4.9 | 56 ± 3 | 1.5 ± 0.1 |
| EtOH | 99.6 ± 0.1 | 17.5 ± 2.9 | 4 ± 0 | 3.6 ± 0.2 |
| MeOH | 92.1 ± 1.4 | 25.3 ± 5.8 | 2 ± 1 | 4.4 ± 0.1 |
| Extraction from CSF with | | | | |
| AA | 97.6 ± 0.2 | 36.7 ± 2.8 | 6 ± 2 | 5.4 ± 0.1 |

## Results

### *Recovery of ET-1 in the different extraction procedures*

Recovery rates for the extraction of $^{125}$I-labelled ET-1 from plasma were more than 65% of total radioactivity, and for extraction from urine or cerebrospinal fluid more than 90% of the total, and were thus satisfactory for all procedures tested. However, extraction efficiency was significantly influenced by the method used (Table 1). It was highest and nearly identical for the methods involving AA or TFA, but significantly lower ($P < 0.05$) for the procedures involving EtOH or MeOH. For the extraction with MeOH in particular, the elution volume of 3 ml used in the other extraction procedures only released 13% of ET-1, and up to 6 ml was necessary to remove ET-1 completely from the columns. Recovery of ET-1 from urine or cerebrospinal fluid was almost complete, independent of the type of extraction procedure (Table 1).

### *Matrix effect on the extraction efficiency*

Recovery was influenced by the matrix, and was significantly lower in plasma than in urine or cerebrospinal fluid. We found that 8–10% of ET-1 was contained in the breakthrough of the plasma applied to the columns, whereas in urine or cerebrospinal fluid only 1–2% of ET-1 could be measured in this fraction. Moreover, addition of either TFA or AA to a plasma sample caused a mild protein precipitation. After centrifugation there was a significant increase in the radioactivity measured in the resulting pellet, from 1.81 ± 0.05% to 2.80 ± 1.11% and 6.44 ± 0.94% of total if 2.5 ml water, 2.5 ml or 5 ml of 0.2%TFA was added, respectively. Very similar values were seen for acidification with AA (data not shown).

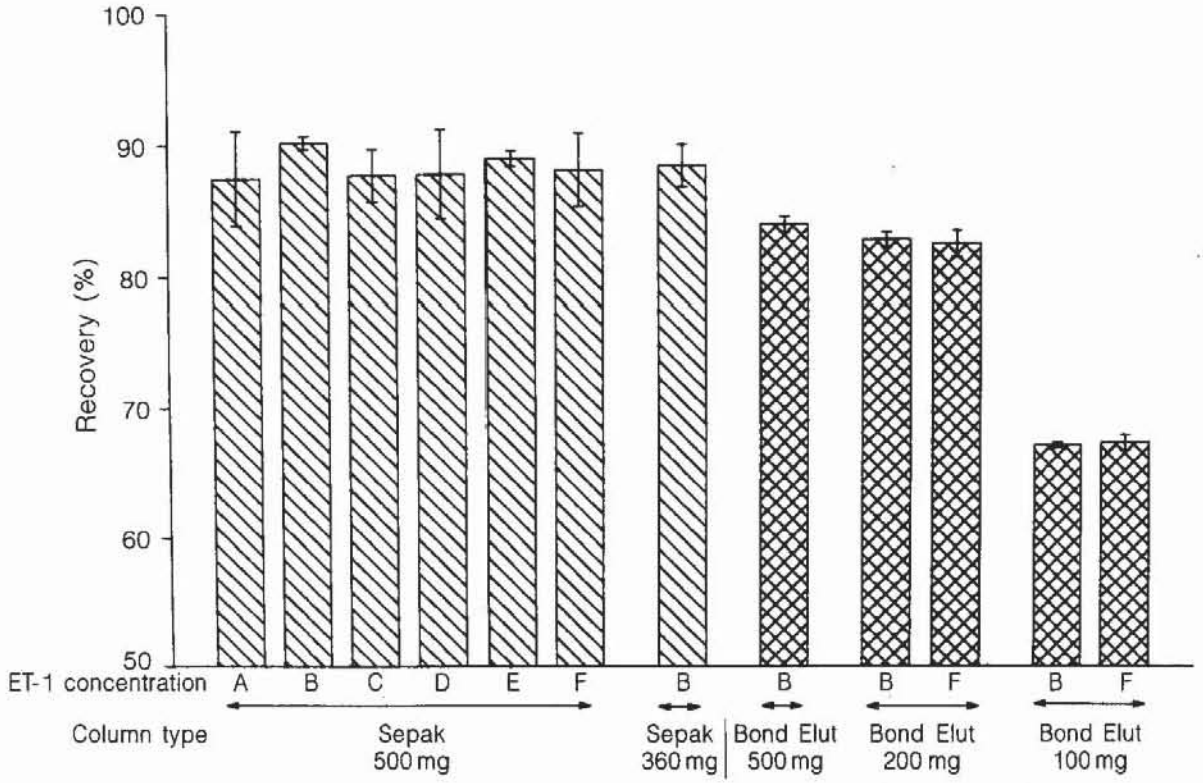



**Fig. 1.** Recovery of ET-1 depended on the column type and size, but not on ET-1 concentration. Extraction from plasma was performed on different C18 columns, indicated at the bottom of the figure. The *letters* represent the different ET-1 concentrations added: *A, B, C*, 0.4, 12 and 120 fmol/ml $^{125}$I-labelled ET-1; *D, E, F*, 12 fmol/ml $^{125}$I-labelled ET-1 + 400, 4,000 and 40,000 fmol/ml unlabelled ET-1. Mean ± SD is shown

### *Other factors influencing recovery*

Recovery was dependent on the flow rate used to pass the sample through the column. It decreased significantly from 83.4 ± 2.6% of total at a flow rate of $1 \times g$ to 71.5 ± 1.4% ($n = 3$ each) if the flow rate was increased by applying reduced pressure of 12 mmHg. Recovery of labelled ET-1 depended on the type and size of C18 column tested (Fig. 1). Sepak cartridges gave a slight but significantly ($P < 0.05$) higher recovery than Bond-Elut of comparable size. Column sizes of less than 200 mg caused a significant loss of ET-1 in the sample fraction. In contrast, the extraction recovery did not depend on the ET-1 concentration in the range tested. For samples containing 0.4, 12, and 120 fmol/ml $^{125}$I ET-1, as well as for samples containing 12 fmol/ml $^{125}$I ET-1 and 400, 4,000, and 40,000 fmol/ml, unlabelled ET-1 recoveries were not significantly different on Sepak 500 mg columns (Fig. 1). Interestingly, the lower recoveries for Bond Elut 200 mg and 100 mg columns were equally observed with samples containing only 12 fmol/ml $^{125}$I-labelled ET-1 or 12 fmol/ml $^{125}$I-labelled ET-1 + 40,000 fmol/ml unlabelled ET-1.

### *Residual matrix components*

The efficiency of the extraction procedures to clear the sample from major plasma or urine components is shown in Table 1. For extraction of plasma, AA proved to be the most effective procedure to remove proteins and reduced the average protein content of plasma (70 g/l) to 50 mg/l in the eluate. In contrast, extraction with

**Table 2.** Influence of urine composition on extraction. Recovery of $^{125}$I-labelled ET-1 and residual matrix components after extraction with AA. Samples 1–5 were prepared from the same urine. 1, 2 and 3 were adjusted to pH 0.7, 5.5 and 9.2, respectively; 4 and 5: addition of 2.6 g/l creatinine, 50 g/l urea and 1 g/l uric acid without (4) and with (5) addition of 50 g/l albumin. Values are given as mean ± SD. Addition of albumin reduced recovery significantly ($P < 0.05$)

| Extraction from urine with acetic acid | Recovery of $^{125}$I-ET-1 (% of total) | Total protein (mg/l) | Osmolarity (mosmol/l) | pH | Creatinine (mg/l) | Urea (mg/l) | Uric acid (mg/l) |
|---|---|---|---|---|---|---|---|
| 1 | 95.0 ± 0.8 | 32.1 ± 1.8 | 0 ± 0 | 5.3 ± 0.1 | 0.7 ± 0.2 | 20 ± 4 | 3 ± 2 |
| 2 | 91.7 ± 0.1 | 56.3 ± 1.6 | 8 ± 3 | 5.5 ± 0.2 | 3.3 ± 0.6 | 50 ± 3 | 354 ± 14 |
| 3 | 94.5 ± 0.3 | 81.8 ± 3.4 | 16 ± 0 | 5.4 ± 0.1 | 10.3 ± 0.9 | 90 ± 4 | 457 ± 12 |
| 4 | 91.0 ± 0.8 | 43.5 ± 2.6 | 15 ± 4 | 5.5 ± 0.1 | 5.7 ± 0.8 | 80 ± 6 | 611 ± 27 |
| 5 | 85.6 ± 0.6 | 71.4 ± 3.8 | 36 ± 2 | 5.2 ± 0.2 | 28.4 ± 1.1 | 280 ± 11 | 147 ± 12 |

TFA resulted in a residual protein content in the eluate of 1,200 mg/l, beside the insoluble particulates in which the presence of protein was also suspected.

Thin-layer chromatography revealed the presence of triglycerides and cholesterol in the eluate after extraction of plasma with TFA or EtOH, and of triglycerides only after extraction with MeOH. No lipids could be detected after extraction with AA. In urine, no lipids could be found with any of the extraction procedures. All extraction procedures, except for that of TFA, reduced the osmolarity of the urine tested (750 mosmol) to < 10 mosmol. The eluate was weekly acidic (pH 5) except for extraction with TFA (pH 2).

Blank plasma samples ($n = 3$ each) extracted with AA, EtOH and MeOH measured in the RIA of NEN yielded concentrations of 6.9 ± 1.2, 5,9 ± 2.1 and 16.2 ± 0.3 fmol/ml, respectively. Parallel samples spiked to 10 fmol/ml resulted in measured values of 20.3 ± 0.6, 17.7 ± 5.9 and 49.0 ± 13.0, respectively. After extraction with TFA, however, blank as well as spiked samples yielded values off the standard curve, so that it was not possible to calculate concentrations. There was a high non-specific binding of the tracer, most probably due to matrix residues, as binding of the tracer in the samples (B/Bo) was higher than in the zero standard (Bo/T).

*Extraction of urine samples*

As tested with AA, the pH of the sample (from 0.7 to 9.2) did not significantly influence either the pH of the eluate or the recovery (Table 2). In contrast, there was a slight increase of total protein, osmolarity, creatinine, urea, and uric acid concentrations in the eluate with pH ($P < 0.05$). Modifying the matrix, simulating an extremely concentrated urine by addition of 2.6 g/l creatinine, 50 g/l urea and 1 g/l uric acid, did not significantly affect the parameters measured as compared with the native urine, except for a slight increase of uric acid content, nor did it influence recovery of ET-1. In contrast, if 50 g/l albumin was additionally added, recovery was significantly ($P < 0.05$) reduced to the range of that of plasma. Creatinine and urea content of the eluate were slightly ($P < 0.05$) increased, but pH, total protein and uric acid concentration were not changed.

**Table 3.** Absorption of ET-1 to particulate material of the eluate. The evaporated eluate from plasma or urine was dissolved in assay buffer and the supernatant was separated from the insoluble particulates. Recovery of $^{125}$I-labelled ET-1 in the different fractions is given as % of total. Mean ± SD is shown

| Extraction procedure | Plasma | | | Urine | | |
|---|---|---|---|---|---|---|
| | Supernatant 1 | Supernatant 2 | Residual pellet | Supernatant 1 | Supernatant 2 | Residual pellet |
| AA | 99.60 ± 0.05 | 0.39 ± 0.12 | 0.00 ± 0.00 | 99.54 ± 0.21 | 0.31 ± 0.30 | 0.00 ± 0.00 |
| TFA | 39.10 ± 3.47 | 14.25 ± 1.89 | 45.35 ± 2.55 | 36.24 ± 3.12 | 14.44 ± 0.56 | 47.61 ± 4.51 |
| EtOH | 75.53 ± 20.87 | 20.87 ± 3.78 | 0.37 ± 0.39 | 40.40 ± 4.60 | 44.82 ± 0.10 | 7.11 ± 2.45 |
| MeOH | 60.40 ± 9.39 | 16.30 ± 2.57 | 20.96 ± 5.41 | 77.15 ± 1.34 | 18.89 ± 0.23 | 1.11 ± 0.19 |

*Recovery of ET-1 from the eluate*

After evaporating the eluates, only small and easily soluble pellets were seen for extraction with AA, whereas for extraction with MeOH or EtOH larger pellets, and for extraction with TFA rather large pellets containing insoluble particulate matter were observed.

After separation of the supernatant from the particulate fraction, ET-1 was almost completely recovered in the case of the extraction with AA, whereas between 25% and 60% of ET-1 was contained in the particulate fraction after extraction with EtOH, MeOH or TFA (Table 3). After a second attempt to dissolve the remaining particulates by vigorous vortexing followed by sonification, a substantial amount of ET-1 still remained firmly bound to the particulate fraction after extraction with MeOH or TFA. Very similar results were obtained for urine samples (Table 3). The loss of ET-1 into the pellet was consistent in at least two sets of experiments. The percentage of ET-1 bound to the pellet was somewhat variable within one extraction procedure, ranging, for instance, from 45.6% to 74.0% of the total for pellets obtained with TFA.

*Absorption of ET-1 to different surfaces*

$^{125}$I-labelled ET-1 adhered to the surface of routinely used test tubes. Absorption (% of total, experiments in triplicate, coefficient of variation < 2%) was 61.9% for borosilicate glass, 61.8% for polystyrol, 65.6% for polypropylene, 47.0% for polypropylene-like HPLC-microvials and 55.1% for Eppendorf reaction vials. Siliconization of polypropylene tubes, or the addition of 0.2 ml of plasma prior to ET-1 reduced absorption to 2.9% and 4.2%, respectively. The average adherence to Gilson pipette tips was 3.6%. If the non-adherent ET-1 was transfered to a new test tube, approximately the same amount of ET-1 was found to be absorbed as in the first experiment (data not shown).

*High performance liquid chromatography*

The HPLC method described resulted in baseline separation of ET-1, -2, -3, and a degradation product of ET-1. Retention times in this system were constant, with a interassay coefficient of variation of 1.0% for ET-1. The method was sufficiently

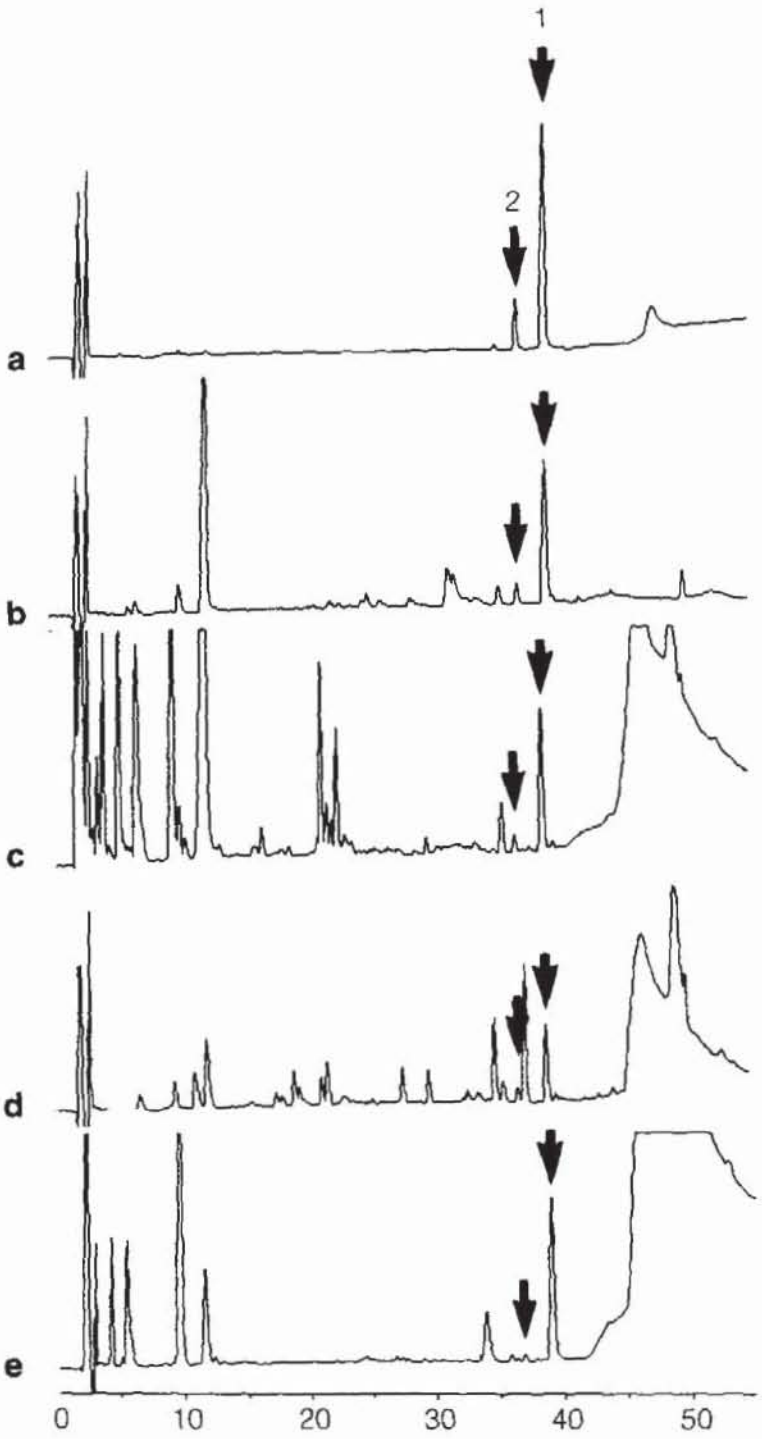



**Fig. 2.** HPLC analysis of (**a**) synthetic ET-1 from Serva, 8 µg/injection, and (**b–e**) a 2.5-ml plasma sample spiked with ET-1 (8 µg/injection) after extraction with: **b** AA; **c** TFA; **d** EtOh; **e** MeOH. *Arrow (1)* indicates the elution position of unchanged ET-1, *arrow (2)* the elution position of the degradation product

precise to allow reliable quantification of ET-1, with intra- and interassay coefficients of variation of 3.6 and 6.1% for injection of 5 µg of ET-1 and quantitation by peak area. Detection limit was 0.1 µg/injection, and the limit of lowest reliable quantification was 1 µg/injection. The determination of ET-1 was linear in the range tested from 1 to 20 µg/injection. ET-1 diluted in PBS or water and injected directly onto the HPLC eluted in two peaks, a major one at 40.1 min (peak 1) and a smaller one at 37.2 min (peak 2) (Fig. 2). The amount of peak 2 increased during storage of ET-1 for several weeks at 4°C. Subsequent experiments showed that peak 1 could be completely converted to peak 2 by oxidation with Chloramin T (data not shown). Therefore, peak 1 most probably represents native ET-1 and peak 2 Meth-sulphoxide ET-1.

When blank samples extracted from PBS or plasma were injected, only small interferences or none at all were seen at the elution times of ET-1 and its degradation product, for all extraction procedures. However, blank samples extracted from urine yielded interfering peaks with areas in the range of those of ET-1 added in the case of extraction with MeOH or EtOH. Recoveries of the extraction procedures as tested with micromolar concentrations with unlabelled ET-1 were significantly lower as tested $^{125}$I ET-1 in picomolar concentrations. Total (native and degraded ET-1) recoveries for extraction with AA, TFA, MeOH and EtOH were 24.7%, 6.7%, 80.2% and 64.9% from PBS, 70.2%, 59.8%, 64.6%, and 41.5% from plasma and 72.0%, 17.1%, 67.2% and 1.2% from urine, respectively. Control experiments with $^{125}$I-labelled ET-1 revealed that the low recoveries of ET-1 from PBS for the extractions with AA or TFA were due to absorption of ET-1 onto the polypropylene wall of the column. Absorption was greatly increased (98.5% of total) when it was extracted with AA over that with MeOH (6.9%). Adherence under acidic conditions was only observed when ET-1 was added onto the columns in a protein-free matrix, whereas recoveries from plasma in this experiment were 87.3% and 81.5% for acidic and neutral conditions, respectively.

Degradation occurring during the extraction was calculated from the amount of degradation product quantitated by peak area, corrected by the recovery of the respective extraction procedure and after subtracting the amount of degraded ET-1 present in the ET-1 standard used to prepare the samples. Degradation was 22.2%, 51.1%, 1.2% and 2.2% of ET-1 when extracted from PBS, but only 4.9%, 3.1%, 0.2%, 10.1% of ET-1 for extraction from plasma, with AA, TFA, MeOH and EtOH, respectively (Fig. 2). In urine the amount of degradation was comparable to that of plasma (7.9%, 0.4% and 4.0% for AA, TFA, and MeOH) except for the extraction with EtOH, when the low overall recovery did not allow calculation of degradation. Thus, degradation of ET-1 was more pronounced for extractions involving acids when investigated in a protein-free matrix, but plasma or urine seem to reduce the oxidative influence of acids on ET-1 in the range of extraction procedures under neutral conditions.

*Immunoreactivity of different ET-1 standards*

Different synthetic ET-1 standards were checked for their immunoreactivity in the respective RIA. Dilution curves of ET-1 proved to be linear, and in most cases there was a good congruency between the curves obtained with samples prepared in RIA buffer and the curves obtained with extracted plasma samples after subtracting the blank plasma value. However, each ET-1 preparation had a very different immunoreactivity, depending on the RIA used, resulting in curves with different slopes (although usually without affecting the intercept). ET-1 obtained from Serva (Fig. 3a) had an extremely strong immunoreactivity in the RIA manufactured by Peninsula, with concentrations measured exceeding those expected up to tenfold. In contrast, in the RIAs from Biomedica, Amersham, NEN or Nichols parallel samples exhibited immunoreactivity only slightly higher, or even lower than expected. For example, samples spiked to a concentration of 50 fmol/ml resulted in measured concentrations of $13.4 \pm 6.8$, $32.3 \pm 2.3$, $34.6 \pm 7.8$, $92.5 \pm 35.3$ and $337.6 \pm 35.6$ fmol/ml in the RIAs from Amersham, NEN, Nichols, Biomedica and Peninsula, respectively. Interestingly, for other ET-1 preparations the relationship between the RIAs was different. ET-1 supplied by NEN (Fig. 2b) exhibited immunoreactivity exceeding the concentration expected in the RIAs produced by

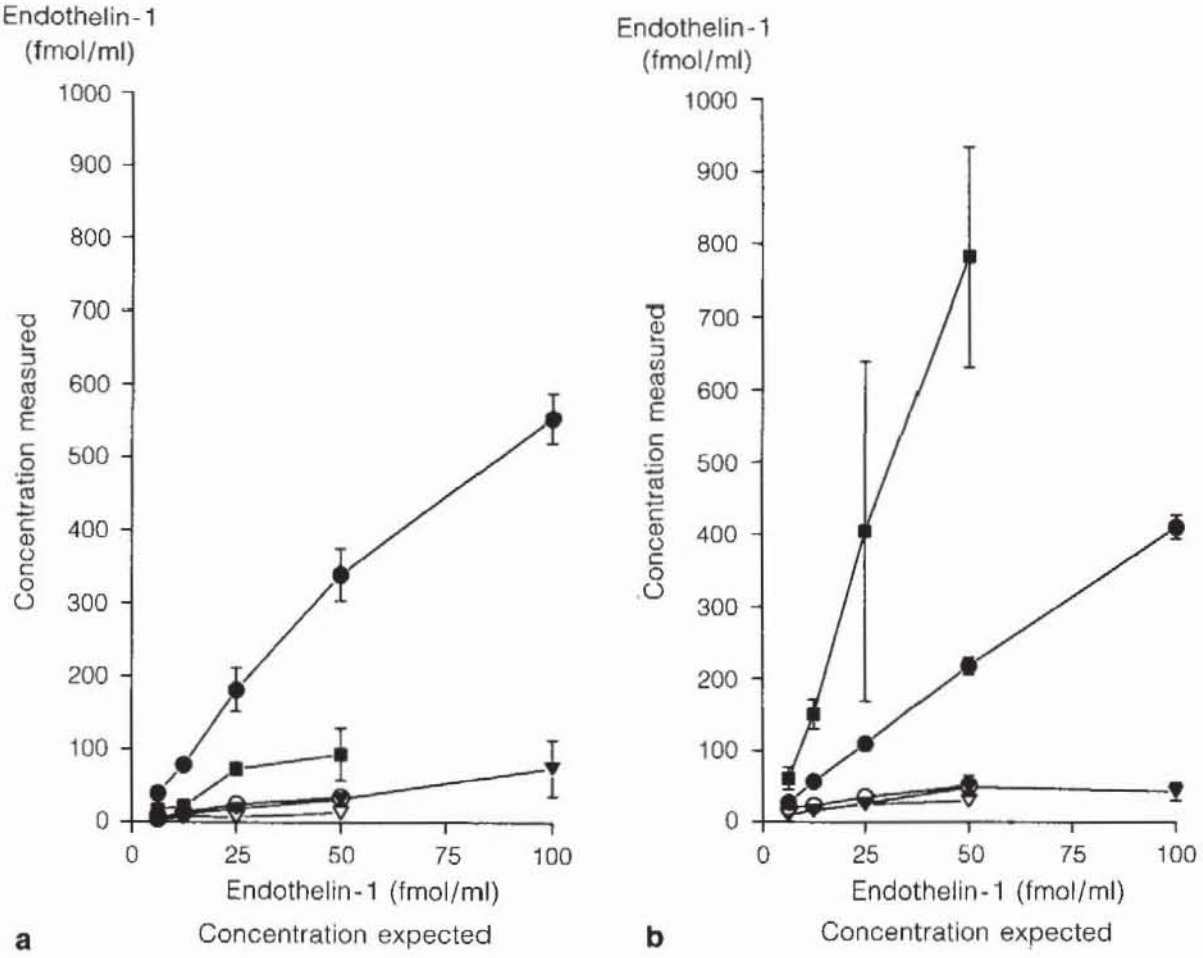
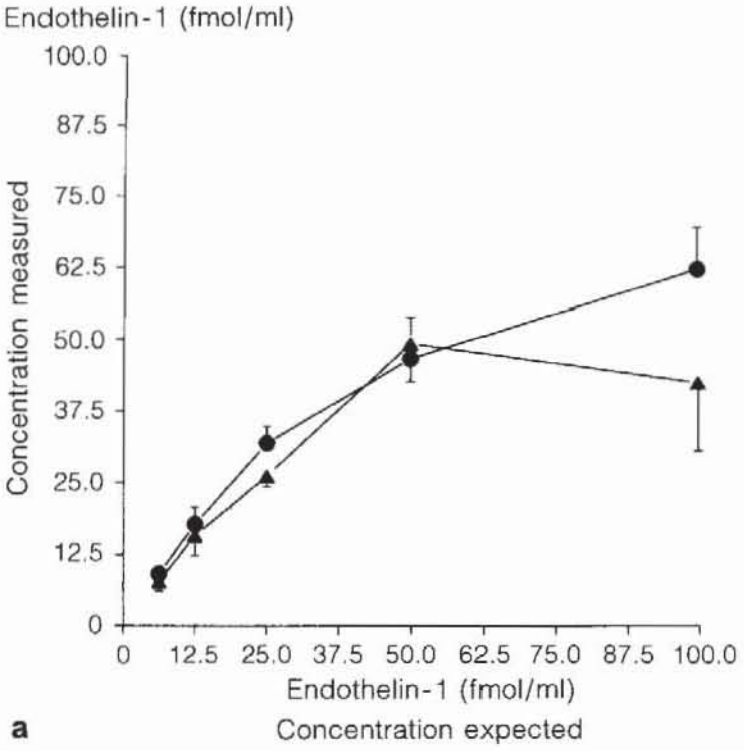



**Fig. 3a, b.** Immunoreactivity of synthetic ET-1 from different suppliers. **a** ET-1 obtained from Serva. **b** ET-1 obtained from NEN. Dilution curves were prepared in plasma, extracted with AA and measured with different radioimmunoassays. Assay obtained from (●) Peninsula, (○) Nichols, (▼) NEN, (▽) Amersham, (■) Biomedica. Results (mean ± SD) are depicted after subtracting the respective blank plasma value. Note the different scales for $x$ and $y$ axes

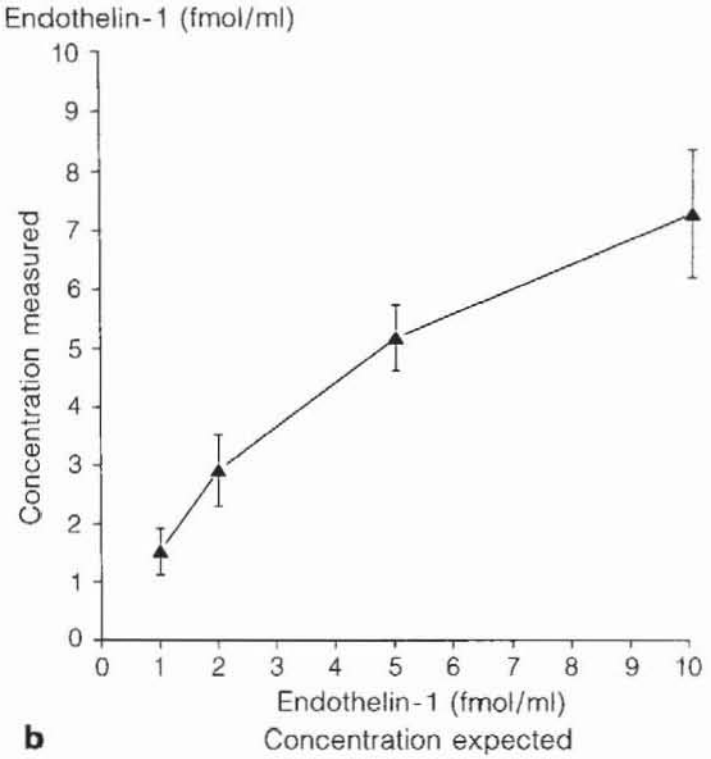
Biomedica and Peninsula, but displayed the expected values in the RIAs of Nichols and NEN, and values lower than expected in the RIA from Amersham. Samples spiked to 50 fmol/ml resulted in concentrations of 29.6 ± 5.7, 49.2 ± 4.6, 51.1 ± 14.1, 217.4 ± 11.7 and 780.2 ± 150.8 fmol/ml in the RIAs of Amersham, NEN, Nichols, Peninsula and Biomedica, respectively. ET-1 obtained from Sigma was not recognized by most of the RIAs, resulting in dilution curves with a slope near zero, but in some instances there was a high background immunoreactivity resulting in a high intercept (20–50 fmol/ml). However, in the RIA supplied by NEN, measured values were approximately 80% of the concentrations expected.

*Performance of the radioimmunoassays*

The overall (including the imprecision of the extraction) intra-assay coefficients of variation for samples extracted with AA from blank plasma ($n$ = 4) were 2.9%, 4.4%, 15.7%, 41.2% and 55.1% for RIAs from Nichols, NEN, Amersham, Biomedica and Peninsula, respectively. The mean overall intra-assay coefficients of variation of a dilution curve from 50 to 6.25 fmol/ml (ET-1 from Serva, four concentrations, each $n$ = 4) for samples extracted from plasma were 7.2%, 11.1%, 11.5%, 20.2% and 22.1%, and for samples prepared in the respective RIA buffer




a




b

**Fig. 4 a, b.** Performance of the radioimmunoassay obtained from NEN. **a** Plateau of the dilution curve observed at higher concentrations of ET-1. In this experiment the influence of the plasma matrix on immunoreactivity was also evaluated. One 2.5-ml aliquot of a plasma sample spiked to 200 fmol/ml was sixfold serially diluted in pooled plasma and subsequently extracted with acetic acid (▲). One other 200 fmol/ml aliquot was extracted and after reconstitution serially diluted in radioimmunoassay buffer to the same concentrations (●). The good congruency between the two curves up to 50 fmol/ml can be seen. **b** Dilution curve prepared in plasma for low ET-1 concentrations. Results are expressed as mean ± SD after subtracting the blank plasma value

were 3.9%, 4.2%, 8.4%, 19.8% 21.8% for RIAs from NEN, Nichols, Peninsula, Biomedica and Amersham, respectively. For the dilution curves prepared with ET-1 from other suppliers similar values were found (data not shown).

The performance of the RIA obtained from NEN was further evaluated in a larger series over a period of 6 months. The overall intra-assay coefficients of variation for a dilution curve prepared in plasma with concentrations of 50, 25, 12.5, and 6.25 fmol/ml of ET-1 from NEN added ($n$ = 6 each) were 5.3%, 2.8%, 2.6% and 4.9%, and the overall interassay coefficients of variation ($n$ = 10 each) for parallel samples were 9.4%, 10.0%, 16.2% and 13.4%, respectively.

Sensitivity of the assay was 0.1 fmol/tube. The mean ($n$ = 10) level of lowest reliable quantification (defined as samples with a tracer displacement < mean tracer displacement of the zero standard – 3 SD) was 0.52 fmol/ml. Determination of ET-1 was linear from 1 to 50 fmol/ml. However, at higher concentrations we observed a considerable flattening or even plateau of the dilution curves prepared with ET-1 from NEN, sometimes with lower values for nominal concentrations of 100 fmol/ml as compared with 50 fmol/ml (Fig. 4a). This was reproducible in several RIAs from different lots and could be observed with extracted plasma samples as well as with samples diluted directly in RIA buffer. ET-1 from other suppliers, however, yielded linear curves up to 100 fmol/ml. In the low range of the assay (1 to 10 fmol/ml) we found a good congruency between values measured and values expected (Fig. 4b).

## Discussion

Extraction recovery of ET-1 from biological samples and the results of ET-1 immunoreactivity measurements are strongly influenced by the experimental conditions. Although most extraction procedures investigated yielded sufficient recovery of ET-1, important differences between the methods could be observed. Extractions based on acidic conditions resulted in higher and more constant recoveries than the one based on neutral conditions. Moreover, differences in the efficiency of clearing the eluate from matrix components were seen. The method based on TFA resulted in the highest residual protein content after extraction of plasma and the highest residual osmolarity after extraction of urine, indicating an incomplete removal of matrix components. Residual lipids could be detected in the eluate after extraction of plasma for all procedures investigated except that of AA, where the additional lipophilic washing step with ethylacetate was able to remove plasma lipids efficiently without eluting ET-1. Residual protein content led to the formation of insoluble particulate material after evaporation of the eluate. Interestingly, in all extraction procedures, except in that of AA, ET-1 was absorbed to these particulates in a considerable amount. After dissolving the dried eluate in assay buffer, even supported by sonification, ET-1 was only partly released into the supernatant. As only soluble ET-1 is accessible to the antisera of the RIA, this phenomenon may greatly influence the results of ET-1 measurement. Moreover, we could demonstrate that matrix components not removed by the extraction can influence the performance of the RIA. High protein content was shown to increase the non-specific binding of the tracer to the antibody, making the measurement less sensitive for low concentrations of ET-1.

Recovery of ET-1 was also influenced by the sample matrix. We found a lower recovery of ET-1 from plasma than from urine or cerebrospinal fluid for all extraction procedures. This, together with the finding that the loss of ET-1 from plasma was contained in the breakthrough of the plasma fraction, suggests that ET-1 is in part bound to plasma proteins with a higher affinity as to the stationary phase of the extraction columns. This hypothesis is further supported by the fact that addition of albumin to urine reduced recovery of ET-1 to the range of plasma. Furthermore, this indicates that albumin might be the main binding protein for ET-1 in plasma. The exact nature of ET-1 binding, however, remains to be established. Small molecules like peptides or drugs are usually bound to plasma proteins by weak hydrophobic interactions and are almost completely released by C18 column

extraction. However, for bilirubin, it could be shown that the fraction of σ-bilirubin is covalently linked to plasma proteins [21]. In this connection it is of interest to note that acidic protein precipitation caused a loss of ET-1 into the pellet and that the amount of ET-1 in the pellet was dependent on the amount of protein precipitated.

The two types of C18 columns tested differed only slightly regarding recovery of ET-1. Recovery of ET-1 did not depend on ET-1 concentration, even if a more than 3,000-fold excess of unlabelled ET-1 was added, indicating a high capacity of the columns to bind ET-1. Furthermore, recovery of ET-1 did not significantly change with column sizes between 500 and 200 mg, if care was taken to optimize the volume of the elution buffer. However, column sizes of less than 200 mg caused a significant loss of ET-1 into the breakthrough of the plasma fraction, regardless of the amount of ET-1 added. This finding suggests that C18 columns have a high binding capacity for ET-1, but a limited one for plasma components. The latter may occupy binding sites of the stationary phase, thereby inhibiting further binding of ET-1. Re-evaluation of recovery, therefore, is recommended if one is decreasing column size or increasing plasma sample size.

Like most small peptides, ET-1 contained in a protein-free matrix was absorbed on all of the tested surfaces. Adherence was greatly increased by acidic conditions. However, absorbtion onto polypropylene was completely prevented by siliconization, or by prior addition of plasma. Even the small residual protein content of the eluate after extraction with AA was sufficient to block adherence to the polypropylene tubes. Therefore, for handling biological samples siliconization of test tubes seems not to be required.

ET-1 concentrations in biological samples are far below the detection limit of UV-absorption, rendering direct HPLC analysis impossible. However, the method described allowed reliable quantification of ET-1 added in micromolar concentrations to biological samples. We investigated samples prepared in PBS, plasma or urine for recovery and for degradation products after extraction. The degraded form, most probably, represents Meth-sulphoxide ET-1, although definitive confirmation by mass-spectrometry could not be performed. Extraction with AA or TFA led to a markedly higher degradation as compared with extraction with MeOH or EtOH in a protein-free matrix. However, when it was extracted from plasma or urine, degradation of ET-1 by acidic conditions was strongly reduced to less than 5% of total. This was comparable to that observed under neutral conditions.

Recovery of ET-1 from plasma was lower than in the experiments with the labelled peptide. This might be caused by the 100-fold higher concentration of ET-1 used in these experiments as compared with the highest amount of unlabelled ET-1 added in the recovery assays with $^{125}$I-labelled ET-1. Moreover, absorption of ET-1 to the particulate matter in the eluate for some extraction procedures may also greatly reduce recovery measured from the supernatant, as compared with total recovery measured with $^{125}$I-labelled ET-1. Recovery from urine was in the range of that for plasma for extraction with AA or MeOH. For extraction with TFA or EtOH, only a small amount of ET-1 added could be detected. In part this might be due to interfering peaks at the elution time of ET-1 and Meth-sulphoxide ET-1, making quantification by subtracting the blank value unreliable.

Each of every RIA evaluated showed a characteristic pattern in responding to different synthetic ET-1 standards. In addition, a high variability in the immunoreactivity of the different ET-1 preparations was observed. This finding had been unexpected in as much as all ET-1 standards used were produced by peptide synthe-

sis and lyophilized from a volatile buffer. According to the manufacturers' information, the protein content of these preparations was at least over 80%, and the protein was confirmed as pure ET-1 to greater than 98% by HPLC analysis. Therefore, the considerable differences observed in their immunoreactivities remain to be elucidated. One possible explanation would be that chemical modifications of the peptide during storage or handling, specific for a given preparation, leads to the formation of degraded peptide. This, even in small amounts, by virtue of a high cross reactivity with the antibody used in the radioimmunoassay, would influence ET-1 immunoreactivity determinations.

The RIA obtained from NEN was characterized by the best congruency between the amount of ET-1 added and the measured concentrations for most of the ET-1 standards tested. This might be due to the fact that in the RIA from NEN a monoclonal mouse antibody was used, whereas all other kits were based on polyclonal rabbit antiserum. Moreover, this assay yielded the smallest coefficients of variation within day, as well as from day to day, for blank plasma samples and for spiked samples up to 50 fmol/ml. At higher concentrations there was an unexplained flattening of dilution curves prepared with ET-1 from NEN which could not be observed with ET-1 from other suppliers. Besides this, we found this assay performing better for physiological concentrations of ET-1 in plasma, cerebrospinal fluid or urine in terms of the coefficients of variation as well as of the correspondence between expected and measured values.

In conclusion, our study clearly demonstrates that ET-1 determination in biological samples is strongly dependent on the assay conditions. The extraction procedures vary with respect to recovery of soluble ET-1 and removal of matrix components, and both factors have impact on ET-1 immunoreactivity measurement. From our data we can conclude that the extraction procedure involving AA is the most efficient, because of the high and constant recovery of ET-1 and the low matrix interference. The oxidative influence of AA on ET-1 seems to be prevented by the protein content of biological samples. The RIAs evaluated showed striking differences in the measurement of ET-1-immunoreactivity for synthetic ET-1 from different suppliers. The RIA from NEN gave the best approximation between added ET-1 concentrations and measured values. In our hands, the assay performed with good precision over more than a year and proved to be suitable for the reliable measurement of ET-1 in plasma and urine in patient samples [11].

The study of ET-1 has become more and more widespread in clinical research. With the availability of ET-1 antagonists in the near future, precise ET-1 measurement may be a crucial prerequisite for therapeutic decisions. Therefore, there is an urgent need for standardization in this field, and quality control and quality-assurance programmes should be an important research issue for future work on endothelins.

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