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THE PHARMACOKINETICS OF ^{125}I-ATRIAL NATRIURETIC FACTOR IN ANAESTHETIZED RATS

EFFECTS OF NEUTRAL ENDOPEPTIDASE INHIBITION WITH CANDOXATRILAT AND OF ANF-C RECEPTOR BLOCKADE

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Abstract—The effects of candoxatrilat (*cis*-4-{[2-carboxy-3-(2-methoxyethoxy)propyl]-1-cyclopentanecarbonylamino}-1-cyclohexane carboxylic acid) and the ring-deleted atrial natriuretic factor (ANF) analogue C-ANF$_{4-23}$ (des[Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}]ANF$_{4-23}$-NH$_2$) on the clearance of (3-[^{125}I]iodotyrosyl^{28})ANF (^{125}I-ANF) were studied in both intact and nephrectomized anaesthetized rats. HPLC analysis was used to verify that the ^{125}I-labelled material isolated by solid phase extraction of rat plasma was intact ANF. In intact animals, clearance of ^{125}I-ANF was biphasic with a $T_{\frac{1}{2}\alpha}$ of 17 sec and $T_{\frac{1}{2}\beta}$ of 95 sec. Volume of distribution (V_d) was 564 mL/kg and plasma clearance (Cl_p) 248 mL/min/kg. Candoxatrilat, over the dose range 0.01–10 mg/kg i.v., increased $T_{\frac{1}{2}\beta}$ (by a maximum of 56%) and decreased Cl_p (by up to 52%) with no effect on $T_{\frac{1}{2}\alpha}$ or V_d. C-ANF$_{4-23}$ (10 μg/kg + 1 μg/kg/min i.v.) reduced V_d (by 57%) and Cl_p (by 54%) with no effect on $T_{\frac{1}{2}\beta}$, whilst abolishing the $T_{\frac{1}{2}\alpha}$ phase in over 50% of animals. Increasing the dose of C-ANF$_{4-23}$ did not increase the effect on any of these parameters, apart from a small increase in $T_{\frac{1}{2}\beta}$. Combining the two agents resulted in a substantial decrease in Cl_p (76%) whilst the reduction in V_d and increase in $T_{\frac{1}{2}\beta}$ were comparable to those seen with C-ANF$_{4-23}$ and candoxatrilat alone, respectively. In nephrectomized rats, the pharmacokinetics of ^{125}I-ANF and the changes induced by candoxatrilat were similar to those observed in intact animals, whilst the effects of C-ANF$_{4-23}$ alone were greater than in intact animals. The combination of C-ANF$_{4-23}$ and candoxatrilat again produced a substantial increase in $T_{\frac{1}{2}\beta}$ (153%) and decreases in V_d (55%) and Cl_p (78%) in nephrectomized animals, although these changes could not be distinguished from those seen in intact animals treated with the same combination. Our studies indicate that neutral endopeptidase and ANF-C receptors are both major, and approximately equal, clearance mechanisms for ^{125}I-ANF, together accounting for at least 75% of the total clearance of this peptide in the rat.

Atrial natriuretic factor (ANF†) is rapidly cleared from the circulation. In man and animals half-lives of between 1 and 4 min have been reported [1–5]. Whilst the process of ANF clearance from the circulatory system is not fully understood, at least three mechanisms are thought to be involved. Firstly, ANF is metabolically degraded by a zinc-dependent neutral endopeptidase (NEP, atriopeptidase, EC 3.4.24.11) [6, 7]. Secondly, ANF can be bound to a clearance or ANF-C receptor which does not possess guanylate cyclase activity. Once bound, the ANF is then internalized and degraded by lysosomal enzymes [1, 8]. Finally, ANF may be cleared by renal filtration [9]. The part played by each of these mechanisms in the overall clearance of ANF *in vivo* has still to be resolved. Thus, we have investigated the relative contribution of these mechanisms by following the disappearance of ^{125}I-ANF from the circulation of the rat under conditions of NEP inhibition, ANF-C receptor blockade and nephrectomy.

In the present series of experiments, degradation of ANF by NEP was blocked using the specific inhibitor candoxatrilat (*cis*-4-{[2-carboxy-3-(2-methoxyethoxy)propyl] - 1 - cyclopentanecarbonylamino}-1-cyclohexane carboxylic acid). The racemic form of this compound (UK-69,578, (±) candoxatrilat) has been shown previously to reduce the clearance and prolong the half-life of ANF in anaesthetized rats [10], whilst natriuretic and diuretic responses together with increases in plasma ANF have been observed following acute administration to conscious mice [11]. The selectivity of the compound has been confirmed by showing a lack of activity against a variety of other peptidases of the zinc- and serine-dependent classes [12], and we now extend these studies to show that it has no effect on type-B and type-C ANF receptors.

ANF-C receptor-mediated clearance was blocked using the ring-deleted ANF analogue C-ANF$_{4-23}$ (des [Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}]rat ANF$_{4-23}$-NH$_2$). This peptide elevates circulating levels of endogenous ANF and increases sodium excretion in anaesthetized rats [8], effects which are consistent with its ability

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† Abbreviations: ANF, atrial natriuretic factor; AUC, area under the plasma concentration curve; Cl_p, plasma clearance; ^{125}I-ANF, (3-[^{125}I]iodotyrosyl^{28})rat ANF; NEP, neutral endopeptidase; V_d, volume of distribution.

to decrease the volume of distribution and clearance rate of ANF in rats [1]. Its selectivity has been demonstrated by virtue of its high affinity for renal and vascular clearance receptors relative to the guanylate cyclase linked biological receptors, and in this present report we have investigated its interaction with another ANF clearance mechanism (i.e. NEP).

Finally, the role of the kidney in ANF clearance has been evaluated using bilateral nephrectomy in the presence and absence of NEP inhibition or ANF-C receptor blockade. The combination of NEP inhibition and ANF-C receptor blockade in nephrectomized animals was employed to investigate whether additional mechanisms for ANF clearance existed as has been suggested from *in vitro* studies [13].

MATERIALS AND METHODS

Plasma clearance of ^{125}I-ANF in anaesthetized rats. Experiments were performed in male Sprague–Dawley rats (Charles River, Manston, Kent, U.K.; 350–400 g body wt; N = 4–9 animals per treatment group) starved for 18 hr and anaesthetized with Inactin (130 mg/kg i.p., BYK Gulden). The trachea was cannulated. A catheter (Portex, 0.6 mm i.d., 6–8 cm in length) was introduced into the aorta via the right carotid artery to allow the rapid sampling of blood, and a second (Portex, 0.6 mm i.d., 16–20 cm in length and dead space of 55–60 μL) placed in the inferior vena cava via the right femoral vein for the administration of labelled peptide, test compounds or vehicle. In some experiments, the right jugular vein was catheterized for the infusion of C-ANF$_{4\text{-}23}$ (ring-deleted rat ANF, Cambridge Research Biochemicals, U.K.). In one set of experiments both kidneys were removed. A recovery period of 60 min was allowed after surgery. The temperature of the animals was monitored and maintained at 37°.

The pharmacokinetics of ANP were investigated by assessing the disappearance of radiolabelled ANF from the circulation. A dose of 2.5 μCi (10 ng/kg) of (3-[^{125}I]iodotyrosyl^{28}) rat ANP(^{125}I-ANF) (Amersham International, U.K.; sp. act. 2000 Ci/mM) in 50 μL of 0.15 M NaCl solution was loaded into the femoral vein catheter and flushed into the circulation with 300 μL of isotonic (0.15 M) NaCl solution. Eight arterial blood samples (300 μL each) were taken at time points between 15 and 300 sec after administration of the labelled peptide and were replaced with an equal volume of isotonic (0.15 M) NaCl solution. This procedure was repeated 60 min later in the presence of test compound, thus allowing each animal to act as its own control. The NEP inhibitor, candoxatrilat (0.1–10 mg/kg) or vehicle (0.15 M NaCl solution) was administered as a bolus (1.0 mL/kg) 10 min before the second administration of ^{125}I-ANF and flushed into the circulation with 300 μL of 0.15 M NaCl solution. The ANF-C receptor ligand C-ANF$_{4\text{-}23}$ or vehicle (0.15 M NaCl solution) was administered as a priming bolus (10 or 100 μg/kg) in 50 μL followed by an infusion (1 or 10 μg/kg/min at a flow rate of 50 μL/min), via the jugular catheter commencing 15 min before the second ^{125}I-ANF administration and continuing until blood sampling had finished.

Extraction of ^{125}I-ANF from plasma. Each blood sample was immediately mixed with EDTA (final concentration 1 mg/mL blood) and aprotinin (1000 KIU/mL blood) and then centrifuged. A volume of 100 μL of plasma was then mixed with 300 μL of ice-cold 4% (v/v) acetic acid (HPLC grade, BDH, Poole, U.K.). ^{125}I-ANF was extracted using C_{18} Sep-Pak cartridges (Waters Associates, U.K.) for solid phase extraction as described previously [14]. Briefly, 0.3 mL of acidified plasma was loaded onto activated Sep-Pak cartridges and then washed with 10 mL of distilled water followed by 10 mL of 4% (v/v) acetic acid. The intact peptide (^{125}I-ANF) was then eluted from the cartridges with 2 mL of a solution containing 86% (v/v) ethanol (HPLC grade, BDH) and 4% (v/v) acetic acid and collected for counting using a gamma counter (LKB).

Recovery of ^{125}I-ANF after Sep-Pak extraction was assessed by spiking 2.5 mL of whole rat blood with 1.0 μCi of ^{125}I-ANF, aliquoting into eight samples and processing as above. The recovery was found to be 67 ± 3%. Data have not been corrected for recovery.

HPLC analysis of plasma samples. To verify that the radioactivity being measured was due to intact ^{125}I-ANF rather than ^{125}I-labelled metabolites, 0.5 μCi of ^{125}I-ANF was added to 100 μL of rat plasma which were processed by Sep-Pak extraction as above. The processed sample was evaporated under nitrogen and reconstituted in 0.5 mL of double distilled water. A small sample of this (180 μL) was then spiked with 20 μL of 0.5 mM unlabelled rat ANF (Cambridge Research Biochemicals) and 150 μL of this mixture injected onto a C_{18} μBondapak column (Waters) and eluted using a gradient of 15–30% acetonitrile in 0.08% phosphoric acid over 15 min at a flow rate of 1.5 mL/min. The eluate was monitored continuously by UV absorption at 214 nm, and fractions were collected every half minute for determination of radioactivity. The time course of the radioactivity profile was adjusted to correct for the dead space (2.3 mL) between the UV detector and fraction collector. A second experiment was performed using plasma samples obtained from an experimental animal at time points 15, 60 and 300 sec after the intravenous administration of 2.5 μCi ^{125}I-ANF. After processing of the plasma on Sep-Pak cartridges, extracted samples were analysed by HPLC as described above.

Pharmacokinetic analysis. Analysis of the log plasma concentration versus time data was performed by linear regression analysis using standard pharmacokinetic equations [15] based on a two-compartment system. For all analyses the correlation coefficient was greater than 0.96 (in most cases 0.98–0.99). Plasma clearance (Cl_p) was calculated by dose/$AUC_{0\text{-}\infty}$. The apparent volume of distribution (V_d) was calculated from the equation $V_d = Cl/\beta$, where β is the rate constant of the terminal elimination phase.

Statistical analysis. Pharmacokinetic data from the ^{125}I-ANF experiments in anaesthetized rats were analysed as follows. Homogeneity between control values (first ^{125}I-ANF administration) in the different groups was determined by analysis of variance (ANOVA), using an F-test to compare the means

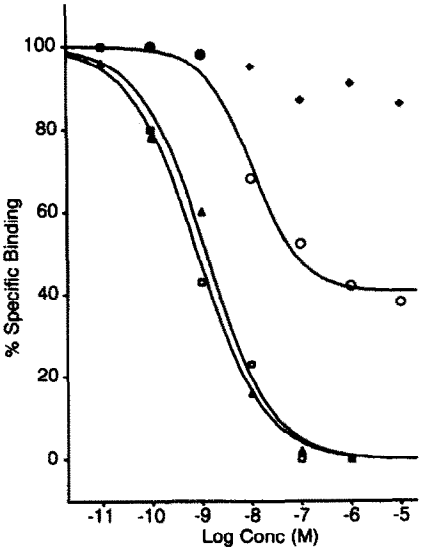

Fig. 1. Displacement of ^{125}I-ANF from rabbit lung membranes by α-ANF (△), ANF_{5-28} (□), $C\text{-}ANF_{4-23}$ (○) and candoxatrilat (◇). ^{125}I-ANF (100 pM) was incubated with rabbit lung membranes (100 μg) and various concentrations of unlabelled analogues as described in Materials and Methods. Binding data are corrected for non-specific binding which was determined in the presence of 1 μM ANF_{5-28} and represented 15% of the total binding. The data presented from a single representative experiment are means of triplicate determinations.

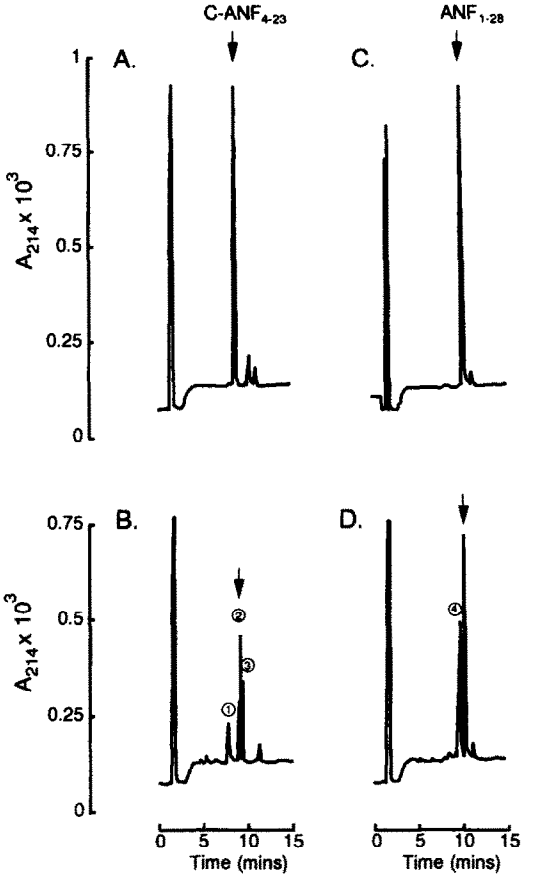

Fig. 2. Hydrolysis of $C\text{-}ANF_{4-28}$ and rat ANF_{1-28} by NEP. Peptides were incubated with NEP and analysed by HPLC as described in Materials and Methods. Panel A represents an HPLC trace of $C\text{-}ANF_{4-23}$ prior to incubation with NEP (time zero) and shows a major UV absorbing peak with a retention time of 9.0 min, corresponding to intact $C\text{-}ANF_{4-23}$. Incubation for 1 hr with NEP, as illustrated in Panel B, results in a decrease in $C\text{-}ANF_{4-23}$ (arrow) and appearance of these new peaks with retention times of 8.0, 9.2 and 9.4 min (peaks 1–3, respectively). Similarly, panel C shows the HPLC trace of rat ANF_{1-28} (retention time 10.4 min) prior to incubation with NEP. After incubation for 1 hr with NEP (as illustrated in Panel D), the line of the ANF_{1-28} peak (arrow) is diminished, and a new peak with retention time of 9.8 min, eluting in the same position as "ring opened" ANF (peak 4), has appeared.

Table 1. Inhibition of specific ^{125}I-ANF binding by ANF analogues in rabbit lung membranes

Compound	N	One or two site binding	IC_{50} (nM) Site 1	Site 2
$C\text{-}ANF_{4-23}$*	5	Two	7.50 ± 1.30	>2000
SC-46,792*	3	Two	1.71 ± 0.13	>2000
α-ANF	9	One	1.10 ± 0.13	
ANF_{5-28}	5	One	1.53 ± 0.20	
ANF_{7-28}	5	One	2.21 ± 0.24	

Binding was determined in crude particulate fractions from rabbit lung as described in Materials and Methods. Inhibition curves were analysed for "best fit" (i.e. one- or two-site binding) by minimizing the sum-of-squares of the errors using a non-linear regression analysis programme [20]. Two analogues* exhibited curves best fitting with a two-site model, confirming their reported selectivity for one of the ANF binding sites (ANF-C receptor sites). The remaining ANF analogues all displayed apparent single-site interactions and thus equal affinity for the two ANF binding sites previously documented in the preparation [21].

of the different groups during the control period. The effects of treatment (second ^{125}I-ANF administration) were determined by analysis of covariance (ANOCOVA) using data from the control period as covariate. The analyses of variance and covariance were carried out using the GENSTAT statistical package (Version 5; Rothamsted, Harpenden, U.K.). In the case of the "dose–response" study with candoxatrilat (Table 2), evidence for a treatment effect was determined using the Williams' test [16], assuming a monotonic dose–response relationship for the drug. For the study in nephrectomized animals (Table 4), the effects of candoxatrilat and $C\text{-}ANF_{4-23}$ were analysed using a factorial treatment structure. Between study analyses were carried out by comparing the 95% confidence intervals of the respective groups, using ANOCOVA to adjust for

differences in pre-dose values. Differences were accepted as significant at the 5% level ($P < 0.05$).

ANF hydrolysis studies. NEP was isolated from dog kidney by the method of Chipkin *et al.* [17]. Aliquots of purified enzyme (0.2 μg) were incubated in duplicate with rat ANF or C-ANF$_{4-23}$ (final concentration 50 μM) in 50 mM in Tris–HCl buffer pH 7.4 at 37° for 60 min. Tubes were immersed in boiling water for 4 min to terminate the reaction, and following centrifugation a 60 μL aliquot of the supernatant was injected onto a C_{18} μBondapak HPLC column (Waters) and eluted using a 10–50% gradient of acetonitrile in 0.1% trifluoroacetic acid over 15 min at a flow rate of 1.5 mL/min. Detection was by UV absorption at 214 nm. Hydrolysis of ANF peptides was quantified by the decrease in peak area, compared to a standard of each peptide. "Ring-opened" ANF (ANF$_{1-7}$[Cys^7-Cys^{23}]ANF$_{8-28}$) was synthesized by Cambridge Research Biochemicals.

ANF binding studies. The competitive binding of ANP was studied in rabbit lung membranes prepared by a standard method [18]. The incubation media consisted of 0.25 mL of a solution containing 50 mM Tris, pH 7.5, 0.1% bovine serum albumin, 100 μg of membrane protein and ^{125}I-ANF (Amersham International) in the absence or presence of unlabelled peptide (10^{-5}–10^{11} M). The competition experiment was initiated by the addition of membrane protein and carried out at 25° for 30 min. The binding reaction was terminated by addition of ice-cold buffer, followed by rapid filtration through GF/B filters that had been presoaked in 0.5% polyethyleneimine. This separation of membrane-bound labelled peptide from free ligand was achieved using a Brandell cell harvester and the filters were counted in a Beckman LS5000CE liquid scintillation counter.

Non-specific binding was defined as binding in the presence of 10^{-6} M ANF fragment 5-28. Specific binding was calculated as total binding minus non-specific binding. All experiments were performed in duplicate and binding data were analysed using a sigmoidal curve fitting program.

The ANF-C receptor ligand SC-46,792 ([Acm-Cys^{105}]ANF (105-114)NH2), a linear decapeptide analogue of ANF [19], was kindly supplied by Dr Phillippe Bovy of Searle R&D (Missouri, U.S.A.). All other peptides were purchased from Peninsula Labs (St. Helens, U.K.).

RESULTS

Effects of C-ANF$_{4-23}$ and candoxatrilat on ANF receptors

Competitive binding of ^{125}I-ANF with unlabelled ANF analogues and candoxatrilat for binding sites on rabbit lung membranes is illustrated in Fig. 1. αANF and ANF$_{5-28}$ (atriopeptin III) displaced ^{125}I-ANF completely while ring-deleted C-ANF$_{4-23}$ only displaced the radioligand from approximately 65% of the total binding sites. Candoxatrilat was entirely without effect on ^{125}I-ANF binding, at concentrations up to 10 μM (highest concentration tested). The relative affinities of several standard ANF analogues, including the ANF-C receptor ligands C-ANF$_{4-23}$ and SC 46542, are summarized in Table 1.

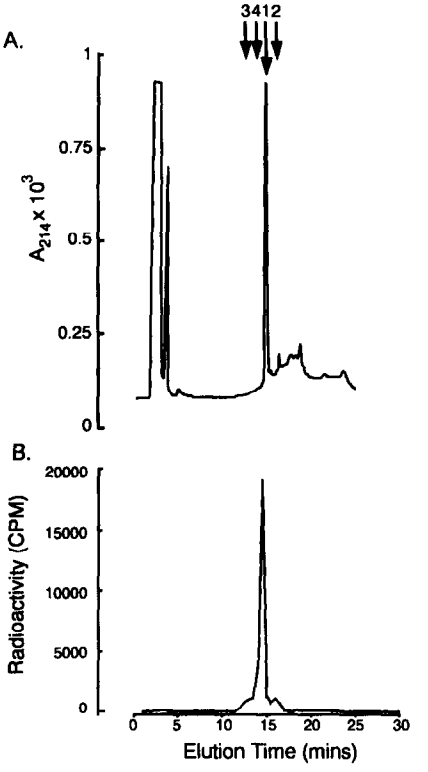

Fig. 3. HPLC analysis of ANF-spiked plasma. Rat plasma was spiked with ^{125}I-ANF and unlabelled ANF as described in Materials and Methods. (A) UV absorbance and (B) radioactivity of the sample. Both profiles show a single peak with a retention time of 14.5 min. The arrows indicate the elution positions of (1) rat ANF$_{1-28}$, (2) ANF$_{5-28}$, (3) ANF$_{5-25}$ and (4) "ring opened" ANF.

Hydrolysis of C-ANF$_{4-23}$ by NEP

Both rat ANF (retention time 10.4 min) and C-ANF$_{4-23}$ (retention time 9.0 min) were rapidly degraded following incubation with 0.2 μg NEP, with hydrolysis rates of 270 and 650 pmol/min, respectively (Fig. 2). In the case of rat ANF, the major product of degradation had a retention time of 9.8 min and co-eluted with "ring-opened" ANF$_{1-28}$. Fragments arising from C-ANF$_{4-23}$ hydrolysis eluted with retention times of 8.0, 9.2 and 9.4 min; however, further characterization of these peptides was not attempted. Inclusion of 1 μM candoxatrilat in the incubation medium significantly inhibited the hydrolysis of both rat ANF and C-ANF$_{4-23}$, and the formation of the corresponding degradation products (data not shown).

HPLC analysis of extracted plasma samples

To confirm that all the radioactivity extracted from plasma samples corresponds to intact ^{125}I-ANF an HPLC analysis of extracted samples was performed. In an initial experiment, ^{125}I-ANF was added to rat plasma *in vitro*, processed by Sep-Pak C18 extraction and assayed by HPLC. The UV absorbance and radioactivity profiles, shown in Fig.

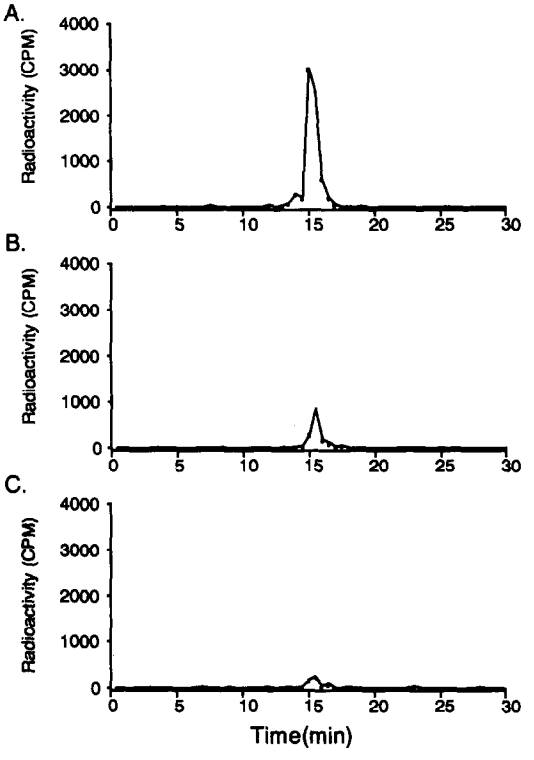

Fig. 4. HPLC analysis of processed plasma samples taken at (A) 15 sec, (B) 60 sec and (C) 300 sec after injection of 2.5 μCi of ^{125}I-ANF into a rat. All the traces show a single peak of radioactivity with a retention time equivalent to ^{125}I-ANF. Thus, all the radioactivity in these samples is associated with intact peptide. The reduction in peak area shows the disappearance of the peptide with time.

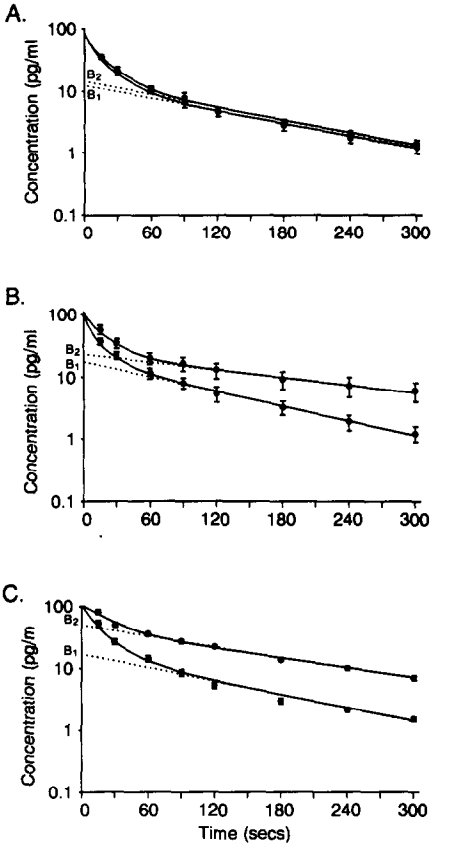

Fig. 5. The disappearance of ^{125}I-ANF from the circulation before (○) and after (●) (A) vehicle (0.15 M NaCl, N = 8), (B) candoxatrilat (3.0 mg/kg, N = 6) and (C) C-ANF$_{4\text{-}23}$ (10 μg/kg + 1.0 μg/kg/min, N = 6). Data are means ± SEM. Dashed lines show the extrapolation of the β-phase, to give the intercepts B_1 and B_2 on the y-axis, for the first and second ^{125}I-ANF administrations, respectively. The intercepts B_1 and B_2 coincide on the y-axis in the case of vehicle (12.5 ± 1.9 vs 12.1 ± 1.7 pg/mL) and candoxatrilat (17.0 ± 3.5 vs 23.1 ± 5.0 pg/mL), but not following C-ANF$_{4\text{-}23}$ (15.2 ± 2.4 vs 47.6 ± 3.2; $P < 0.05$, unpaired Student's t-test).

3, show that the peak for the labelled peptides was coincident with unlabelled ANF. In a subsequent experiment, plasma samples, taken from a rat 15 sec, 1 min and 5 min after injection of 2.5 μCi ^{125}I-ANF, were processed as above and analysed by HPLC. The resulting traces are shown in Fig. 4. At each time point there is a single peak with a retention time corresponding to intact ^{125}I-ANF. The size of the peak diminishes with time as the peptide is removed from circulation. No other peaks are apparent on the radioactivity profile, indicating that any radiolabelled fragments formed in the circulation are not extracted by the Sep-Pak procedure. Using identical HPLC conditions we can clearly differentiate between intact ANF and its ring-opened metabolites (Fig. 3). Thus, we can conclude that all the radioactivity in the Sep-Pak-processed samples corresponds to intact peptide and that this can be used to follow reliably the clearance of the peptide from the circulation.

Pharmacokinetics of ^{125}I-ANF in intact rats

In anaesthetized rats, the clearance of ^{125}I-ANF is biphasic with an initial rapid phase ($T_{½\alpha}$ 16.5 ± 1.3 sec) lasting 30–60 sec, followed by a slower, more prolonged phase ($T_{½\beta}$ 95 ± 4 sec, Fig. 5A and Table 2). By 60 sec, approximately 60% of the administered dose had been eliminated. The clearance, calculated from the dose and $AUC_{0\text{-}\infty}$, is 248 ± 30 mL/min/kg with an apparent V_d of 564 ± 74 mL/kg. The administration of vehicle 10 min before the second injection of ^{125}I-ANF had no significant effect on the clearance or other pharmacokinetic parameters (Fig. 5A).

Candoxatrilat significantly reduced the clearance of ^{125}I-ANF with maximal effects observed at 3.0 mg/kg where clearance was reduced by 52%; increasing the dose to 10 mg/kg did not increase the responses further (Table 2). There were no consistent changes in the apparent V_d, whilst $T_{½\beta}$ increased significantly at all doses tested. The increases in $T_{½\beta}$ can be seen as a flattening of the log plasma concentration versus time curve (Fig. 5B) from 60 sec onwards. In

Table 2. Effects of candoxatrilat on the pharmacokinetics of ^{125}I-ANF (10 ng/kg) in anaesthetized rats

Dose of candoxatrilat	N	$T_{\frac{1}{2}\alpha}$ (sec)	$T_{\frac{1}{2}\beta}$ (sec)	$AUC_{0-\infty}$ (pg.sec/mL)	V_d (mL/kg)	Cl (mL/min/kg)
Vehicle (0.15 M NaCl)	8	16.5 ± 1.3	95 ± 4	2817 ± 280	564 ± 74	248 ± 30
0.01 mg/kg	5	18.4 ± 6.1	126 ± 16*	2798 ± 543	779 ± 143	278 ± 45
0.03 mg/kg	5	18.0 ± 0.5	122 ± 8*	2479 ± 232	880 ± 54	306 ± 29
0.3 mg/kg	9	14.6 ± 0.6	148 ± 14*	3202 ± 660	919 ± 112	267 ± 32
3.0 mg/kg	6	12.8 ± 0.7	142 ± 12*	6713 ± 1904*	381 ± 61	118 ± 21*
10.0 mg/kg	5	12.7 ± 1.5	147 ± 7*	5172 ± 987*	459 ± 78	131 ± 23*

The apparent V_d, Cl, plasma half life ($T_{\frac{1}{2}\alpha}$ and $T_{\frac{1}{2}\beta}$) and $AUC_{0-\infty}$ were derived by analysis of the disappearance curves of ^{125}I-ANF in anaesthetized rats receiving a bolus of vehicle (0.15 M NaCl, 1.0 mL/kg) or candoxatrilat at various doses administered 10 min before the second administration of ^{125}I-ANF as described in Materials and Methods.

Values are raw means ± SEM.

*$P < 0.05$ compared to vehicle (Williams' test following analysis of covariance).

Table 3. Effect of an ANF-C receptor ligand on the pharmacokinetics of ^{125}I-ANF (10 ng/kg) in anaesthetized rats

Treatment	N	$T_{\frac{1}{2}\alpha}$ (sec)	$T_{\frac{1}{2}\beta}$ (sec)	$AUC_{0-\infty}$ (pg.s/mL)	V_d (mL/kg)	Cl (mL/min/kg)
Vehicle (0.15 M NaCl)	4	14.9 ± 0.8	114 ± 10	3994 ± 452	445 ± 45	164 ± 18.3
C-ANF$_{4-23}$ (10 μg/kg + 1 μg/min/kg)	6	ND	106 ± 6	8567 ± 554*	192 ± 14*	75 ± 5*
C-ANF$_{4-23}$ (100 μg/kg + 10 μg/min/kg)	5	ND	113 ± 4	7752 ± 238*	254 ± 11*	94 ± 3*
C-ANF$_{4-23}$ (10 μg/kg + 1 μg min/kg) + candoxatrilat (3 mg/kg)	8	ND	198 ± 14*	16484 ± 787*	180 ± 7*	39 ± 2*

The apparent V_d, Cl, plasma half-life ($T_{\frac{1}{2}\alpha}$ and $T_{\frac{1}{2}\beta}$) and $AUC_{0-\infty}$ of ^{125}I-ANF in the presence of an infusion of vehicle (0.05 mL/min), or ANF-C receptor ligand (C-ANF$_{4-23}$), or of an infusion of ANF-C ligand plus candoxatrilat given as a bolus 10 min before the second sampling run. Total infusion time was 15 min preceding and including the second sampling run.

Values are raw means ± SEM.

ND, could not be determined in over 50% of animals.

* $P < 0.05$ compared to vehicle (t-test following analysis of covariance).

contrast, the close coincidence of the curves up to 60 sec after ^{125}I-ANF administration shows that the initial rapid clearance phase was unaffected by candoxatrilat. There were no significant changes in $T_{\frac{1}{2}\alpha}$ following candoxatrilat.

C-ANF$_{4-23}$ was administered as a continuous infusion following an initial bolus dose in accordance with the procedure of Maack *et al.* [8]. Vehicle administered in exactly the same manner did not significantly alter ^{125}I-ANF clearance from that obtained during the first administration of the peptide. In the presence of C-ANF$_{4-23}$ (10 μg/kg + 1 μg/kg/min infusion), clearance was reduced by 54% (Table 3). which was comparable to the maximal change seen with candoxatrilat (Table 2). However, unlike candoxatrilat there was a significant reduction in the apparent V_d (57%). Thus, with clearance and V_d changing by a similar amount, there was little change in $T_{\frac{1}{2}\beta}$. Increasing the dose of C-ANF$_{4-23}$ 10-fold did not result in further reductions in clearance or V_d. There was a small increase in $T_{\frac{1}{2}\beta}$ although this failed to achieve statistical significance (Table 3). The effect these changes have on the disappearance profile are shown in Fig. 5C. C-ANF$_{4-23}$ causes an upward but parallel shift in the curve and the initial rapid clearance phase is attenuated. Under these circumstances, there are insufficient data points for a reliable estimate of $T_{\frac{1}{2}\alpha}$ to be made.

Combining candoxatrilat (3 mg/kg) and C-ANF$_{4-23}$ (10 μg/kg + 1 μg/kg/min) resulted in a 4-fold increase in $AUC_{0-\infty}$ and a substantial (76%)

Table 4. Effect of candoxatrilat or ANF-C receptor ligand on the pharmacokinetics of ^{125}I-ANF (10 ng/kg) in nephrectomized rats

Treatment	N	$T_{\frac{1}{2}\alpha}$ (sec)	$T_{\frac{1}{2}\beta}$ (sec)	$AUC_{0-\infty}$ (pg.sec/mL)	V_d (mL/kg)	Cl (mL/min/kg)
Vehicle (0.15 M NaCl)	9	16.1 ± 0.7	98 ± 6	3645 ± 359	434 ± 52	183 ± 18
Candoxatrilat (3 mg/kg)	8	14.4 ± 0.7	140 ± 5	6021 ± 514	365 ± 33	109 ± 10
C-ANF$_{4-23}$ (10 μg/kg + 1 μg/min/kg)	4	ND	138 ± 7	16293 ± 141	129 ± 6	39.0 ± 0.2
C-ANF$_{4-23}$ (10 μg/kg + 1 μg/min/kg) + candoxatrilat (3 mg/kg)	9	ND	248 ± 23	16314 ± 1697	239 ± 21	42.2 ± 5
Candoxatrilat effect			*	*	†	*
C-ANF$_{4-23}$ effect			*	*	†	*

The apparent V_d, Cl, plasma half-life ($T_{\frac{1}{2}\alpha}$ and $T_{\frac{1}{2}\beta}$) and $AUC_{0-\infty}$ of ^{125}I-ANF in the presence of a bolus of vehicle (1.0 mL/kg) or candoxatrilat given 10 min before the second sampling run, or infusion of ANF-C receptor ligand (C-ANF$_{4-23}$) or infusion of ANF-C ligand plus candoxatrilat given as above. Total infusion time was 15 min proceeding and including the second sampling run.

Values are raw means ± SEM.

ND, could not be determined in over 50% of animals.

* $P < 0.05$, P values obtained from analysis of covariance with factorial treatment structure.

† C-ANF$_{4-24}$–candoxatrilat interaction was statistically significant.

reduction in clearance (Table 3), which was greater than that evoked by either compound alone (as judged by comparison of 95% confidence intervals for the treatment effects). Apparent V_d was also reduced but only to the same degree as C-ANF$_{4-23}$ alone, whilst the increase in $T_{\frac{1}{2}\beta}$ was not different from that seen with candoxatrilat alone (comparison of 95% confidence intervals).

Pharmacokinetics of ^{125}I-ANF in nephrectomized rats

A bilateral nephrectomy was performed before the first administration of ^{125}I-ANF to enable the effects of nephrectomy *per se* on ^{125}I-ANF pharmacokinetics to be examined. It was found that whilst this manoeuvre had minimal effects on any of the parameters measured in the control run, there was residual radioactivity in extracted plasma samples taken immediately before the second ^{125}I-ANF administration, although this amounted to less than 1% of the total radioactivity administered to the animal. Since this had not been observed previously in samples from intact rats, HPLC analysis of extracted plasma from nephrectomized animals was carried out; however, the residual radioactivity could not be attributed to intact ANF or ANF fragments. In all experiments using nephrectomized animals, the residual radioactive count was subtracted from those of the second sampling run. After this correction, vehicle administration before the ^{125}I-ANF administration did not affect the clearance of ^{125}I-ANF (Table 4).

In nephrectomized animals the changes in the pharmacokinetics of ^{125}I-ANF induced by candoxatrilat were similar to those observed in intact rats (Table 4), with an approximate doubling of $AUC_{0-\infty}$, a halving of clearance and a 43% increase in $T_{\frac{1}{2}\beta}$. Since there was evidence for an interaction between candoxatrilat and C-ANF$_{4-23}$ on volume of distribution (i.e. candoxatrilat produced a small reduction in V_d when administered alone, but increased V_d when administered with C-ANF$_{4-23}$) it was not possible to use the factorial analysis to examine the effects of treatment on volume of distribution. However, comparison of the 95% confidence intervals between treatment groups indicated that the effect of candoxatrilat on volume of distribution in nephrectomized rats was not different from that seen in intact animals.

Infusion of C-ANF$_{4-23}$ (10 μg/kg + 1 μg/kg/min) to nephrectomized rats evoked a 4.5-fold increase in $AUC_{0-\infty}$ and 79% reduction in clearance of ^{125}I-ANF (Table 4), which was greater than that observed for intact animals (comparison of 95% confidence intervals). Although V_d decreased (by 70%), this change was smaller than the observed reduction in clearance; hence, unlike the observations in intact rats there was a significant increase in $T_{\frac{1}{2}\beta}$.

NEP inhibition with candoxatrilat (3 mg/kg) combined with ANF-C receptor blockade (10 μg/kg + 1 μg/kg/min) produced a similar increase in $AUC_{0-\infty}$ to that seen in intact rats. Clearance was reduced to the same value as had been observed in intact rats (77% reduction); however, V_d was only reduced by 45%. Although the proportionately greater fall in clearance than apparent V_d resulted in a large increase in $T_{\frac{1}{2}\beta}$ (to 248 ± 23 sec), this effect on $T_{\frac{1}{2}\beta}$ could not be clearly distinguished from that seen in intact rats (comparison of 95% confidence intervals). With the exception of the large increase in $T_{\frac{1}{2}\beta}$, the effects of a combination of ANF-C receptor blocker and NEP inhibitor closely resemble those of ANF-C receptor blocker alone in nephrectomized rats.

DISCUSSION

The present study shows that NEP inhibition with candoxatrilat and blockade of ANF clearance receptors with C-ANF$_{4-23}$ reduces the clearance of ^{125}I-ANF to similar extents, suggesting that both mechanisms play major and almost equal roles in the rapid clearance of ^{125}I-ANF from the circulation of the anaesthetized rat. However, the kidney contributes little to these processes over the range of ANF concentrations (i.e. 10–100 pg/mL) achieved in this study. The major role played by NEP in ANF clearance is consistent with our earlier observations that (±) candoxatrilat (UK-69,578) elevates circulatory ANF levels and elicits natriuretic and diuretic responses in conscious rodents [11] and dogs with atrio-ventricular heart block [22]; furthermore, the natriuretic and antihypertensive responses to (±) candoxatrilat are attenuated by pretreatment with ANF antiserum [23].

In a previous study of ANF clearance [10], we relied on the assumption that all the radioactivity recovered from the Sep-Pak extraction columns is intact ^{125}I-ANF and is not contaminated by ^{125}I-labelled ANF degradation products. The HPLC analysis of the plasma samples, taken after injection of ^{125}I-ANF into rats, showing a single radioactive peak that elutes at the same retention time as unlabelled ANF verifies that this assumption is correct. The present method also involves taking a total of 5 mL of blood from the animals in two separate sampling runs separated by 1 hr. The finding that the pharmacokinetic parameters are unchanged by vehicle administration indicates that this is a reliable method for determining the effects of compounds on the clearance of ANF. Since this method allows each animal to act as its own control and permits plasma ANF concentrations to be maintained within the physiological/pathophysiological range, it appears to offer significant advantages over methods which use a radioimmunoassay end point [23, 24].

The clearance of ANF is clearly biphasic, with a rapid early (α) phase followed by a more prolonged late (β) phase. Although these may be classically described as distribution and elimination phases [15], the present experiments indicate that these are distinct clearance processes. Thus, the rapid decline in the concentration of ANF after a bolus dose can largely be attributed to the binding of ANF to ANF clearance receptors. In agreement with Almeida *et al.* [1], we conclude that these receptors contribute to the large apparent V_d for ANF. However, we now demonstrate that metabolic degradation of ANF by NEP also contributes significantly to ANF clearance under these conditions, but that this is a slower process.

The finding that candoxatrilat reduces clearance without changing V_d whereas C-ANF$_{4-23}$ reduces both clearance and V_d suggests that these are independent processes that can be differentiated by their respective pharmacokinetic profiles. The NEP inhibitor SCH 39370 has also been reported to reduce the clearance of ANF in rats without changing V_d, although the dose of SCH 39370 used (30 mg/kg s.c.) had no significant effect on the $T_{\frac{1}{\beta}}$ of ANF in intact animals [25]. This finding is in contrast to the consistent increases in $T_{\frac{1}{\beta}}$ observed following doses as low as 0.01 mg/kg i.v. candoxatrilat, and may reflect differences in tissue penetration and/or *in vivo* potency of the two compounds.

Our data would indicate that NEP-mediated degradation and binding to ANF-C receptors each account for approximately half of the total clearance of ^{125}I-ANF. From our enzyme hydrolysis studies, however, we find that C-ANF$_{4-23}$ is a good substrate for dog kidney NEP, being degraded at a rate close to that of ANF itself. Furthermore, whilst this manuscript was in preparation, Seymour *et al.* [26] reported that C-ANF$_{4-23}$ was also degraded by NEP isolated from rat kidney. Therefore, we are unable to attribute the effects of C-ANF$_{4-23}$ entirely to ANF-C receptor blockade, and the proportion of total clearance due to ANF-C receptors may in fact be overestimated, as a result of competition between ^{125}I-ANF and C-ANF$_{4-23}$ for the NEP. In contrast, binding studies have demonstrated that candoxatrilat at concentrations up to 10 μM does not displace ^{125}I-ANF from rabbit lung membranes (a preparation containing both ANF-B and ANF-C receptors [21]). Thus, we can conclude that the reductions in clearance seen with candoxatrilat are not due to ANF-C receptor blockade, but appear specific to NEP inhibition. Whilst metabolism by NEP and binding to ANF-C receptors are biologically significant clearance mechanisms for ANF, evidence for the physiological importance of both mechanisms comes from studies we have performed on renal function in rats. Thus whilst candoxatrilat or C-ANF$_{4-23}$ alone induced significant changes in renal function, a marked increase in sodium excretion and urine flow was observed when the two agents were combined [27].

Although filtration at the glomerulus is thought to contribute to the removal of ANF from the circulation [9], our results from nephrectomized animals suggest that renal clearance contributes little to the total clearance of ^{125}I-ANF in the rat. Whilst this is in agreement with other studies in rats [2, 24, 28], dogs [3] and humans [4], it contrasts with our earlier findings [10] and those of Luft *et al.* [29] using high doses of atriopeptin III (ANF$_{5-28}$), which suggested that the kidney was responsible for nearly 60% of the clearance. Taken together, our results indicate that either the structure or more importantly the dose of ANF administered may determine the role of the kidney in ANF clearance. Thus, at the low doses of ANF employed in the present study, any effect of removing the kidneys may be compensated for by an increase in (for example) ANF-C receptor mediated clearance, and this would be consistent with the apparently greater effects of C-ANF$_{4-23}$ we observe in nephrectomized animals. In contrast, high doses of ANF may saturate ANF-C receptor-mediated uptake and unmask the role of the kidney in ANF clearance. Saturation of ANF-C receptors may also be of clinical significance, particularly in those disease states where endogenous ANF levels are elevated (e.g. congestive heart failure).

The ability or candoxatrilat to reduce ^{125}I-ANF clearance to the same extent in intact and

nephrectomized animals is entirely in agreement with the observations made using atriopeptin III [10]. Although the kidney is known to contain a high concentration of NEP, this appears to play no role in ANF clearance from plasma, consistent with its intra-luminal distribution in the proximal segments of the nephron. Thus, the ANF would only be metabolized by renal NEP after filtration in the glomerulus. However, although inhibition of tubular NEP may be unimportant for the clearance of ANF, it may play a significant role in the pharmacological activities of NEP inhibitors. Thus, filtered ANF is normally degraded rapidly and little intact ANF appears in the urine [9]. However, in the presence of candoxatrilat, ANF would be permitted to reach more distal segments of the nephron where, acting upon receptors in the collecting duct for example [30], it could produce the natriuresis and diuresis characteristic of this compound [23]. Thus, whilst extra renally located NEP is important for the changes in plasma ^{125}I-ANF clearance seen following administration of candoxatrilat, the pharmacological responses to candoxatrilat may result from localized inhibition of NEP within the target organs themselves. This is consistent with our earlier findings [10] and consonant with the wide distribution of NEP [31] and the involvement of many organs in the clearance of ANF [2, 32].

In addition to the ANF-C receptor and NEP mechanisms discussed above, there is a residual clearance (42 mL/min/kg, approximately 16% of total clearance) that remains after nephrectomy, ANF C-receptor blockade and NEP inhibition. This represents a high clearance rate with a $T_{\frac{1}{2}\beta}$ of 4.0 min and is not likely to be due to insufficient doses of candoxatrilat or C-ANF$_{4\text{-}23}$, as in both cases increasing the dose was without further effect. Whilst the sites and mechanisms responsible for this residual clearance are presently unknown, Johnson *et al.* [13] have described a peptidase in vascular smooth muscle cells which specifically removes the C-terminal tripeptide of ^{125}I-ANF and is not affected by inhibitors of NEP. The possibility that this mechanism is responsible for the residual clearance observed after nephrectomy, ANF-C receptor blockade and atriopeptidase inhibition merits further investigation.

In conclusion, degradation by NEP and binding to ANF-C receptors are major and almost equal clearance mechanisms for ANF in anaesthetized rats. Their specific blockade, by candoxatrilat and C-ANF$_{4\text{-}23}$, respectively, results in similar reductions in ANF clearance, but with characteristically different effects on $T_{\frac{1}{2}\beta}$ and V_d. Both agents represent valuable tools with which to elucidate the physiological and pathophysical role of ANF. However, the utility of C-ANF$_{4\text{-}23}$ is limited by its peptidic nature and instability to proteases, requiring that it be administered by intravenous infusion. Candoxatrilat, in contrast, is not metabolized extensively *in vivo* and its indanyl ester pro-drug (candoxatril) is active upon oral administration [33]. This latter agent may have clinical utility in the pharmacological manipulation of ANF levels for the treatment of cardiovascular disorders.

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REFERENCES

1. Almeida FA, Suzuki M, Scarborough RM, Lewick JA and Maack T, Clearance function of type C receptors for atrial natriuretic factor in rats. *Am J Physiol* **256**: R469–475, 1989.
2. Widimsky J, Debinski W, Kuchel O and Buu NT, ANF disappearance and distribution in rats. *Am J Physiol* **258**: H134–139, 1990.
3. Woods RL, Contribution of the kidney to metabolic clearance of atrial natriuretic peptide. *Am J Physiol* **255**: E934–947, 1988.
4. Tonolo G, McMillan M, Polonia J, Passola A, Montorsi P, Toro A, Glarisso N and Richards AM, Plasma clearance and effects of α-hANP infused in patients with end-stage renal failure. *Am J Physiol* **254**: F895–899, 1988.
5. Yandle TG, Richards AM, Nicholls MG, Cuneo R, Espiner EA and Livesey HM, Metabolic clearance rate and plasma half life of alpha-human atrial natriuretic peptide in man. *Life Sci* **38**: 1827–1833, 1986.
6. Sonnenberg JL, Sakane Y, Jeng AY, Koehn JA, Ansell JA, Wennogle LP and Ghai RD, Identification of protease 3.4.24.11 as the major atrial natriuretic factor degrading enzyme in the rat kidney. *Peptides* **9**: 173–180, 1988.
7. Stephenson SL and Kenny AJ, The hydrolysis of α-human atrial natriuretic peptide by pig kidney microvillar membranes is initiated by endopeptidase-24.11. *Biochem J* **243**: 183–187, 1987.
8. Maack T, Suzuki M, Almeida FA, Nussenzveig D, Scarborough RM, McEnroe GA and Lewicki JA, Physiological role of silent receptors of atrial natriuretic factor. *Science* **238**: 675–678, 1987.
9. Espiner EA, Nicholls MG, Yandle TG, Crozier TG, Cuneo RC, McCormick D and Ikram H, Studies on the secretion, metabolism and action of natriuretic peptide in man. *J Hypertens* **4**: 585–591, 1986.
10. Barclay PL, Bennett JA, Samuels GMR and Shepperson NB, The atriopeptidase inhibitor (±) candoxatrilat reduces the clearance of ANF in both intact and nephrectomised rats: evidence for an extra-renal site of action. *Biochem Pharmacol* **41**: 841–844, 1991.
11. Danilewicz JC, Barclay PL, Barnish IT, Brown D, Campbell SF, James K, Samuels GMR, Terrett NK and Wythes MJ, UK-69,578, a novel inhibitor of EC 3.4.24.11 which increases endogenous ANF levels and is natriuretic and diuretic. *Biochem Biophys Res Commun* **164**: 58–65, 1989.
12. Barclay PL, Peters CJ, Bennett JA, Danilewicz JC, Samuels GMR and Shepperson NB, The natriuretic and diuretic responses to the atriopeptidase inhibitor UK-69,578 in volume expanded rats. *Br J Pharmacol* **98**: 824P, 1989.
13. Johnson GR, Arik L and Foster CJ, Metabolism of ^{125}I-Atrial natriuretic factor by vascular smooth muscle cells. *J Biol Chem* **264**: 11637–11642, 1989.
14. Lang RE, Tholken H, Ganten D, Luft FC, Ruskoaho H and Unger T, Atrial natriuretic factor—A circulating hormone stimulated by volume loading. *Nature* **314**: 264–266, 1985.
15. Gibaldi M and Perrier D, Multicompartment models. In: *Pharmacokinetics 2nd Edn, Drugs and the Pharmaceutical Sciences* (Ed. Swarbrick J), Vol. 15, pp. 45–111. Marcel Dekker, New York, 1982.
16. Williams DA, The comparison of several dose levels with a zero dose control. *Biometrics* **28**: 519–531, 1972.
17. Chipkin RE, Berger JG, Billard W, Iario LC, Chapman R and Bennett A, Pharmacology of SCH 34826, an

orally active enkephalinase inhibitor analgesic. *J Pharmacol Exp Ther* **245**: 829–838, 1988.
18. Olins GM, Patton DR, Tjoeng FS and Blehm DJ, *Biochem Biophys Res Commun* **140**: 302–307, 1986.
19. Bovy PR, O'Neal JM, Olins GM and Patton DR, Identification of structural requirements for analogues of Atrial Natriuretic Peptide (ANP) to discriminate between ANP receptor subtypes. *J Med Chem* **32**: 869–874, 1989.
20. Munson PJ and Rodbard D, LIGAND: a versatile computerised approach for the characterisation of ligand-binding systems. *Anal Biochem* **107**: 220–239, 1980.
21. Olins GM, Patton DR, Bury PR and Mehta PP, A linear analog of atrial natriuretic peptide (ANP) discriminates guanylate cyclase-coupled ANP receptors from non-coupled receptors. *J Biol Chem* **263**: 10989–10993, 1988.
22. Alabaster CT, Machin I, Barclay PL and Samuels GMR, The effect of UK-69,578, an atriopeptidase inhibitor, in a conscious dog model of cardiac insufficiency. *J Am Coll Cardiol* **13**: 75A, 1989.
23. Shepperson NB, Barclay PL, Bennett JA and Samuels GMR, Inhibition of neutral endopeptidase (EC 3.4.24.11) levels to an ANF mediated natriuretic, diuretic and antihypertensive response in rodents. *Clin Sci* **80**: 265–269, 1991.
24. Katsube N, Schwartz D and Needleman P, Atriopeptin turnover: quantitative relationship between *in vivo* changes in plasma levels and atrial content. *J Pharmacol Exp Ther* **239**: 474–479, 1986.
25. Chiu PJS, Tetzloff G, Romano MT, Foster CJ and Sybertz EJ, Influence of C-ANF receptor and neutral endopeptidase on pharmacokinetics of ANF in rats. *Am J Physiol* **260**: R208–R216, 1991.
26. Seymour AA, Norman JA, Asaad MM, Fennell SA, Offei BA-, Little DK, Kratuius VJ, Delaney NG, Hunt JT and Donato GD, Possible regulation of atrial natriuretic factor by neutral endopeptidase 24.11 and clearance receptors. *J Pharmacol Exp Ther* **256**: 1002–1009, 1991.
27. Barclay PL, Bennett JA, Greengrass PM, Shepperson NB and Samuels GMR, Comparison of the natriuretic and diuretic activity of an ANF-C receptor ligand (C-ANF_{4-23}) and an atriopeptidase inhibitor (candoxatrilat) in anaesthetised rats. In: *A Decade of ANF Research, Abstracts of the International Society of Hypertension Satellite Symposium, Ottowa, Canada, 21–23 June 1990* (Ed. de Bold AJ), p. 20.
28. Murphy KK, Thibault G, Schiffrin EL, Garcia R, Chartier L, Gutkowska J, Genest J and Cantin M, Disappearance of atrial natriuretic factor from circulation in the rat. *Peptides* **7**: 241–246, 1986.
29. Luft FC, Lang RE, Aronoff GR, Ruskoaho H, Toth M, Ganten D, Sterzel RB and Unger T, Atriopeptin III kinetics and pharmacokinetics in normal and anephric rats. *J Pharmacol Exp Ther* **236**: 416–418, 1986.
30. Zeidel ML, Renal actions of atrial natriuretic peptide: regulation of collecting duct sodium and water transport. *Annu Rev Physiol* **52**: 747–759, 1990.
31. Ronco P, Pollard H, Galceran M, Delauche M, Schwartz JC and Verroust P, Distribution of enkephalinase (membrane metalloendopeptidase EC 3.4.24.11) in rat organs. Detection using a monoclonal antibody. *Lab Invest* **58**: 210–217, 1988.
32. Krieter PA and Tripani AJ, Metabolism of atrial natriuretic peptide. *Drug Metab Dispos* **17**: 14–19, 1988.
33. Samuels GMR, Barclay PL, Ellis P and Shepperson NB, The natriuretic and diuretic efficacy of an orally active atriopeptidase inhibitor (API), UK-79,300 in mice. *Am J Hypertens* **2**: 119A, 1989.