PAIRED-ION REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN AND RAT CALCITONIN

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SUMMARY

Improved methods for isolation, characterization, and quantitation of immunochemically heterogeneous forms of calcitonin (CT) in tissue and plasma must be developed before the biological origins and clinical importance of CT moieties can be elucidated. We are now proposing reversed-phase high-performance liquid chromatography (RP-HPLC) as one possible means of achieving high recovery, high resolution of CT moieties. In this paper we report RP-HPLC analyses of trace amounts of radiolabeled and unlabeled synthetic human and rat CT. We have systematically evaluated our application of RP-HPLC by employing several elution modes, including isocratic and gradient elution, as well as several elution reagents. We determined that high recovery and high resolution were best achieved with alkyl ion-pairing reagents, such as tetrabutylammonium phosphate, pH 7.5, or sodium sulfonyl hexane, pH 3.5. The most sensitive UV detection of trace amounts of CT was achieved with tetrabutylammonium phosphate buffer (TBAP). We recommend for RP-HPLC of CT a C_{18}-bonded silica column and elution with a 20-min linear gradient of methanol–water (20:80 to 80:20, v/v) containing 0.005 M TBAP. Combined with appropriate extraction procedures, such as silica adsorption or immunoadsorbant chromatography, this paired-ion RP-HPLC method can be an important aid in achieving more accurate and extensive information about CT moieties in biological samples. This method will also allow the rapid, optical detection and quantitation of CT moieties recovered from tissues, and perhaps from plasmas.

INTRODUCTION

Improved immunoassay methods have demonstrated elevations of plasma calcitonin (CT) in many physiological and pathophysiological states, notably cancer, renal failure, and hypercalcemia[1–7]. Despite some discrepancies in apparent size and number of immunochemical forms of CT detected in hypercalcitoninemic states, gel chromatography analyses do confirm the existence of multiple forms[1–6]. The supposition is that the specific forms comprising hypercalcitoninemia differ in various pathophysiological states, and that these differences among the CT moieties reflect the

pathogenesis of these states[1,2]. Despite the potential importance of this concept, its proof remains elusive. Sensitive immunoassays in concert with new methods for extracting and concentrating CT moieties could support extensive studies of CT heterogeneity[7-9]. But the labor involved in gel chromatography analysis and the limited resolution it now offers do not make this procedure a likely choice for accurately characterizing and elucidating the complex mixture of CT moieties expected to be recovered from plasma and CT-producing tissues.

We previously reported reversed-phase high-performance liquid chromatography (RP-HPLC) on octadecylsilyl-bonded silica for purification of certain vitamin D metabolites[10,11]. Recently, RP-HPLC methods have evolved for high resolution and efficient recovery of peptides[12-14]. These methods employ a stationary phase of octadecyl-bonded silica[12-14] and a mobile phase containing hydrophobic ion-pairing reagents[12,13]. The RP-HPLC method for CT we are reporting has developed systematically from our evaluation of ion-pairing reagents, solvent-buffer composition, pH, elution mode (isocratic vs. gradient), and flow rate. These components were studied not only in respect to their effects on the resolution and recovery of CT, but also in relation to their effects on sensitive detection of CT by UV absorbance and by radioimmunoassay.

MATERIAL AND METHODS

Spectroanalytic grade methanol (Burdick & Jackson Lab., Muskegon, MI, U.S.A.) and distilled–deionized water were filtered through fluoropore filters with Z-μm pore size (Millipore, Bedford, MA, U.S.A.) and thoroughly degassed prior to use. The ion-pairing reagents (Water Assoc., Milford, MA, U.S.A.) were tetrabutylammonium phosphate (TBAP) and sodium sulfonyl n-hexane (SSH); the final concentration of the ion-pairing reagents in methanol and in water was 0.005 M. In addition to paired-ion chromatography buffers, we also studied the following buffer systems: 0.01 M ammonium acetate, pH 4.5–6.0; 0.01 M Tris, pH 6.5–7.5; and 0.05 M sodium phosphate, pH 6.0–7.5.

CT preparations, radioimmunoassay and immunoextraction

Synthetic human CT (hCT) was obtained from Ciba-Geigy (Basel, Switzerland); synthetic rat CT (rCT) was analyzed for amino acids and obtained from Drs. D. Ontjes and C. Cooper at the University of North Carolina, Chapel Hill, NC, U.S.A.[7]. The synthetic CTs were radioiodinated by chloramine-T oxidation[15]. ^{125}I-CT monomer was purified by gel chromatography (30 × 0.7 cm column of Sephadex G-50, fine) in 0.2 M ammonium acetate, pH 5.8, containing 0.03% Brij; preparations of purified CT monomer ($K_d = 0.44$) had specific radioactivities ranging between 125 and 350 μCi/μg. Purified CT was lyophilized, stored for several days, and solubilized by overnight incubation in solvent-buffers used for RP-HPLC. Radioimmunoassays and immunoextractions of HPLC fractions employed rabbit antibodies to hCT with affinities for intact hCT and rCT and for carboxyterminal fragments of CT[1,7,16].

High-performance liquid chromatography

The HPLC apparatus consisted of the following components (Waters Assoc.):

U6K injector, 2 6000A pumps; 660 programmer; and 450 variable wavelength UV detector. Full-scale UV absorbance was expressed as a.u.f.s. A Hewlett-Packard 3380A recording integrator was used to register retention times and to obtain integrated UV-absorbance peak areas. A reversed-phase μBondapak C_{18} column (10 μm) (Waters Assoc.) was used in tandem with a CO:PELL PAC guard column (Whatman, Clifton, NJ, U.S.A.).

Both isocratic and gradient elution modes were examined for their effects on resolution and quantitation of hCT and rCT. The isocratic solvent systems we considered ranged from methanol–aqueous buffer (35:65 to 100% methanol with a pH range of 3.5 to 7.5. Constant flow-rates studied were between 1 and 2 ml/min. Both linear and asymptotic gradients of methanol–aqueous buffer (20:80 to 80:20) were run over a 20-min period (after 20 min the methanol:buffer ratio was maintained at 80:20) at a selected constant flow-rate of 1–2 ml/min. The radioactive and non-radioactive CT samples were applied to RP-HPLC in 20–50-μl volumes.

Polyacrylamide gel electrophoresis (PAGE)

Samples of ^{125}I-hCT dissolved in 50 μl sodium dodecyl sulfate (SDS)–urea–mercaptoethanol were electrophoresed by previously described methods[17]. Acrylamide concentration was 10%; bisacrylamide concentration was 1%; gels contained 8 M urea and 0.1% SDS.

RESULTS

Fig. 1 shows the elution profile of trace amounts of hCT and ^{125}I-hCT, previously purified by gel chromatography (Sephadex G-50)[28], following their application to an isocratic RP-HPLC system of methanol–TBAP (70:30), pH 7.5. The first radioactive peak (retention time of 3 min) had the same retention time as ^{125}I, was not immunoreactive, and could be eliminated by prior treatment of samples with an anion exchange resin, AG1-X8[15]. Compared to isocratic elution with other buffer systems (0.01 M ammonium acetate, pH 4.5–6.0; 0.01 M Tris, pH 6.5–7.5; and
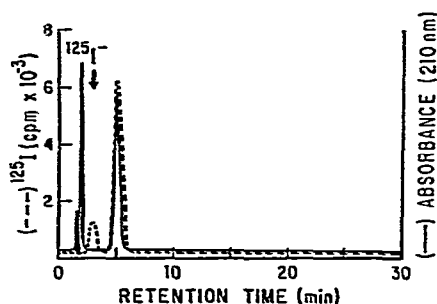

Fig. 1. Isocratic paired-ion RP-HPLC elution profiles of trace amounts of ^{125}I-hCT and hCT. Samples (10,000 cpm of ^{125}I-hCT or 100 ng of hCT) were chromatographed using a μBondapak C_{18} column in tandem with a CO:PELL PAC guard column and as eluent methanol–water (70:30) which contained 0.005 M TBAP, pH 7.5. Elution rate was 1.5 ml/min at 100 bar. Radioactivity (– – –) and UV absorbance at 210 nm (——) are the mean $\pm$ S.D. of 9 RP-HPLC procedures, with retention times of 3.0 $\pm$ 0.1 and 5.0 $\pm$ 0.2 min.

0.05 M sodium phosphate, pH 6.0–7.5), this system gives better resolution (narrower peak width and relatively longer retention time for hCT). Radioactive and non-radioactive hCT coeluted close to the solvent front with a retention time of 5.0 $\pm$ 0.2 min (mean $\pm$ S.D.). We achieved better hCT recovery (71 $\pm$ 12%, $n = 9$) with ion-pairing reagents (TBAP or SSH) than we did with other buffer systems. Systems without phosphate gave low (11.7 $\pm$ 1.6%) recovery; sodium phosphate buffer gave 44.9 $\pm$ 3.5% recovery, but the retention time was less than that obtained with TBAP or SSH (3.5 $\pm$ 0.1 min for sodium phosphate $vs.$ 5.0 $\pm$ 0.2 min for TBAP or SSF).
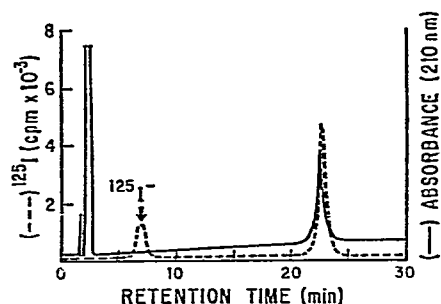

Fig. 2. Gradient paired-ion RP-HPLC elution profiles of trace amounts of ^{125}I-hCT and hCT. Samples (10,000 cpm of ^{125}I-hCT or 100 ng of hCT) were chromatographed on the columns described in Fig. 1 using a linear gradient of methanol–water (20:80 to 80:20) containing 0.005 M TBAP, pH 7.5, run over a 20-min period at an elution rate of 1.5 ml/min. Elution profiles were determined as in Fig. 1; results are the mean $\pm$ S.D. of 22 RP-HPLC procedures, with retention times of 6.5 $\pm$ 0.4 and 22.0 $\pm$ 0.3 min.

The elution profiles from 22 RP-HPLC analyses of hCT and ^{125}I-hCT using a 20-min linear gradient of methanol–water (20:80 to 80:20) in TBAP, pH 7.5, are summarized in Fig. 2. As in the isocratic system (Fig. 1), there was narrow peak width without double peaks or tailing[14]. With gradient elution, the retention time of hCT increased: hCT and ^{125}I-hCT eluted at 22.0 $\pm$ 0.3 min with good recovery (75.1 $\pm$ 9.9%, $n =$ 22). We performed similar RP-HPLC using 0.005 M SSH, pH 3.5, instead of TBAP. In isocratic and in gradient elution modes the use of SSH gave hCT recovery comparable to TBAP and a retention time slightly longer, without peak widening. Acetic acid, used to adjust the pH of the SSH buffer, interfered with UV-absorbance monitoring below 230 nm[12,13,18]; we found TBAP much less UV-absorbing, even at 210 nm. A further advantage of TBAP over SSH emerged in radioimmunoassay studies. To detect possible interference of the ion-pairing reagents in CT radioimmunoassays, 10–100-μl aliquots of aqueous TBAP or SSH at various concentrations were tested for their effects on antibody–tracer binding[15,19]: 10 μl of 0.15 M SSH markedly decreased specific binding by 75%; 10 μl of 0.15 M TBAP caused 45% decrease in tracer–antibody binding. At lower concentrations (0.015 M), neither buffer interfered with radioimmunoassay performance. Subsequent studies were done only with TBAP.

Fig. 3 summarized 7 RP-HPLC elution profiles for purified rCT and ^{125}I-rCT applied to the same type of methanol–water gradient (0.005 M TBAP, pH 7.5) used to fractionate hCT (Fig. 2). The retention time (23 $\pm$ 0.2 min) for rCT was nearly identical to the retention time (22.2 $\pm$ 0.5 min) for hCT (Fig. 2).

The RP-HPLC elution profiles of purified hCT and ^{125}I-hCT after 2 months of storage in 0.01 M ammonium acetate buffer at −20°C are summarized in Fig. 4. Samples were lyophilized just before RP-HPLC. Radioiodide and hCT eluted with retention times similar to those noted for fresh samples (Fig. 2); recovery of radioactivity applied to HPLC was 71 ± 11%, n = 6. For both labeled and unlabeled hCT preparations there was good resolution of 3 additional peaks (9.0 ± 0.1, 11.0 ± 0.8, 16.3 ± 0.6 min), presumably fragments or aggregates of hCT. The radioactive peaks from RP-HPLC of fresh (Fig. 2) and stored (Fig. 4) ^{125}I-hCT were further characterized by pooling, lyophilizing, and immunoextracting specific peaks from several HPLC runs. The only immunoreactive peak was the latest eluting (22 min) and major peak, previously identified as hCT (Fig. 2). SDS–urea–polyacrylamide gel electrophoresis of the two-month-stored CT preparation showed fragments and higher molecular weight aggregates in addition to CT monomer (not shown). The resolution of these additional peaks was poor, and recovery of radioactivity was low, ranging between 24 and 40%.
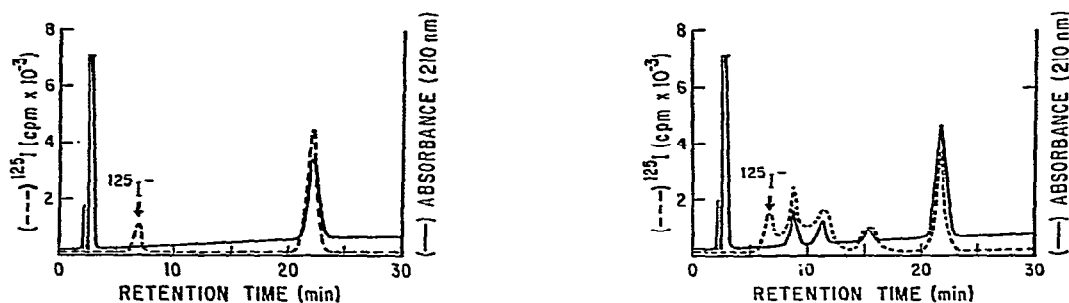

Fig. 3. Gradient paired-ion elution profiles of trace amounts of ^{125}I-rCT and rCT. Sample amounts (10,000 cpm or 100 ng), chromatography conditions, and radioactivity (– – –) or UV-absorbance (——) measurements were as described in Fig. 2. Results are the mean ± S.D. for 7 RP-HPLC procedures, with retention times of 7.0 ± 0.2 and 23.0 ± 0.2 min.

Fig. 4. Gradient paired-ion-elution profiles of trace amounts of ^{125}I-hCT and hCT stored frozen for 2 months in 0.01 M ammonium acetate. Samples (15,000 cpm of stored ^{125}I-hCT or 200 ng of stored hCT) were chromatographed and radioactivity (– – –) or UV absorbance (——) measured as described in Fig. 2. Results are the mean ± S.D. for 6 RP-HPLC procedures, with retention times of 6.5 ± 0.8, 9.0 ± 0.1, 11.0 ± 0.8, 16.3 ± 0.6, and 22.2 ± 0.5 min.

DISCUSSION

Gel chromatography, cation exchange chromatography and electrophoresis have been primary methods for partial separation of CT moieties[1–6,19–22]. To achieve resolution/purification of CT moieties it has been necessary to combine several of these time-consuming procedures, each associated with significant loss of immunoreactive CT moieties[20,22]. We initiated the present studies with the impression that RP-HPLC could well be an eventual means for efficient, high-resolution isolation, and perhaps even for direct quantitation, of CT moieties in biological samples. We have assessed recovery, peak resolution, and retention time of synthetic CTs (human and rat) with a variety of protein- and detergent-free solvent–buffer combinations and elution modes. CT recovery and CT resolution were decreased with standard chroma-

tography buffers: Resolution values* for the separation of ^{125}I and hCT were at best 3.7 for ammonium acetate, sodium phosphate, and other standard buffer systems examined; alkyl ion-pairing reagents, tetrabutylammonium phosphate (TBAP) or sodium sulfonyl hexane (SSH), characteristically gave resolution values of 4.0 or greater. In contrast to the poor recoveries noted with phosphate and other standard buffers (44.9 ± 3.5% or less), good recovery was achieved with alkyl ion-pairing reagents (75.1 ± 9.9%). The best resolution was obtained by gradient, as opposed to isocratic, elution mode (compare Figs. 1 and 2).

Comparisons of the two ion-pairing reagents, TBAP and SSH, revealed that each has certain advantages and limitations. SSH, pH 3.5, provided slightly longer retention times and better resolution. The improved resolutions with SSH could be due to alkyl-ion pairing with protonated amino groups of CT combined with partial ionic suppression of the free carboxyl ($pK = 3.65$) group of the aspartic acid residue[12,23,24]. It is very likely that lowering the pH, perhaps to 2, which decreases ionization of aspartate and further reduces hydrophilic interactions, would lengthen retention times, as a result of relatively increased hydrophobicity. Both effects, ion pairing- and ionic suppression, reduced hydrophilic interactions and made more dominant hydrophobic interactions between the CT–alkyl–ion complexes in the mobile phase and the stationary organic phase (octadecyl groups bonded to silica). At pH 7.5 there can be no ionic suppression of CT's major protonated sites[23], such as the epsilon-amino group in lysine and the alpha-amino group in the amino-terminal cysteine residue[23,24]. Both amino groups have pK values close to 10.5[23]. Still, TBAP at pH 7.5, nearly the upper pH limit for column-packing stability, gave nearly as good a resolution as did SSH at pH 3.5. This suggests that the increased retention time of CT during RP-HPLC with alkyl ion-pairing reagents is due mainly to the increased hydrophobicity of alkyl–ion–CT complexes and not to ionic suppression[12,13].

In comparison to SSH, TBAP is relatively UV-transparent at low wavelengths. This fact has permitted UV monitoring of peptide (amide) bonds at 210 nm, a wavelength close to the peak molar absorbance of peptide bonds[18]. Based on the height and resolution of UV-absorbance peaks with 100 ng of CT, there should be no problem detecting as little as 20 ng of CT by UV absorbance[18]. Appropriate derivatization of CT moieties could further decrease the amount of peptide needed for optical detection or make possible detection by sensitive fluorimetry[25]. This would make feasible rapid, direct detection of several ng of CT. Derivatization of free amino groups[25] should not affect immunoreactivity, which resides in the carboxyl-terminal region of CT[6,7,19,26]; this will allow immunological identification of trace amounts of peptides purified and quantitated by RP-HPLC of biological samples[18,27,29].

Further studies will be needed to assess paired-ion RP-HPLC for specific isolation and for quantitation of the multiple immunochemical forms of CT in tissue and plasma[1–6,18,27,28]. Based on our present results, such investigation should include the use of a microanalytical, octadecylsilyl-bonded silica column and methanol–water gradient elution employing TBAP, or some other UV-transparent quaternary ammonium cation, as a hydrophobic counter-ion. We are currently working out methods of

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* Resolution values are derived from the equation $R_s = 2(V_r - V_r)/(W_1 + W_2)$ where V_r is the peak retention time and W is the peak width in min.

sample preparation and of peptide derivatization that will enable us to use this RP-HPLC method to study specific CT moieties in biological samples[7–9,25].

ACKNOWLEDGEMENTS

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