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PRODUCTION OF UNIFORMLY STABLE ISOTOPE LABELLED PROTEINS FROM MAMMALIAN CELLS†

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SUMMARY

In order to implement a new strategy for determining the kinetics and metabolism of therapeutic proteins, we had to produce milligram quantities of a uniformly stable isotope labelled species. To produce the labelled protein, the rat pituitary tumor cell line GH_3 was cultured in Celtone-M, a commercially prepared medium that contains all the necessary ^{13}C,^{15}N- labelled amino acids as well as a uniformly ^{13}C-labelled carbohydrate source to produce uniformly ^{13}C,^{15}N-labelled rat growth hormone. The growth hormone secreted into the culture medium was purified using ultrafiltration followed by reversed-phase chromatography. The yield of labelled protein was approximately 10 mg/L. SDS-PAGE and western blot analyses confirmed that the labelled growth hormone migrated similarly to the unlabelled material, and that both labelled and

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unlabelled growth hormone were immunoreactive. Electrospray ionization mass spectrometry (ESI) of the purified material revealed that the molecular weight of the labelled material was 995 units higher (22804 Da) than its unlabelled counterpart (21809 Da). This indicated labelling at 81% of all possible carbon and nitrogen sites throughout the protein. An alternative strategy using HPLC/CRIMS indicated that the protein was 89% ^{13}C-labelled. It is possible to quantitate this stable-isotope labelled protein following iv injection in a rat, using HPLC/CRIMS. These results show that once cells have been adapted to growth in Celtone-M, the preparation of highly and uniformly labelled proteins from mammalian cells appears no more difficult than production of their unlabelled counterparts. This will make a much wider range of proteins available for NMR and other applications that employ stable-isotope labelled proteins.

INTRODUCTION

In this laboratory, we are investigating a new paradigm for studying the pharmacokinetics and metabolism of therapeutic macromolecules. Our method involves the production of a uniformly stable-isotope labelled protein and the subsequent selective detection of the stable-isotope enrichment of the parent molecule and any of its metabolites using a newly-developed technique, high performance liquid chromatography combined with chemical reaction interface mass spectrometry (HPLC/CRIMS) [1]. The present paper describes use of a stable-isotope enriched cell culture medium to generate uniformly labelled rat growth hormone from a mammalian cell line. We are using this ^{13}C, ^{15}N-labelled growth hormone to conduct *in vivo* studies of pharmacokinetics and metabolism in rats.

The strategy of using uniformly labelled protein with HPLC/CRIMS has several advantages over the currently employed methods of radioiodine labeling, bioassay or immunoassay. With a uniformly incorporated label, not only the parent compound, but also the metabolites are detectable. Cleaving the protein into smaller fragments cannot result in the generation of unlabelled, and therefore undetectable, protein fragments, as can occur if a label is incorporated at only a few sites within the macromolecule. Furthermore, using carbon labelling

that is incorporated into the amino acids of the protein should be superior to radioiodine labelling, in that radioiodine can be enzymatically cleaved from its site on tyrosine thereby losing the "tag". The rate of deiodination can then be interpreted as the rate of clearance of the protein, leading to incorrect conclusions about the pharmacokinetics of the macromolecule. With uniformly incorporated stable-isotope labels, no alteration in a macromolecule renders it undetectable. Like radioactivity, the detection in HPLC/CRIMS is structure independent, so no assumptions about the potential metabolites need to be made prior to detection. The stable-isotope label allows endogenous protein to be distinguished from exogenously administered material which neither immunoassays nor bioassays can accomplish. Stable isotopes do not present the same health risks or disposal problems as radioactive isotopes. Because the United States Food and Drug Administration is continuing to move the criteria by which it evaluates bioengineered materials to parallel those used for other drugs, the need for more effective techniques for studying the disposition of these compounds is increasing. We believe that the stable-isotope labelled macromolecule-HPLC/CRIMS approach can meet this need.

To evaluate the utility of HPLC/CRIMS to study pharmacokinetics and metabolism of macromolecules it was necessary to obtain a supply of a uniformly stable-isotope labelled protein. Although it has been possible to generate stable isotope labelled proteins using bacterial hosts for several years, many of the proteins of pharmaceutical interest must be synthesized in mammalian cells, where the generation of uniformly labelled proteins has been much more difficult. Hansen *et al.* produced uniformly ^{13}C, ^{15}N-labelled urokinase in Sp 2/0 cells by supplementing an amino acid free cell culture medium with labelled amino acids prepared from bacterial and algal hydrosylates [2]. This procedure was successful; however, it was labor intensive. Archer et al. were successful in producing a partially ^{13}C,^{15}N-labelled TGF-β1 protein in Chinese hamster ovary cells [3]. This was accomplished by supplementing medium with ^{15}N-choline and unspecified ^{13}C-labelled amino acids. Lustbader et al. reported using a serum free medium (CHO-S-SFM-I) supplemented with ^{13}C-^{15}N labelled amino acids, ^{13}C glucose and ^{13}C sodium pyruvate to culture bioengineered CHO cells and produce uniformly labelled human

chorionic gonadotropin (hCG) [4]. This medium is commercially available as Celtone M from Martek Biosciences (Columbia, MD) and makes preparation of the labelled protein less time consuming. Although this medium was designed to support CHO cells, in this study we have used it to produce highly ^{13}C and ^{15}N-labelled rat growth hormone from a rat pituitary tumor cell line.

MATERIALS AND METHODS

Cell Culture and Protein Isolation

Cell Culture - The cell line GH_3, used in these studies, was derived from a rat anterior pituitary tumor [5]. These cells secrete growth hormone and prolactin [6]. The synthesis and secretion of growth hormone can be stimulated and the prolactin secretion depressed by treatment of the cells with dexamethasone and triiodothyronine [7]. GH_3 cells were obtained from American Type Culture Collection (ATCC) and were initially grown in DMEM with 15% horse serum (Sigma, St. Louis, MO) in the presence of penicillin and streptomycin (Penstrep, Sigma Chemical Co.).

The uniformly labelled protein was prepared by growing cells using a proprietary cell culture medium (Celtone-M). The medium we chose for our experiments was serum-free CHO-SSFM-1 which contained highly labelled amino acids as well as a labelled carbohydrate source. Celtone-M is available labelled with any combination of ^{13}C, ^{15}N, and ^{2}H. The medium in our studies was double labelled with ^{13}C and ^{15}N. Cells were adapted to growth first in unlabelled Celtone-M. Previous reports in the literature indicated that GH_3 cells grew poorly and did not secrete growth hormone in serum-free medium [6]. In order to test whether GH_3 cells would divide in serum-free Celtone-M, growth curves comparing the doubling rates of these cells grown in DMEM plus 15% horse serum versus Celtone M were obtained. Cells ($1x10^5$) were plated in 60 mm culture dishes. Every two days, three dishes were removed and the cells were scraped off and counted.

Isolation of stable-isotope labelled growth hormone - Once cultures were sufficiently established in the unlabelled Celtone-M, the production of stable-isotope labelled growth hormone was

initiated. Cells were seeded into 150 cm^2 flasks at 1.4 x 10^6 cells in 15 mL of serum-free ^{13}C,^{15}N-labelled Celtone-M. All medium contained 1% Penstrep, 1 μM dexamethasone and 1 nM triiodothyronine. While the cells generally adhered to the flasks in DMEM, they were generally nonadherent, but viable (as determined by trypan blue exclusion) in Celtone-M. Every 3-4 days cells and medium were aseptically removed from each flask and centrifuged. The medium was decanted and stored frozen. The cells were replated back into the flasks. Care was taken to avoid introducing sources of unlabelled carbon and nitrogen into the cultures. For example, cells were scraped off of flasks rather than using trypsin, which would contain both unlabelled carbon and nitrogen.

Prior to HPLC purification of the labelled growth hormone, the medium underwent partial fractionation and concentration using ultrafiltration. Ten mL aliquots of the medium were ultrafiltered first through a 100 kDa molecular weight cut-off filter, and the filtrate was centrifuged through a 10 kDa molecular weight cut off filter (both Centriplus, Amicon, Beverly, MA). The retentate (about 2 mL) from the 10 kDa molecular weight cut-off filter (the fraction >10 and <100 kD) was further purified using reversed-phase HPLC. A Waters 600E HPLC and 490E UV detector were used for the purification. The method was initially worked out using a Waters Delta-Pak 8 x 100 mm C18 column. The mobile phases were HPLC grade water (solvent A) and acetonitrile (solvent B) (both EM Science, Gibbstown, NJ), each containing 0.1% trifluroacetic acid (TFA; J.T. Baker, Phillipsburg, NJ). Three mL aliquots of the concentrated cell culture medium were loaded onto a Waters Delta Pak C18 radial compression cartridge (8 x 100 mm, 300 Å pore size), and eluted with a linear gradient of 30-70% B over 40 minutes [8] using a 1 mL/min flow rate. In order to purify larger amounts of growth hormone, this method was adapted to preparative chromatography using a 25 x 100 mm Waters Delta-Pak C18 radial compression cartridge. The larger column allowed the processing of 30 mL aliquots of the concentrated cell culture medium. The medium was pumped onto the column at 9 mL/min using a Perkin-Elmer HPLC pump. Once all of the medium was loaded, the column was reconnected to the Waters 600E HPLC. For preparative chromatography a flow rate of 10

mL/min was used. The gradient was held at the initial conditions until the nonretained material had passed through the column. Once the UV detector (because of the higher flow rate a Linear Systems model 200 was used for the preparative chromatography) was no longer detecting unretained material, the same 30-70% gradient was started.

A standard of pituitary-derived rat growth hormone (National Hormone and Pituitary Program, NIDDK, NICHHD, USDA), and growth hormone spiked into cell culture medium were both shown to elute at 37 minutes using this gradient. The ultrafiltered, concentrated cell culture medium from the GH_3 cells showed a peak at 37 minutes. The peak eluting between 36 and 38 minutes on both the 8 mm and the 25 mm diameter columns was collected, dried in a Speed-Vac (Savant Instruments, Hicksville, NY), and stored at -20°C until used.

Rate of Incorporation of Label - Cells initially grown in DMEM were cultured with stable-isotope labelled Celtone M. Every two days the medium was removed and growth hormone was isolated using the ultrafiltration and preparative HPLC method described above. The extent of labeling was determined using HPLC/CRIMS. A Brownlee C18 4.6 x 30 mm (300Å) column was used, and the mobile phases were the same as those described above. The growth hormone eluted at 59% B using a gradient of 30-70% B in 40 minutes.

Characterization of growth hormone

SDS-PAGE and Western Blot Analysis - Samples of labelled and unlabelled cell-derived growth hormone, and rat growth hormone standard all reconstituted in 0.15M NaCl, 0.03M $NaHCO_3$, pH 10.8, were loaded onto a 15% polyacrylamide gel for SDS-PAGE electrophoresis according to the method of Laemmli [9]. The gel was stained with coomassie blue for protein detection. A second gel, run for western blot analysis, was transferred to a nitrocellulose membrane (25 volts for 30 minutes). The membrane was blocked in 0.1% bovine serum albumin in Tris base, sodium chloride and Tween-20 (TBS-T) for one hour prior to the addition of the primary antibody (a 1:500 dilution of monkey anti-rat growth hormone serum in TBS-T; National Hormone and Pituitary Program). After incubating with the primary antibody for one hour and

washing for one hour in TBS-T, the membrane was incubated with goat anti-monkey IgG conjugated to horseradish peroxidase (1:4000 dilution in TBS-T, Sigma Chemical Co.), and washed for one hour. The membrane was then treated with the chemiluminese reagents (Dupont, NEN Research Products, Boston, MA). To reduce background, a 1:1 mixture of the reagents was diluted 1:5 after mixing, and the filter was exposed to film for 15 seconds [10].

Analytical high performance liquid chromatography - To evaluate the purity of the growth hormone from the preparative chromatography, samples of pituitary-derived and cell-derived (both labelled and unlabelled) growth hormone were subjected to analytical HPLC using a Brownlee (Applied Biosystems) C4 4.6 x 30 mm (300Å) column and a linear 15-80% acetonitrile gradient over 20 minutes. The growth hormone was detected using a uv detector set at 280 nm. Solvents were prepared as described in the previous section.

Since the protein was being produced for use in a pharmacokinetic study, it was necessary to be able to detect the stable isotope labelled growth hormone in plasma. Blood samples were collected, following iv injection of rat growth hormone to a rat. The collected plasma samples were chromatographed on a Poros R1 column (2.1 x 30 mm; PerSeptive Biosystems, Framingham, MA) , using a linear 15-80% ACN gradient over 10 minutes.

Electrospray mass spectrometry - In order to obtain a more precise determination of molecular weight, and to evaluate isotopic incorporation, samples of the pituitary-derived rat growth hormone standard, and both labelled and unlabelled versions of rat growth hormone isolated from the GH_3 cells were analyzed by electrospray ionization (ESI) using a Perkin Elmer Sciex triple quadrupole mass spectrometer.

HPLC/CRIMS

High performance liquid chromatography combined with chemical reaction interface mass spectrometry (HPLC/CRIMS; [1]) was also used to determine the extent of ^{13}C incorporation into the growth hormone. In HPLC/CRIMS, an analyte is separated from its matrix (e.g. plasma, culture medium, etc) using reversed-phase HPLC. The analytes are first desolvated

and then dry analytes flow into a microwave reaction chamber where they dissociate to their component atoms. These atoms are then oxidized by a reactant gas so that all carbons in the analyte molecule were monitored as CO_2. When any carbon is stable-isotope labelled, the molecular weight of the oxidation product is increased by one unit. Thus, $^{12}C^{16}O_2$ has a molecular weight of 44, while the stable-isotope labelled version ($^{13}C^{16}O_2$) weighs 45. The amounts of material detected at m/z 44 and m/z 45 were quantified using an Extrel C50/400 quadrupole mass spectrometer with a Technivent (Maryland Heights, MO) Vector 2 data acquisition system. For these experiments the reactant gas was SO_2. The ratio of the total amount of CO_2 produced (the peaks at m/z 44 plus 45) compared to the area under the peak at 45 ($^{13}CO_2$), allows us to calculate the extent of ^{13}C labeling. Although the protein was both ^{13}C and ^{15}N labelled, the background amount of nitrogen is much higher than the carbon background (due to solvents and air), the carbon label was preferentially studied in these experiments.

RESULTS

The GH_3 cells grew in both the DMEM and in the Celtone-M medium. The doubling time in DMEM appeared to be about 2 days, and was initially about 4 days for the cells in Celtone-M. As the cells adapted to the unlabelled Celtone-M their doubling time decreased, although they never grew as rapidly as cells in serum supplemented DMEM (data not shown). The cells grown in Celtone-M were viable, and were capable of cell division and secretion of growth hormone. The growth hormone was purified as described in the Methods. Figure 1 shows a chromatogram of the concentrated cell culture medium using the 8 x 100 mm Waters C18 Deltapak column.

The overall yield of growth hormone was about 10 mg/liter of labelled Celtone-M. We also found that the medium continued to support cell growth if the rententate for the 100 kDa MWCO filter and the filtrate from the 10 kDa cut-off filter (i.e. the non-growth hormone containing fractions) were combined and resterilized. Cells continued to divide and to secrete growth hormone in this "recycled" medium.

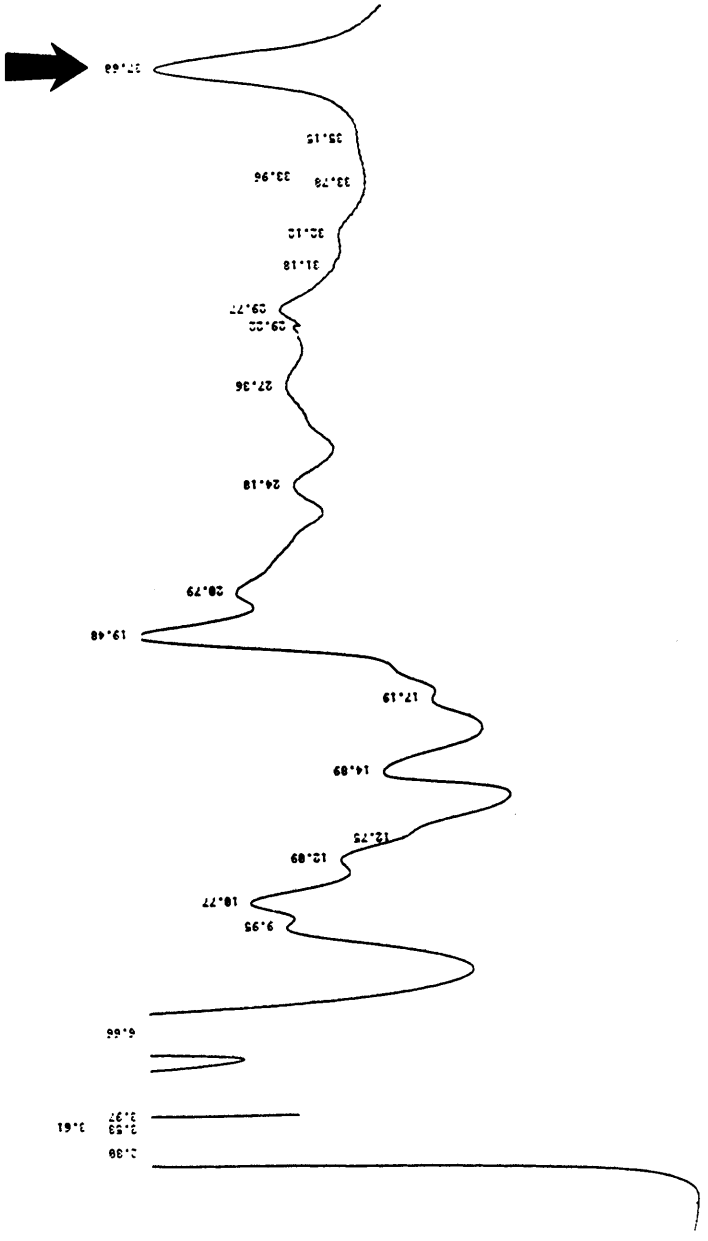

Figure 1. Reversed phase chromatography of the concentrated cell culture medium using a Waters Delta Pak C18 radial compression cartridge (8 x 100 mm), 30-70% ACN gradient and absorbance at 280 nm. Growth hormone elutes at 37 minutes, marked by the arrow.

The growth hormone isolated after 2 days growth in Celtone-M was 10% labelled with ^{13}C. The amount of ^{13}C increased in samples up to about day 14 when HPLC/CRIMS analysis (Figure 2) indicated that the growth hormone was 89% ^{13}C labelled.

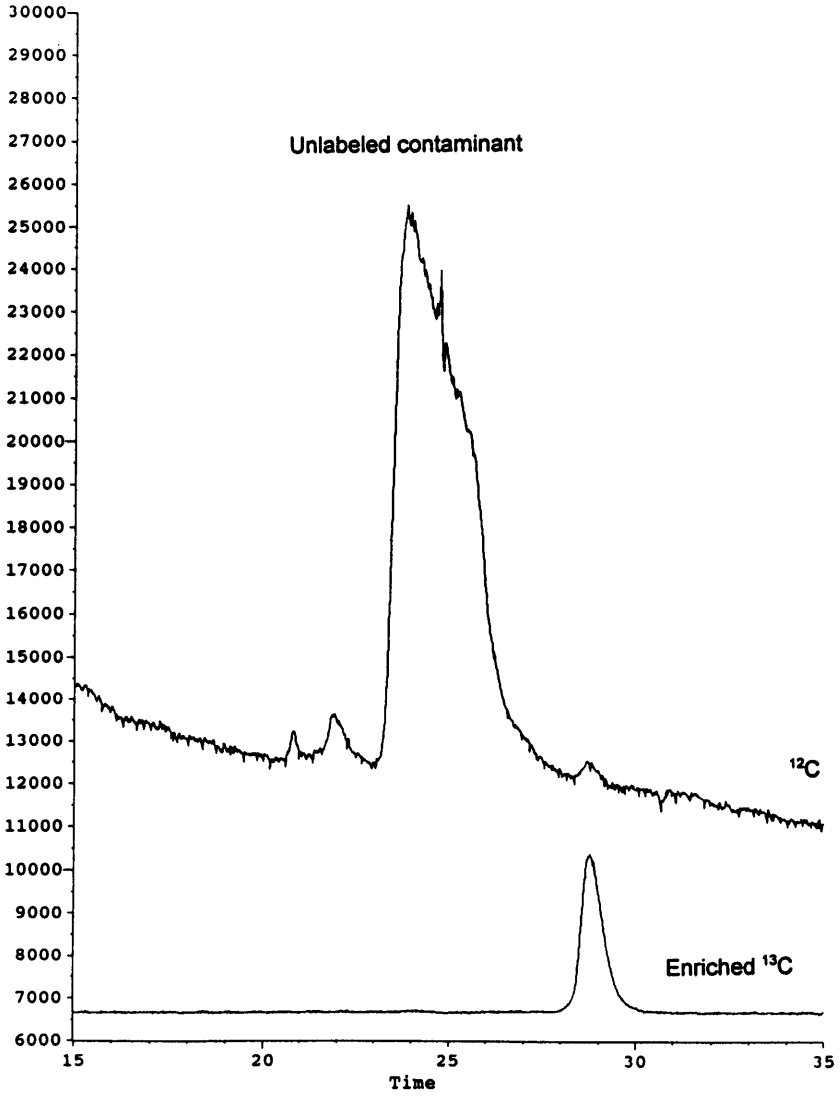

Figure 2. HPLC/CRIMS analysis of labelled growth hormone using a Brownlee C18 (4.6 x 30 mm) column. The upper trace represents ^{12}C and the lower trace ^{13}C. As can be seen, the majority of the carbon from the dissociation of the protein is ^{13}C rather than ^{12}C, indicating a high degree of ^{13}C labelling in the protein. The large peak on the upper trace is an unlabelled carbon-containing, but not UV-absorbing, contaminant.

Several methods were used to determine that the desired protein had been produced. The 15% SDS-polyacrylamide gel shows that the pituitary growth hormone standard and both the unlabelled and labelled cell-derived growth hormone all produce bands of the same apparent molecular weight (Figure 3), and that this weight is consistent with the size of growth hormone determined from its amino acid sequence [11]. There have been reports in the literature of growth hormone molecules forming disulfide dimers that migrate at 36 kD [12]. The predominant band on the gel does run at the appropriate molecular weight, however, if the reduction of the protein was insufficient, the larger molecular weight bands may represent disulfide dimers of growth hormone. The western blot analysis shows that the pituitary-derived standard and both of the cell-derived versions of growth hormone are immunoreactive (Figure 4).

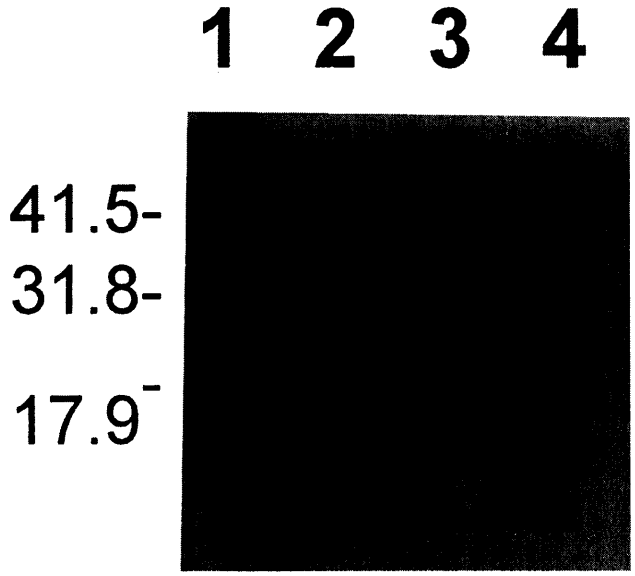

Figure 3. SDS-PAGE of rat growth hormone samples run on a 15% gel. Lane 2 - pituitary-derived growth hormone; Lane 3 - unlabelled cell-derived growth hormone; Lane 4 - labelled cell-derived growth hormone. All three samples form bands at the same apparent molecular weight, and that weight is consistent with the weight of unlabelled growth hormone (M.W. 21809) when compared to the molecular weight markers in Lane 1.

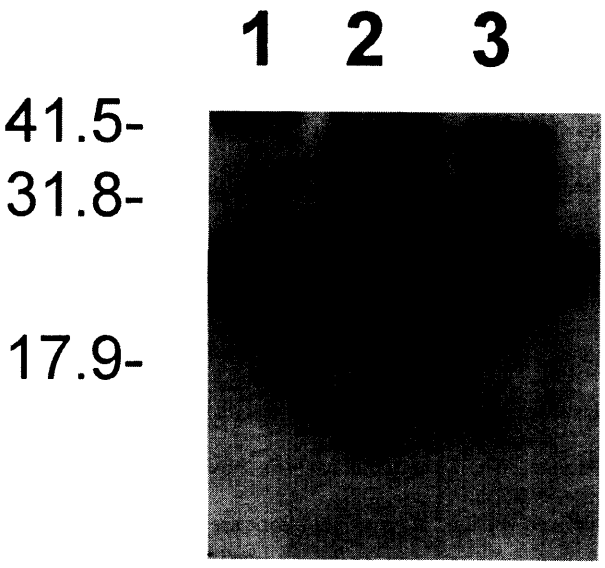

Figure 4. Western blot analysis of the same three samples as in figure 3. All three samples immunoreact with the monkey anti-rat serum. Lane 1 - pituitary-derived growth hormone; Lane 2 - unlabelled cell-derived growth hormone; Lane 3 - labelled cell-derived growth hormone.

The pituitary-derived (Figure 5 upper chromatogram) and cell-derived labelled (Figure 5 lower chromatogram) growth hormone had coincident retention times in the analytical HPLC system. The UV trace shows that there were few other peaks present in the chromatogram from the cell-derived labelled protein indicating that the preparative HPLC system was providing growth hormone that was relatively free of contaminating proteins. The HPLC/CRIMS trace in Figure 2 does show that an unlabelled contaminant is present. This contaminant peak represents a considerably larger amount of the carbon in this sample than the growth hormone does. Since the contaminant is not labelled, it is probably not something generated by the cells in culture. The impurity does not absorb at 280 nm, and therefore does not contain aromatic rings, making it unlikely to be a contaminating protein. The SDS-PAGE gel did not show evidence of an additional protein present in larger amounts than the growth hormone. This impurity is only detectable using the general carbon-detecting abilities of CRIMS. It may be something from the centrifuge filters, the column during preparative chromatography or from the centrifuge tubes used to store the growth hormone.

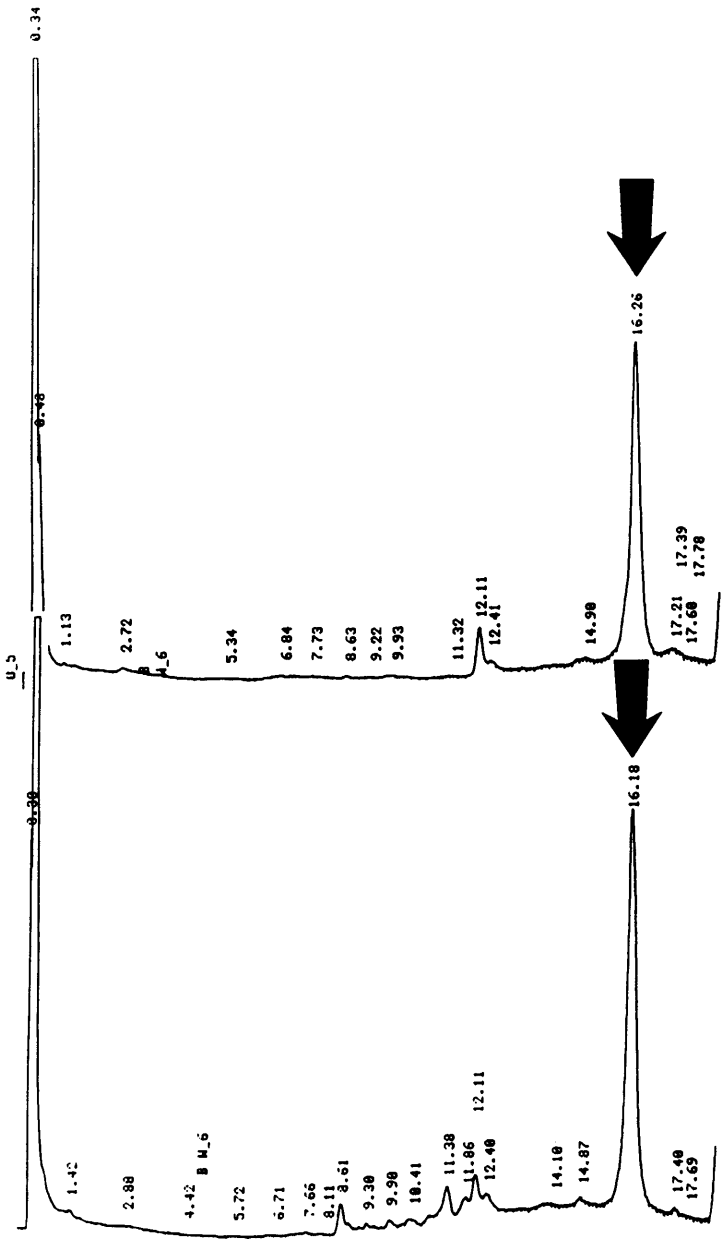

Figure 5. Analytical chromatography of the fraction collected from the preparative procedure. A Brownlee C4 4.6 x 30 mm column and a 15-80% gradient over 20 minutes was used. **Upper Chromatogram**. Pituitary-derived growth hormone standard. **Lower Chromatogram**. Cell-derived labelled growth hormone.

The ESI analysis revealed that the pituitary-derived rat growth hormone standard and the unlabelled growth hormone derived from the GH_3 cells both had a molecular weight of 21809. The sequence for rat growth hormone, as obtained from the Protein Identification Resource (release 47.00) [13], and calculated using the General Protein Mass Analysis program (Lighthouse Data, Denmark) indicates that the correct weight for isotopically normal growth hormone is 21806.9, a difference of less than 0.01%. The labelled material from the GH_3 cells had a molecular weight of 22804, an increase of 995 units. There are 975 carbons and 262 nitrogens in growth hormone for a total of 1237 potential sites of labeling. In unenriched growth hormone it is expected that 11 of the carbons (1.12% natural abundance) and 1 of the nitrogens (0.4% natural abundance) will be ^{13}C or ^{15}N, respectively. Therefore, a completely labelled growth hormone species could actually only increase by 1225 mass units. The observed increase of 995 in molecular weight indicates that 81.2% of the possible carbon and nitrogen sites were labelled.

We also investigated the labeling of the amino acids in the medium. We established HPLC/CRIMS conditions for three amino acids (phenylalanine, tyrosine and leucine) that were present in relatively high amounts in the culture medium and could be retained by a column. A SynChropak, 4.6 x 250 mm, RPP-100, C18 column (Micra Scientific, Northbrook, IL), and reversed-phase HPLC were used. Solvent A was water with 0.1% TFA, and solvent B was isopropanol with 0.05% TFA. A 0-35% gradient was run at 1% per minute. Once the elution times of these amino acids were determined, HPLC/CRIMS studies of the Celtone-M were done under these same conditions. Phenylalanine eluted at 15.6 minutes, tyrosine at 13.4 minutes and leucine at 13.9 minutes. Peaks with retention times matching the amino acid standards were integrated both on the ^{12}C and ^{13}C channels so that the extent of labeling could be calculated as described previously. ^{13}C-labeling of these amino acids was between 89 and 95%. These results are in reasonable agreement with the calculated 89% ^{13}C labeling for the rat growth hormone.

Detection of this labelled growth hormone in a rat plasma sample following iv injection of rat growth hormone is shown in Figure 6. The upper chromatogram represents ^{12}C, which is

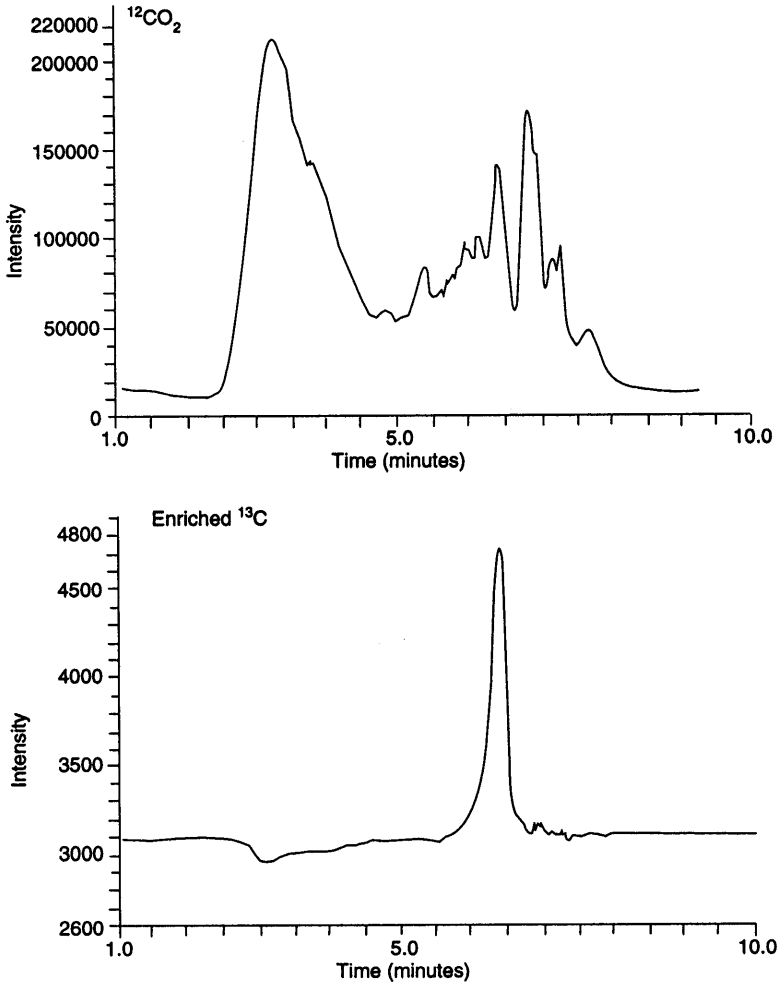

Figure 6. Detecting ^{13}C-labelled rat growth hormone in rat plasma. An anesthetized rat was dosed iv with 4 mg of ^{13}C rat growth hormone. A 200 µL blood sample was taken via an intraarterial catheter at 1.5 minutes after dosing was completed. The plasma was immediately separated by centrifugation. A 20 µL aliquot of plasma was chromatographed using the Poros R1 column (2.1 x 30 mm) as described in the methods. The upper chromatogram represents ^{12}C and the lower chromatogram represents enriched ^{13}C. In an isotopically normal sample the result is a flat line, however any component enriched in ^{13}C gives a peak on this channel. In CRIMS, the peak areas are proportional to the amount of enriched ^{13}C in the plasma sample. The retention time of the one peak observed is identical to that of authentic rat growth hormone.

the predominant isotope for carbon in the plasma sample. The lower chromatogram shows ^{13}C enrichment (m/z 45 minus the naturally occurring 1.12% ^{13}C). The labelled growth hormone is detected on this trace, without interferences from the plasma. This peak represents 66 picomoles of growth hormone.

DISCUSSION

Celtone-M is capable of supporting growth of GH_3 cells. Despite published reports that these cells grow poorly and do not secrete growth hormone in serum-free medium [6], in Celtone-M the cells were capable of growth and secretion of hormone in quantities that permit isolation of the labelled protein.

Growth of mammalian cells in Celtone-M provides a practical method for the production of stable-isotope labelled proteins that is less labor intensive than the method proposed by Hansen *et al.*, [2], and can be used to prepare larger molecules than synthetic methods allow. The availability of a commercially prepared highly labelled medium simplifies the process of preparing uniformly labelled material for use in NMR and mass spectrometry studies. Once the cells have been adapted to grow and secrete in this medium, production and isolation of the labelled protein should be no more difficult than the production of its unlabelled counterpart.

To obtain the highest degree of enrichment in any protein, one must use materials of the highest possible enrichment, and culture the cells through enough cycles that the unlabelled materials initially present in the cells are totally replaced by labelled versions. To accomplish this latter point, our cells were continuously cultured in the labelled medium for 4 months, which means that there were more than 30 changes of medium. Our data showed a plateau in ^{13}C enrichment after just 2 weeks of growth in labelled medium.

By electrospray MS of the intact protein in unlabelled and labelled forms, we found that 81% of the available C and N positions in rat growth hormone were labelled. This was computed from the difference in masses of the two species, each of which was determined to better than 0.01%. Using HPLC/CRIMS we found that 89% of the carbons were labelled. This

measurement is usually precise to better than ± 1% relative standard deviation. For the unusual situation of this high a level of enrichment, the errors might have been larger because of what is always a higher background of ^{12}C than ^{13}C. We further found that the enrichment of three amino acids from the Martek medium were 89-95% ^{13}C-labelled. Clearly, the final product cannot have a higher enrichment than the starting materials. Thus all our measurements are fairly consistent with each other.

Lustbader *et al.* [4] prepared hCG with a similar strategy. They did not examine the isotopic content of the intact protein, but rather three tryptic fragments from the α subunit of hCG. Their results showed that the extent of label incorporation ranged from a low of 75% to a high of 98.9% with precisions ranging from ± 8% to ± 2% respectively. Their analysis of two amino acids that were liberated from the protein by acid hydrolysis showed 95-96% ± 10% labeling. While we are somewhat puzzled by the rather large errors reported for each of these measurements, their ranges include the enrichments we measured.

This technique greatly simplifies the process of obtaining uniformly labelled proteins from eukaryotic cells, both for the more prevalent use of tertiary protein structure NMR studies, and for our application of employing a highly labelled protein in studies of physiological disposition. Figure 6 demonstrates that performing studies of physiologic disposition using HPLC/CRIMS is feasible. Production of labelled proteins using this methodology will allow a much wider variety of proteins to be made available for study since these will now include materials isolated from mammalian cells, not just from bacteria and yeast.

This was our first attempt at growing these cells and isolating the protein. The yield of protein was acceptable, but there should be ways of both increasing the amount of labelled protein secreted into Celtone-M, and of more efficiently purifying the protein. The amount of labelled protein in the culture medium might be increased by growing cells in suspension culture, so that a greater density of cells could be achieved than is possible in flasks. Purification of the protein might be less laborious if other methods of isolating the secreted protein from the Celtone-M, such as immunoaffinity columns, are used. Ultrafiltration of larger volumes might also decrease the time required to get to the final product.

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