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EFFECT OF TRANSITION METALS ON RECOVERY FROM PLASMA OF THE GROWTH-MODULATING TRIPEPTIDE GLYCYLHISTIDYLLYSINE

L. PICKART and M. M. THALER*

Department of Pediatrics and the Liver Center, University of California, San Francisco, Calif. 94143 (U.S.A.)

and

M. MILLARD

Western Regional Laboratories, U.S. Department of Agriculture, Berkeley, Calif. (U.S.A.)

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SUMMARY

Isolation and purification of growth-modulating peptides from biological sources is often accompanied by excessive losses of bioactive material. During the isolation of a growth-modulating tripeptide glycylhistidyllysine (GHL) from human plasma, copper and iron were found to co-isolate with the peptide. Studies with [^{3}H]GHL demonstrated that these metals interfere at several steps of the procedure for the isolation of GHL from plasma (gel filtration chromatography, high-pressure silica-gel). Removal of these metals with an insoluble chelating resin (Cellex 100) enhanced recovery of [^{3}H]GHL from plasma 8-fold. These results suggest that removal of transition metals may aid in the recovery of peptides which are difficult to isolate from biological sources.

INTRODUCTION

Various low-molecular-weight factors extracted from peptone digests, serum, and tissues have been reported to accelerate or inhibit growth in cultured cells and tissues[1–8]. Most such factors are simple peptides, 300 to 2000 daltons in size[1–6], or contain peptide moieties, as in the case of liver or mammary chalone[7,8]. Investigation of these "growth-modulating" molecules has been hampered by extreme losses of active material incurred in the course of isolation.

Glycyl-L-histidyl-L-lysine (GHL) is a tripeptide present in normal human plasma at *ca.* 200 ng/ml[9,10]. When added to cell culture medium in nanomolar concentrations, synthetic GHL enhances the growth rate of hepatoma cells[11], prolongs the survival of non-replicating hepatocytes[11], neurons and glial cells[12], L-929 fibroblasts[13], lymphocytes[14], macrophages[15], eosinophils[16], mast-cells[17], T-strain mycoplasma[18] and

* To whom correspondence should be addressed.

ascaris larvae[19]. The peptide was found to co-isolate from plasma with copper and iron. Similarly, Fernandez-Pol[20] has reported that a peptide of 1600 daltons which stimulates the growth of a line of 3T3 cells (siderophore-like growth factor) co-isolates with iron, while Duntze *et al.*[21] observed that a peptide of 1400 daltons which stimulates sexual conjugation in yeast (alpha-factor), co-isolated as a complex with copper. Taken together, these results suggested that some of the difficulties involved in the isolation of low-molecular-weight, growth-modulating peptides may be due to the presence of transition metals in the isolates. In this paper we describe experiments which demonstrate that copper and iron reduce the overall recovery of GHL from plasma. The presence of such metals has deleterious effects on gel filtration and silica gel chromatographic purification steps. Based on these findings, measures may be taken which may reduce losses due to the presence of transition metals, and may improve the recovery of bioactive peptides from biological extracts.

EXPERIMENTAL

Materials

Sources of material were as follows: Non-soluble chelating resin (Cellex 100), Bio-Rad Labs, Richmond, Calif., U.S.A.; synthetic GHL, Pierce, Milwaukee, Wisc., U.S.A.; Sephadex G-10, Sigma, St. Louis, Mo., U.S.A.; ultrafiltration membranes, Amicon, Lexington, Mass., U.S.A.; metal salts and formic acid, reagent grade, J. T. Baker, Van Water and Rogers, San Francisco, Calif., U.S.A.; thin-layer silica gel (0.1 mm) plates, Brinkmann, Burlingame, Calif., U.S.A.

[^{3}H]GHL was prepared by catalytic tritiation of the synthetic peptide by Amersham-Searle, Chicago, Ill., U.S.A., and purified by high-performance liquid chromatography (HPLC) on silica gel by methods previously described[22]. Final specific activity of [^{3}H]GHL was 1600 mCi/m*M*.

The instrument used was a high-performance liquid chromatograph, ISCO Model 384, Omaha, Nebr., U.S.A. Silica gel columns were packed with 5-μm LiChrosorb, Altex Labs, Berkeley, Calif., U.S.A.

Isolation of GHL from plasma

The procedure followed is outlined in Fig. 1. Fresh human plasma (500 ml) was dialyzed against 3 volumes of double distilled water which was changed every 12 h for 7 days. A 2-g amount of a non-soluble, metal-ion chelating resin (Cellex 100) was then added to the dialysate and the mixture stirred for 1 h. The pH was adjusted to 4.5 with 1 *M* HCl, the solution boiled for 20 min, then cooled to 5°, and the coagulated proteins sedimented by centrifugation at 10,000 *g* for 30 min at 2°. The precipitate was discarded, and the supernatant was adjusted to pH 7.0 then passed through an Amicon ultrafiltration cell (UM-05 filter) at 60 p.s.i. and lyophilized. The lyophilizate was cleared of salt on a Sephadex G-10 column (100 × 2.5 cm) in 1% formic acid. The fraction containing GHL eluted immediately before the salt fraction. This fraction was lyophilized, and the powder extracted with 5 ml of dichloromethane–methanol–formic acid (12:8:1).

Approximately 50 mg of total peptides could be dissolved in solvent. Undissolved material was removed by centrifugation at 2000 *g* for 30 min, then the supernatant was injected in 0.5 ml aliquots on HPLC columns (three 25 × 1 cm columns

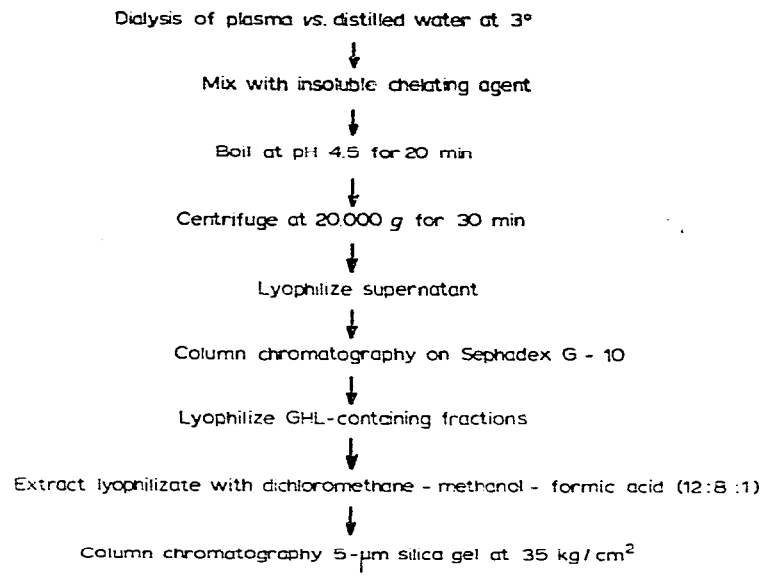

Fig. 1. Steps in the extraction and purification of GHL.

in sequence) and eluted in the extraction solvent with an applied pressure of 35 kg/cm^2.

X-ray photoelectron spectral analysis of fractions

The technique of X-ray photoelectron spectral analysis (XPS)[23,24] was employed for elemental analysis of isolates containing GHL. The procedure, which is non-destructive to the samples, measures the energy of electrons which are ejected by low energy X-rays from elements in the outer 40 Å of a surface layer. Thus it is suitable for analysis of material in the 1 μg range.

For analysis, samples in water were plated on 0.5 cm diameter glass discs and allowed to air-dry. The sample-covered discs were then analyzed in a DuPont Model 650 spectrometer.

Effects of chelating resin, copper and iron on overall recovery of GHL from plasma

The recovery of GHL from plasma at various stages of the isolation procedure was monitored by following tracer amounts of [^{3}H]GHL (500,000 cpm) added to 50-ml batches of human plasma. The effect of omitting the chelating resin at various stages of the isolation procedure on the recovery of [^{3}H]GHL was studied, and final recovery of [^{3}H]GHL after addition of 10^{-4} *M* $CuSO_4$ or 10^{-4} *M* $FeSO_4$ to starting plasma in the presence or absence of chelating resin determined.

Effect of copper on the elution pattern of GHL on Sephadex G-10

The effect of copper on the elution pattern of synthetic GHL on Sephadex G-10 was determined. GHL (1 m*M*) containing 50,000 cpm [^{3}H]GHL was dissolved in 1 ml of phosphate-buffered saline with or without 0.2 m*M* $CuSO_4$, and the solution chromatographed on a 50 × 0.5 cm Sephadex G-10 column. Peptide elution was monitored by measuring absorbance at 210 nm. Sodium chloride concentrations were estimated from the precipitate formed with 1% $AgNO_3$.

Effect of transition metals on silica-gel chromatography of GHL

Thin-layer plates were used in experiments surveying the effects of metal ions on silica gel chromatography of GHL. Equimolar (10^{-3} *M*) amounts of synthetic GHL, and chloride or sulfate salts of Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mo^{2+}, Mn^{2+}, Na^{+}, Ni^{2+}, Zn^{2+}, were prepared in aqueous solution. The mixtures were chromatographed on thin-layer silica gel plates in the solvent used for HPLC (dichloromethane–methanol–formic acid, 12:8:1) or in a solvent at pH 7.0 (methanol–chloroform–0.05 *M* Tris buffer, 2:2:1). After development, the tripeptide was located by ninhydrin–isatin staining[9].

The effects of cupric, ferrous, and ferric ions on yields of GHL from HPLC on silica gel were determined by mixing 5 ml of 10^{-3} *M* synthetic GHL, containing 500,000 cpm as [^{3}H]GHL, with aqueous $CuSO_4$, $FeSO_4$, or $FeCl_3$ at final concentrations of 10^{-5}, 10^{-4}, 10^{-3} or 10^{-2} *M*. The solutions were lyophilized, then extracted into 5 ml of the HPLC solvent. The extract was centrifuged at 2000 *g* for 30 min, and 0.5-ml portions were used for HPLC. Elution peaks were monitored by absorbance at 254 nm, collected, and radioactivity determined by liquid scintillation counting.

RESULTS

Elemental analysis of isolates at various stages of purification

XPS analysis of fractions obtained at each step in the purification procedure revealed that at the early stages (*e.g.* after boiling and ultrafiltration) extracts contained large amounts of nitrogen, oxygen, carbon, sodium, and chloride. After chromatography on the Sephadex G-10 column, the GHL fraction was associated with substantial amounts of copper and iron (Fig. 2). These transition metals were present even in samples previously treated with the insoluble chelating agent.

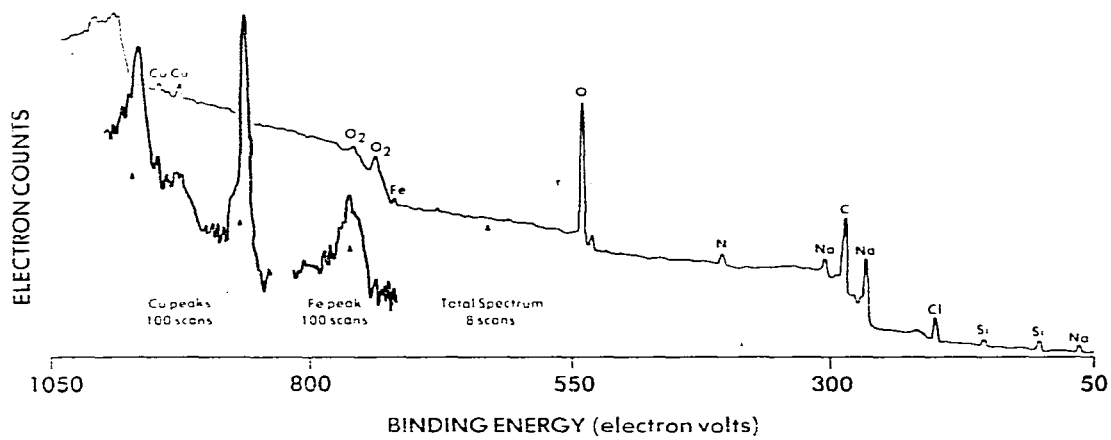

Fig. 2. X-ray photoelectron spectra of GHL-containing fraction at the Sephadex G-10 stage of purification. The line graph of electron counts *versus* binding energies from 50 to 1050 eV represents the elemental composition of 1 μg of isolate, as determined by a computer average of eight scans of the sample. The inserts for the copper and iron peaks represent a computer average of 100 scans of the sample.

Silica gel chromatography removed the metals from the peptide. GHL emerged as the leading peak in this system while the copper and iron eluted primarily in the area associated with peaks D and E (Fig. 3).

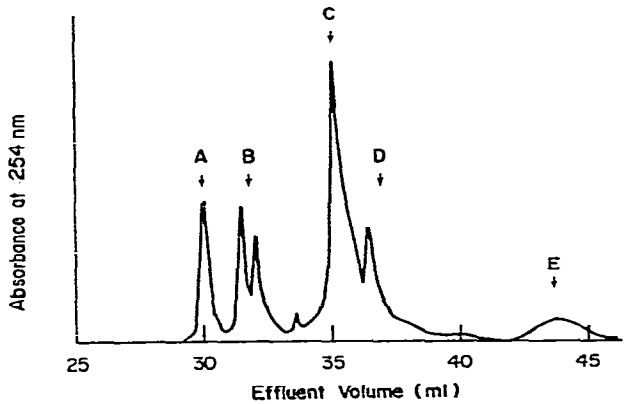

Fig. 3. HPLC on silica gel of GHL-containing isolates from the G-10 column. The center of peak A is 97% pure GHL by amino acid analysis. Copper and iron associate with peaks D and E.

Effect of chelating resin, $CuSO_4$ and $FeSO_4$ on recovery of [^{3}H]GHL from plasma

The overall recovery of [^{3}H]GHL from plasma when using the standard isolation procedure (Fig. 1) was 2.25± 0.15% (Table I). In the absence of chelating resin recovery was only 0.31 ± 0.11%. Omission of resin influenced recovery primarily at the initial fractionation step (extraction of GHL into the supernatant during after boiling of plasma) and at the final step (HPLC on silica gel) (Fig. 4).

TABLE I

EFFECT OF CHELATING RESIN ON RECOVERY OF GHL FROM PLASMA

Methods as detailed in text. Values represent mean ± standard deviation.

Additions to plasma	*Recovery of [^{3}H]GHL (%)*
None	0.32 ± 0.11
Cellex 100 (normal procedure)	2.25 ± 0.15
Cellex 100 + $CuSO_4$ (10^{-4} *M*)	0.37 ± 0.10
Cellex 100 + $FeSO_4$ (10^{-4} *M*)	0.18 ± 0.06
$CuSO_4$ (10^{-4} *M*)	0.28 ± 0.05
$FeSO_4$ (10^{-4} *M*)	0.23 ± 0.09

$CuSO_4$ or $FeSO_4$ blocked the effect of chelating resin on recovery of GHL (Table I). However, addition of these metals to plasma in the absence of the chelating resin did not appreciably reduce the extractability of GHL, suggesting that in normal plasma GHL may be already saturated with these metals.

Effect of copper on elution of GHL from Sephadex G-10

$CuSO_4$ also altered the elution pattern of [^{3}H]GHL on Sephadex G-10

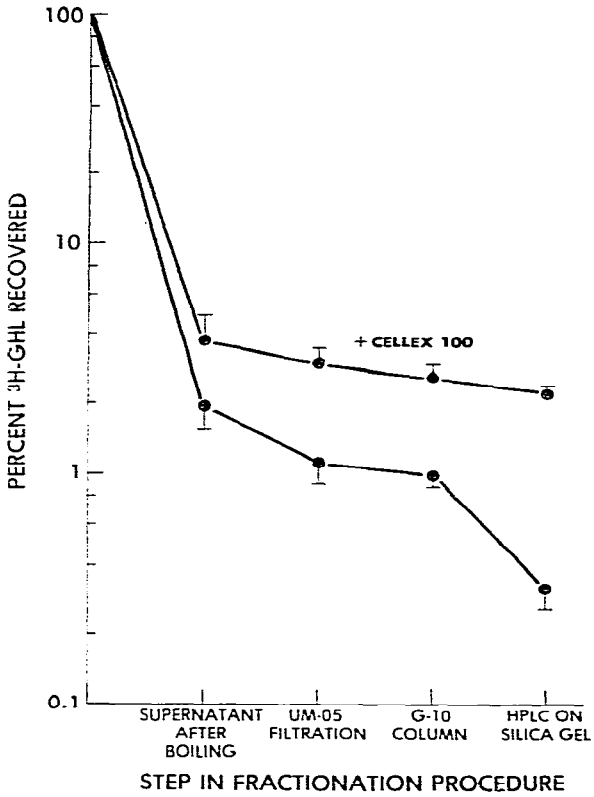

Fig. 4. Effect of chelating resin on recovery of [^{3}H]GHL from plasma at various stages of the isolation procedure. The addition of the chelating resin (Cellex 100) improved recovery of [^{3}H]GHL at most stages of the isolation procedure. Values are mean ± standard deviation. Details of procedure in text.

columns. In the absence of added copper, the bulk of tripeptide eluted immediately before the sodium chloride peak (Fig. 5). In the presence of copper, a substantial portion of the label emerged with the elution peak (>700 daltons). This pattern suggests that in the presence of copper, a portion of GHL elutes as either a bichelate of 2 GHL and 1 copper molecule or a higher-molecular-weight complex.

Effect of transition metals on silica gel chromatography of GHL

Chromatography of equimolar GHL and metal ions on thin-layer silica gel plates demonstrated that transition metals alter the R_F of GHL. The R_F value of GHL when chromatographed in dichloromethane–methanol–formic acid (12:8:1) was 0.21, but in the presence of equimolar Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mo^{2+}, Mn^{2+}, Ni^{2+} or Zn^{2+} the R_F values were, respectively, 0.05, 0.03, 0.05, 0.05, 0.03, 0.09, 0.02, 0.05. The R_F value of GHL when chromatographed in the other solvent at pH 7.0 (methanol–chloroform–0.05 *M* Tris buffer, 2:2:1), was 0.28, but in the presence of equimolar Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mo^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}, the R_F values were, respectively, 0.09, 0.01, 0.06, 0.07, 0.09, 0.14, 0.06, 0.06. There was no

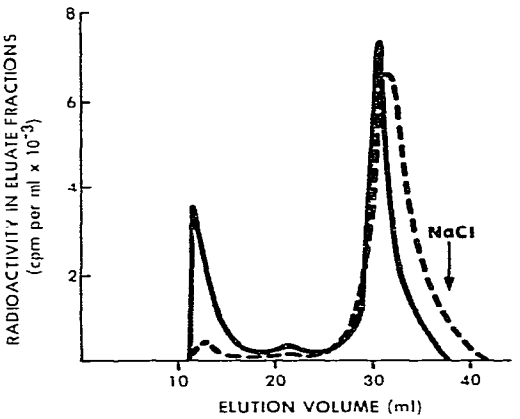

Fig. 5. Effect of copper sulfate on elution pattern of GHL from a Sephadex G-10 chromatography column (50 × 0.5 cm). The dashed line represents GHL alone; the continuous line, GHL plus $CuSO_4$. The presence of cupric ion increases the amount of [^{3}H]GHL recovered in the exclusion peak.

evidence of interaction between GHL and Ca^{2+}, K^+, Mg^{2+}, or Na^+ in either solvent.

In the HPLC system, the presence of Cu^{2+}, Fe^{2+}, or Fe^{3+} markedly reduced the recovery of the tripeptide in peak A (Fig. 3). Increasing the amount of any one of the three metals resulted in correspondingly decreased yields of the tripeptides. Equimolar amounts of any of the three metals and GHL reduced recovery by about 95% (Table II).

TABLE II

EFFECT OF COPPER OR IRON ON RECOVERY OF [^{3}H]GHL FROM PEAK A USING HPLC ON SILICA GEL

[^{3}H]GHL was chromatographed on silica gel HPLC columns with added metal ions. Recovery of radioactivity refers to cpm in peak A of Fig. 3. Further details are in the Experimental section.

Metal added to [^{3}H]GHL	*Recovery (cpm)*
Control ([^{3}H]GHL, 10^{-3} *M*)	46,500 ± 1100
10^{-5} *M* Cu^{2+}	42 700 ± 2000
10^{-4} *M* Cu^{2+}	34,900 ± 1500
10^{-3} *M* Cu^{2+}	4000 ± 300
10^{-2} *M* Cu^{2+}	2600 ± 700
10^{-5} *M* Fe^{2+}	38,400 ± 1400
10^{-4} *M* Fe^{2+}	27,200 ± 600
10^{-3} *M* Fe^{2+}	3100 ± 900
10^{-2} *M* Fe^{2+}	400 ± 100
10^{-5} *M* Fe^{3+}	33,500 ± 2300
10^{-4} *M* Fe^{3+}	28,200 ± 1200
10^{-3} *M* Fe^{3+}	3000 ± 500
10^{-2} *M* Fe^{3+}	500 ± 200

DISCUSSION

These results indicate that copper and iron co-isolate from plasma with GHL and interfere with numerous stages of the isolation procedure. The findings reported here, and reports of other small growth-modulating peptides such as siderophore-like growth factor[20] and alpha-factor[21], which co-isolate with transition metal ions, suggest that metal–peptide interactions may play a role in the isolation of other small-molecular-weight growth-modulating factors. Despite extensive efforts at isolation of such growth-modulators, few factors have been purified and characterized structurally. Growth-stimulatory and inhibitory activities which alter replication rates of cultured cells have been attributed to small peptides of 300 to 2000 daltons obtained from a variety of peptone digests, and ultrafiltrates of serum and tissues[16]. Peptides such as the liver chalone (450 daltons)[7] or the mammary chalone (2000)[8] specifically inhibit proliferative growth of their target organs *in vivo*. The isolation and structural determination of such factors is of special interest since their small size should readily permit synthesis of these factors and of bioactive analogs. However, the high losses associated with the purification of many of these factors have inhibited progress toward these goals. For example, 99.95% of bioactive material is lost during isolation of somatomedin-C[22]. Similarly, 1 mg of thyrotropin releasing factor, a tripeptide, has been extracted from 270,000 sheep pituitaries[25].

Our work suggests that transition metals in isolates may cause various complications which may contribute to losses of bioactivity during isolation. Transition metal ions may form chelates with growth-modulating peptides, which may cause precipitation of the peptide or its adsorption to other contaminating factors. Such metals may also catalyze air oxidation of peptides. Rapid and irreversible oxidation of iron(II) to iron(III) by histidine, and of cobalt(II) to cobalt(III) by carnosine (β-alanylhistidine) with formation of amino acid and peptide decomposition products, has been previously reported[26,27]. Finally, bioactivity associated with a peptide may be due to a complex of the peptide and a transition metal, and neither factor alone may possess the bioactivity of the complex. Growth-promoting effects of transition metal complexes with peptides or proteins on cultured cells have been reported for the iron complex of siderophore-like growth factor on SV-40 3T3 cells[20], for iron-transferrin on V79 Chinese hampster cells[28] and mouse 3T6 cells[29] and zinc-transferrin on human lymphocytes[30]. Also, GHL functions synergistically with copper and iron to stimulate the growth of hepatoma cells, an effect which may be mediated through GHL–metal ion complexes[31]. Thus, purification of bioactive metal–peptide complexes may reach a stage at which the metal–peptide complex is separated with a subsequent loss of bioactivity.

These considerations suggest three procedures which may potentially improve the recovery of growth-modulating peptides during isolation from biological sources. First, isolates should be assayed for the presence of transition metals. XPS is especially suitable for this purpose since the method is non-destructive, and requires only a few micrograms of isolated material. Second, the concentration of transition metals in the isolates may be reduced with non-soluble chelating resins or ion exchange chromatography. However, many peptides, especially those containing histidine, bind tightly to ion-exchange resins and often require extreme pH or salt applications for removal. Finally, peptide-containing solutions should be cleared of oxygen since

transition metals can catalyze oxidative processes destructive to amino residues in peptides.

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