inflammation. A clinical study has shown that COX-2-specific inhibition improves endothelial-dependent vasodilation and reduces low-grade chronic inflammation and oxidative stress in severe coronary artery disease (22). It has been observed that celecoxib produces a dose-dependent increase in nitrous oxide release and reduces the oxygen radical antioxidant capacity in primary cultures of human endothelial cells (23).

The Vioxx Gastrointestinal Outcomes Research (VIGOR) trial (24), however, showed that statistically more thromboembolic cardiovascular events occurred in individuals receiving a COX-2-specific inhibitor, rofecoxib. These observations have led to debates on the effects of COX-2 on cardiovascular events. By contrast, there was no increase in risk for patients receiving celecoxib when evaluated in either a prospective trial (25) or retrospective analyses (26–28). The basis for the observed differences in thrombotic risk among COX-2 inhibitors may be related to their distinct physicochemical properties (29). Rofecoxib is a sulfone with relatively poor tissue distribution, whereas celecoxib is a sulfonamide that has extensive tissue distribution. It has been shown that rofecoxib can cause a marked increase in nonenzymatic generation of isoprostanes and reduce the oxygen radical antioxidant capacity (23). One possible explanation of the results of VIGOR, therefore, may be that the increased risk for thromboembolic cardiovascular events is based on a nonenzymatic mechanism of rofecoxib and is not related with COX-2 inhibition.

References
cient specificity can be difficult because of the close sequence homology of CNP with atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP). Cross-reacting antibodies are particularly troublesome in heart failure patients, in whom plasma concentrations of ANP and BNP are increased. Plasma extraction before measurement has also been necessary to avoid protein interference \((I, 2)\); however, extraction can reduce assay performance because of low and inconsistent peptide recovery. The aim of the present study was to develop an RIA for measurement of proCNP in human plasma. Plasma was extracted by a simple procedure before immunoanalysis with antibodies raised against a unique epitope in proCNP. The proCNP concentrations in venous plasma from healthy individuals and heart failure patients were assessed. Additionally, proCNP concentrations were measured in plasma selectively sampled from the coronary sinus in heart failure patients.

Human proCNP 1–10 extended C-terminally with 4 alanyl and 1 tyrosyl residue was custom synthesized for tracer preparation and standards. ProCNP 1–10 extended C-terminally with 4 alanyl and 1 cystyl residue was used for directional carrier coupling (Cambridge Biochemical Research Ltd.). In addition, human proCNP 1–7, proCNP 2–7, and mouse proCNP 1–10 were synthesized for specificity testing. The purities and identities of all peptides were verified by amino acid analysis and reversed-phase HPLC.

Antisera against the N-terminal sequence 1–10 of human proCNP were produced by use of 10 mg of proCNP 1–10-Ala-Ala-Ala-Ala-Cys coupled to 20 mg of bovine serum albumin \((3)\). The rabbit immunization procedure used has been described previously \((4)\).

We iodinated 5 μg \((2.1 \text{ nmol})\) of the tyrosine-extended fragment \((\text{proCNP 1–10-Ala-Ala-Ala-Ala-Tyr})\), using a mild chloramine-T method \((5)\). The iodinated peptide was purified by reversed-phase HPLC \([\text{Cg column; 250} \times 4.6\text{ mm (i.d.)}\); Vydac] and eluted by a linear ethanol gradient \((10\%–80\%)\) in 10 g/L trifluoroacetic acid. We evaluated the separation of labeled and nonlabeled peptide by reapplying a 1-mL mixture of the moniodinated peak fraction and 10 pmol of nonlabeled proCNP 1–10-Ala-Ala-Ala-Ala-Tyr to the HPLC column. The radioactivity and immunoreactivity were then measured. Calibrators were prepared from synthetic proCNP 1–10-Ala-Ala-Ala-Ala-Tyr in 20 mmol/L barbital buffer \((\text{pH 8.4})\) containing 1.1 g/L bovine serum albumin and 0.6 mmol/L thiomersal.

Venous blood was obtained from 14 chronic heart failure patients undergoing right heart catheterization \((2\) females, 12 males; median age, 51 years; range, 26–68 years) \();\) the characteristics of these patients have been reported previously \((6)\). The left ventricular ejection fraction was severely reduced on ventriculography \((\text{median, 20\%; range, 10\%–35\%})\). All patients were in a stable phase and receiving standard medical treatment including an angiotensin-converting enzyme inhibitor, beta-blocker, and spironolactone. In addition, 18 control individuals were included \((9\) females, 9 males; median age, 68 years; range, 60–79 years). All participants had normal findings on 2-dimensional echocardiography, pulmonary function tests, ergometry, blood screening, and physical examination.

Both the heart failure patients and the controls gave informed, written consent for participating in the study, and the local ethics committee had approved the study protocol \((\text{KF 01-231/99})\).

Right heart catheterization was performed in the heart failure patients with a French 8 Swan-Ganz catheter or a French 6 multipurpose catheter. Blood \((20 \text{ mL})\) was sampled from the inferior caval vein and the coronary sinus and was collected in chilled 10-mL Vacutainer Tubes containing disodium EDTA \((1.5 \text{ g/L})\). The position of the catheter tip was verified by a small retrograde infusion of contrast agent under fluoroscopic guidance.

Plasma \((750 \mu\text{L})\) was extracted by use of ultracentrifugation filter devices \((\text{Amicon Ultra-4 100 000 molecular weight cutoff; Millipore})\) at 4700g at 4°C for 1 h. The ultrafiltrate was collected and stored at −20°C until analysis. For RIA, both calibrators and extracted plasma \((150 \mu\text{L})\) were mixed with tracer peptide \((3000 \text{ cpm})\) and antisizer \((\text{final dilution, 1:376 000})\) in 200 μL of 20 mmol/L barbital buffer \((\text{pH 8.4})\) containing 1.1 g/L bovine serum albumin and thiomersal \((0.6 \text{ mmol/L})\). Synthetic proCNP 1–10-Ala-Ala-Ala-Ala-Tyr was used as calibrator. After 4 days of incubation at 4°C, the antibody-bound and free tracer was separated by addition of 0.5 mL of plasma-coated charcoal. The mixture was left for 20 min at room temperature and centrifuged for 10 min \((3450g)\). Controls, buffer blanks, and sample blanks \((\text{without antisiser added})\) were included in the assays, and samples were always assayed in duplicate.

A single report has suggested that seminal plasma contains high amounts of bioactive CNP \((7)\). To explore the impact of ultrafiltration on both recovery and protein interference, we subjected human seminal plasma and seminal plasma extracted with ultrafilters to size-exclusion chromatography on Sephadex G-50 Superfine columns \((1000 \times 10 \text{ mm}; \text{Pharmacia}); samples were eluted with assay buffer supplemented with 0.5 mol/L NaCl. The void and total volumes were determined by elution of \(^{125}\text{I}^-\text{labeled albumin and} \ ^{22}\text{Na}^-\text{labeled NaCl, respectively.}

The incorporation of \(^{125}\text{I}^-\text{labeled} \text{proCNP 1–10-Ala-Ala-Ala-Ala-Tyr was 70\%–90\%}. \text{Labeled and nonlabeled peptides were completely separated, and the dilution curves for labeled and nonlabeled antigens were parallel. Of the 24 immunized animals, only 1 rabbit produced an antisiser with a sufficient titer. This antisiser revealed a high degree of sensitivity in the assay with an analytical detection limit of 0.4 pmol/L, calculated as the mean of 10 replicates of the zero calibrator + 3 SD. The equilibrium constant was calculated to 0.15 × 10⁻² L/mol, and the Sips index of heterogeneity was 0.92 \((8)\). The antisiser specificity was then expressed as the median inhibitory dose needed for 50% tracer displacement \((\text{ID}_{50}\) ) of calibrator peptide, 15 pmol/L; \ID_{50} of proCNP 1–7, 82 pmol/L. In contrast,
removal of the N-terminal lysyl residue completely abolished antiserum binding, as did substitution of alanine with threonine in position 4 (mouse proCNP). Consequently, the N-terminal lysyl and the alanyl residue in position 4 constitute an essential part of the antiserum epitope, whereas residues 7–10 are of only moderate importance for antibody binding. A search in the SwissProt database for sequences resembling human proCNP 1–10 identified only the corresponding N-terminal proCNP sequence in mammals. The between-assay CVs (controls) were 6.9% at 8 pmol/L, 5.7% at 15 pmol/L, and 3.8% at 40 pmol/L (n = 10), and the within-assay CVs were 5.6% at 8 pmol/L, 8.9% at 15 pmol/L, and 4.1% at 40 pmol/L (n = 10). The CVs of the plasma assay, including ultrafiltration, were 19% at 9 pmol/L, 4.2% at 21 pmol/L, and 5.7% at 55 pmol/L (n = 6). The mean (SD) volume recovery after plasma ultracentrifugation was 78 (1)% (n = 10). Assay buffer or normal plasma supplemented with 1000 pmol/L synthetic proCNP 1–10-Ala-Ala-Ala-Ala-Tyr followed by extraction with ultrafilters gave recoveries of 76 (9)% and 62 (3), respectively (n = 6).

To assess the extraction procedure, we subjected untreated and ultrafiltered seminal plasma (a rich source of proCNP) to gel-filtration chromatography. The elution profile of untreated seminal plasma revealed an immunoreactive peak near the void volume and a distinct peak (K_D = 0.89; Fig. 1A). In contrast, ultrafiltration of seminal plasma before gel filtration completely abolished the

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Fig. 1. Elution profiles of proCNP immunoreactivity (A and B), and proCNP concentrations in plasma (C and D).
(A), elution profile for crude seminal plasma; (B), elution profile for seminal plasma extracted by ultrafiltration. (C), proCNP concentrations in plasma from heart failure patients and controls; (D), proCNP concentrations in plasma from different cardiovascular regions in heart failure patients. Connected points in D represent data obtained from the same patient. Horizontal bars indicate median concentrations. NS, not significant.
immunoreactivity in the void volume but preserved the later peak (Fig. 1B).

The proCNP concentrations in venous plasma from the controls and from the heart failure patients are shown in Fig. 1C. There was no difference between the 2 groups [median (range), 8.3 (7.0–12.0) pmol/L vs 8.0 (4.9–11.0) pmol/L; \( P = 0.47 \)]. The proCNP concentrations in plasma from different cardiovascular regions in the heart failure patients are shown in Fig. 1D. In plasma from the inferior caval vein, the proCNP concentration was marginally higher than in plasma from the coronary sinus [median (range), 7.5 (6.2–11.0) pmol/L vs 8.3 (7.0–12.0) pmol/L; \( P = 0.013 \)].

The present study demonstrates that measurement of precursor peptide is a feasible way to achieve gene product specificity. Extraction by Sep-Pak cartridges, which has been applied in other studies (1, 2, 9), offers the advantage that samples can be concentrated. On the other hand, peptide recovery and, consequently, measurements can be highly variable. To our knowledge, no previous studies have used ultrafiltration for plasma extraction. We established that the recovery is reproducible and sufficient to allow accurate measurement of proCNP.

The proCNP concentration in ultrafiltered venous plasma was not increased in heart failure patients compared with controls. The use of plasma proCNP as a marker in stable heart failure patients is thus not supported by our findings. However, patients with destabilized, severe exacerbations were not included in our study. Whether proCNP could be a marker in such patients remains to be investigated.

The proCNP concentration was marginally higher in peripheral venous plasma than in plasma sampled from the coronary sinus. Interestingly, cardiac secretion of CNP has been reported recently (10). Although our results do not exclude a minor cardiac secretion, they suggest that circulating proCNP is not secreted from the failing heart. Referring to CNP as a “cardiac” natriuretic peptide is thus misleading (11).

The expert technical assistance of Lone Olsen is gratefully appreciated. This study was supported by a research fellowship position from the Lundbeck Foundation.

References


DOI: 10.1373/clinchem.2005.053488

False-Positive Results in the Detection of Methadone in Urines of Patients Treated with Psychotropic Substances, Frédérique Lancelin, Linda Kraoul, Nadia Flatischler, Sophie Brovedani-Roussel, and Marie-Liesse Piketty (Laboratoire Central de Biologie, Centre Hospitalier Sainte Anne, 1 rue Cabanis, F 75014, Paris, France; *author for correspondence: fax 33-1-45657446, e-mail m.piketty@ch-sainte-anne.fr)

In the context of methadone maintenance programs, immunochemical detection of methadone in urine has evolved, with rapid and sensitive techniques gaining impact in clinical laboratories because of their cost-effectiveness and suitability for automation (1). However, immunochemical methods may lack the specificity necessary for accurate measurement. We report spurious methadone results encountered in a psychiatric hospital.

We have been detecting urinary methadone with the Kinetics Interaction of Microparticles in Solution (KIMS) methodology on the Roche Integra 800 since April 2003 (Roche Diagnostics). The test is used in the qualitative mode, with a methadone concentration exceeding 0.300 mg/L being reported as positive. This technology is known for its steep dose–response curve around the cutoff of the assay (1). In its first formulation, the reagent included a polyclonal antibody that remained free in solution and captured either methadone or a methadone multivalent conjugate coupled on microparticles. In September 2004, Roche Diagnostics switched from the polyclonal antibody to a monoclonal antibody and modified the original KIMS technology by coupling the antibody to the microparticles and keeping the multivalent drug conjugate in solution. This second-generation inhibition assay was expected to improve the sensitivity and specificity of the test (2).

Soon after this change, some clinicians contacted the laboratory about a mismatch between the clinical information and the methadone detection result. Urines from some patients tested positive for methadone without a