Journal of Chromatography, 562 (1991) 625–637
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5544

Microderivatization of 4-[^{18}O]hydroxyproline and quantitation with a benchtop mass spectrometer

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ABSTRACT

Accurate estimation of *in vivo* turnover rates of collagen is complicated by amino acid reutilization. It was previously shown that the ideal, non-recycling tracer was [^{18}O]hydroxyproline synthesized *in vivo*. The analytical method for measuring turnover rates with [^{18}O]hydroxyproline must include analyte quantitation for pool size determination and isotope ratio measurement for determining levels of label incorporation. For ease of use and widest availability, a benchtop gas chromatograph–mass spectrometer in the electron-impact ionization mode was chosen. Here we present a versatile procedure for hydroxyproline derivatization that is well suited for routine, large-scale determination of analyte concentrations and relative levels of ^{18}O incorporation.

INTRODUCTION

Accurate estimation of the rates of turnover of collagens *in vivo*, as obtained by analysis of the change in radioactivity of collagen following administration of labeled glycine or proline, for example, is complicated due to the reutilization of the labeled amino acid. To circumvent this problem the method of ^{18}O labeling *in vivo* of hydroxyproline in collagens was proposed [1] and the utility of this procedure has been confirmed [2,3]. Hence, we have further examined the measurement of [^{18}O]hydroxyproline labeling in an amino acid matrix via mass spectrometry (MS). For purposes of convenient analysis a benchtop gas chromatographic–mass spectrometric (GC–MS) system in the electron-impact (EI) ionization mode

0378-4347/91/$03.50

would appear to offer a valuable choice. Derivatization of amino acids is a prerequisite for GC separation, so additional care must be used in selecting a derivative which fragments in a manner conducive to isotopic analysis. Thus, although a previously reported method [4] was successfully utilized in *in vivo* ^{18}O labeling studies [1,5], it is limited by the types of esters the method can produce and the ease by which the esterification reagent can be prepared and stored. Here we present a more versatile procedure for hydroxyproline derivatization to its amide and mono-ester analogue that is well suited for routine determinations of analyte concentrations and relative levels of ^{18}O incorporation.

EXPERIMENTAL

Reagents

Methanolic HCl (4:1 molar ratio) was prepared by slowly dripping the appropriate amount of acetylchloride (Aldrich, Milwaukee, WI, U.S.A.) into cold, stirring reagent-grade methanol. The solution was stored at −20°C for several months with no loss of activity. All other esterification reagents were prepared and stored in the same fashion. Trifluoroacetic anhydride, pentafluoropropionic anhydride and heptafluorobutyric anhydride, all 99% (Aldrich), were used without further purification.

Amberlite IRA-93 resin (Sigma, St. Louis, MO, U.S.A.) was packed in water when received and washed several times with dry methanol and then oven-dried at 100°C for 30 min. Prior to use the dried resin was slurried in methanol and stored for no more than two weeks. AG50W-X2 cation-exchange resin, 100–200 mesh, hydrogen form (Bio-Rad Labs., Richmond, CA, U.S.A.), was washed several times with deionized water and kept at 10°C in a 1:1 slurry of resin–water acidified to a pH of 5. No degradation in performance was observed with long-term storage.

Quik-Sep 5.5-ml polypropylene screening columns with plastic filter discs (Isolab, Akron, OH, U.S.A.) were first rinsed with 5 ml of deionized water before the addition of a 1-ml volume of the AG50W-X2 resin.

The compound 5-hydroxypipecolic acid (5-hydroxypiperidine-2-carboxylic acid), 99% (Sigma), was used as an internal standard (I.S.). This and 4-hydroxyproline, 99% (Sigma), were used to prepare various aqueous analyte concentration standards in the 14 μM to 14 mM range. The concentration of I.S. was held constant. These are reported in Results and discussion as the molar ratio of analyte to internal standard.

GC conditions

The gas chromatograph used was a Hewlett-Packard (HP) 5890 Series II (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with flame-ionization detector and split, heated injector port. Injector port and detector were maintained at a temperature of 250°C.

The column (15 m × 0.25 mm I.D., film thickness 0.25 μm) used was a fused-silica capillary with a bonded polyethylene glycol stationary phase (DB-WAX, available from J&W Scientific, Folsom, CA, U.S.A.). Helium was the carrier gas and the linear velocity was set to 38 cm/s (volumetric flow-rate = 1.1 ml/min). The split ratio was set to 6:1 and septum purge flow-rate to 0.6 ml/min.

The temperature profile was as follows: initial temperature, 150°C (1 min); rate, 5°C/min, final temperature, 200°C; then the column was heated rapidly to 250°C and maintained at this temperature for an additional 3 min. Under such conditions the retention time of the I.S., 5-hydroxypipecolic acid, was 7.0 min and that of the analyte 9.3 min. The peaks were typically 8 s wide.

Chromatograms were recorded with an HP 3396A integrator.

GC–MS conditions

An HP 5890 gas chromatograph linked to an HP 5970 mass-selective detector via a heated, direct transfer line (250°C) was used. GC conditions were the same as listed above. Data collection and mass spectrometer control were via an HP 1000 computer equipped with RTE-6/VM software. Injections of 1 μl were made with an HP 7673A autosampler. The mass spectrometer was tuned daily to maximize *m/z* 219 of perfluorotetrabutylamine (PFTBA) in order to increase detection limits of ions produced in this region.

The mass spectrometer was operated in the selected-ion monitoring (SIM) mode and ions monitored were *m/z* 175–181 ($M - CO_2CH_3 - H_2O = 178$ a.m.u.) for the I.S. and *m/z* 180–186 ($M - CO_2CH_3 = 182$ a.m.u.) for the analyte. Dwell times were 10 ms for *m/z* 178 and 182, 20 ms for *m/z* 179 and 183 and 25 ms for all others in order to optimize peak-area determinations [6,7].

Sample preparation and derivatization

Rat skin collagen samples, separated previously into its Type I and III components and hydrolyzed [8], were acidified with 1 ml of 1 *M* acetic acid and added to a 5.5-ml screening column containing 1 ml of the AG50W-X2 cation-exchange resin. The resin was then washed with an additional 5 ml of deionized water and the amino acids eluted with 3.5 ml of 3 *M* NH_4OH into a 3.7-ml glass vial. The samples were evaporated to dryness in a heating block at 80°C with a gentle stream of dry nitrogen gas blowing over the solutions in order to facilitate evaporation. Approximately 0.5 ml of the esterification reagent was then added to the cooled samples; the vials were then sealed with a Teflon-lined screw-top cap, vortex-mixed and heated to 80°C for 60 min. Excess esterification reagent was evaporated off at 60°C with nitrogen gas.

Acetylation was accomplished by adding 200 μl of the selected anhydride to the sample, vortex-mixing and letting stand at room temperature for 30 min. In order to prevent any loss of the volatile derivative, excess reagent was evaporated at room temperature.

Solvolysis of the O-trifluoracetyl group of hydroxyproline was expedited by

pipetting 100 μl of the methanol–Amberlite IRA-93 slurry such that approximately 10–15 μl (approximately 10 mg dry weight) of resin was transferred. The vials were capped, vortex-mixed and heated at 60°C for 10 min. The methanol solution containing the imino acid derivative was diluted to an approximate analyte concentration of 1 μ*M* and transferred without resin to a 1-ml microvial.

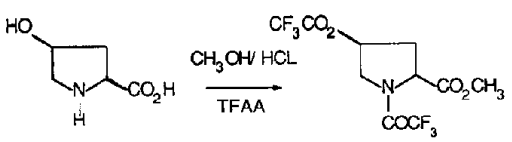
RESULTS AND DISCUSSION

A typical amino acid derivative for GC and GC–MS analysis is the N-trifluoroacetylmethyl (TAM) ester [9]. This derivative is obtained by esterification with methanolic HCl followed by acetylation with trifluoroacetic anhydride. When this procedure is used with hydroxyproline the hydroxyl group will additionally undergo acetylation to give its N,O-ditrifluoroacetylmethyl (TAM-A) ester (Fig. 1). The mass spectrum of the TAM-A derivative of hydroxyproline shows a base peak at *m/z* 164, formed by loss of trifluoroacetic acid and elimination of the carbomethoxy group. No ions retaining the ^{18}O label are observed, precluding this derivative from use for estimating collagen turnover.

Earlier work by Jackson and Heininger [1,5] showed that the derivative of choice for measuring ^{18}O incorporation by EI-MS is the free hydroxyl analogue of this derivative (TAM-B). Fragmentation of TAM-B hydroxyproline yields *m/z* 182 (Fig. 2A) by the loss of the carbomethoxy group (Fig. 3).

The derivatization procedure for the formation of TAM-B hydroxyproline, in order to avoid derivatizing the free hydroxyl, calls for post-acetylation esterification with the reagent diazomethane [4]. Extreme care must be taken in diazomethane's preparation, handling and storage [10] and, in all practicality, this type of reagent is limited to the formation of methyl, ethyl and propyl esters. The primary goal of this investigation, then, was to elucidate a TAM-B derivatization procedure more amenable to repeated, multi-sample analysis.

Chromatograms of TAM-A derivatives of hydroxyproline standards and rat skin collagen samples measured on a DB-WAX column reveal a peak with a longer retention time than the TAM-A derivative suggesting a more polar compound. The mass spectrum of this compound is identical to that of the TAM-B (Fig. 2A). Methyl, ethyl and propyl esters of the trifluoroacetyl derivative gave the same ion at *m/z* 182 confirming its origin via the loss of a carbomethoxy,



TAM-A

Fig. 1. Reaction of hydroxyproline with methanolic HCl and trifluoroacetic anhydride (TFAA).

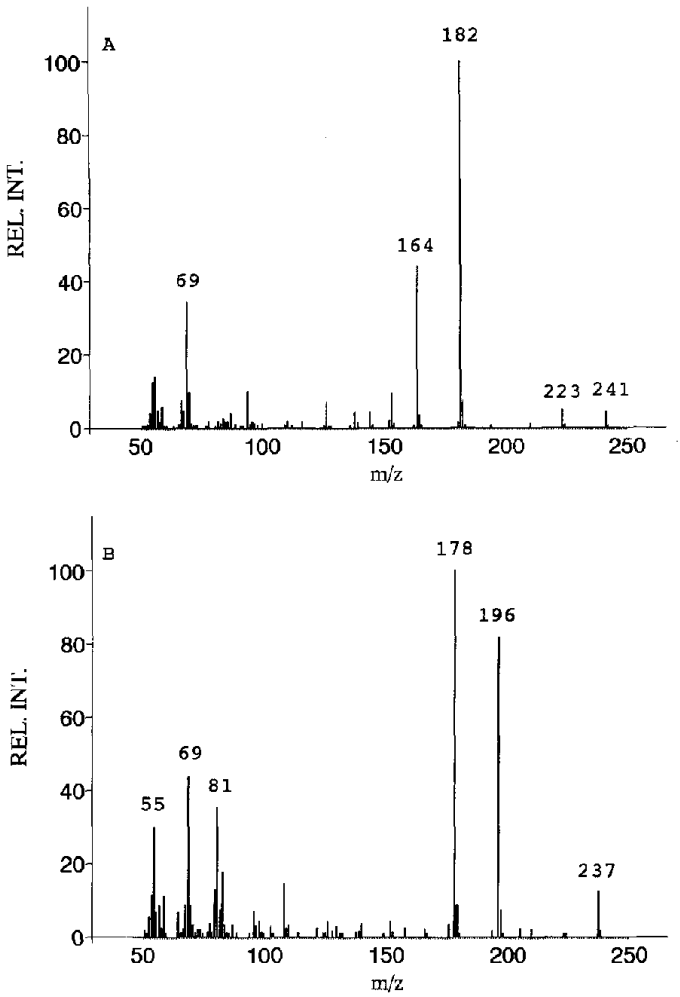


Fig. 2. Mass spectrum of N-trifluoroacetylmethyl derivatives of (A) hydroxyproline and (B) hydroxypipecolic acid.

carboethoxy or carbopropoxy group, respectively, from the molecular ion. Additional confirmation of the ion chemistry was observed with the methylpentafluoropropionyl and heptafluorobutyryl derivatives. These derivatives gave an homologous ion incremented by the expected number of CF_2 mass units.

The chromatogram of freshly derivatized hydroxyproline shows only a small amount of this compound (TAM-B). The compound was also observed when methanol solutions of TAM-A were permitted to stand for a long time. The peak areas were irreproducible, though, so other conditions were sought which would give large peak areas suitable for repeated analysis.

Optimization

A number of approaches were used to improve TAM-B yield. It is clear from the mass spectra and from the change in retention time that the TAM-B forms by solvolysis of the alkoxy group from the ester. Simply adding water was slow and did not give reproducible results. Addition of dilute ammonium hydroxide followed by mild heating gave better yields, but the chromatography was unacceptable due to tailing across the first few minutes of the chromatogram, presumably by ammonium acetate formed in the reaction.

Base-catalyzed methanolysis was attempted (Fig. 4) since TAM-B forms slowly from TAM-A in methanol. Methanolysis using triphenylamine, quinoline and triethylamine was investigated, but none of these were as effective as the catalyst eventually selected. In addition, samples treated with these bases were plagued with poor chromatography due to salt formation. Yields for the three bases are shown in Table I.

These problems were avoided altogether by using a solid-phase base catalyst for the methanolysis (Fig. 4). Amberlite IRA-93 resin is a cross-linked polystyrene resin with tertiary amine groups attached. When this catalyst is added to a methanol solution of the TAM-A, methanolysis occurs efficiently and reproducibly. No TAM-A peaks were observed in chromatographic profiles of hydroxyproline standards and samples treated this way.

The derivatization procedure was then optimized to give high yields of TAM-B. In all these experiments, the same amount of benzophenone was used as an external standard (E.S.) and was added as a solution in methanol before each sample was analyzed. Relative yields from each treatment were then assessed by measuring the ratio of the TAM-B to external standard (TAM-B/ES).

Sonication of the TAM-A derivative in methanol–resin solution gave good yields of TAM-B (Table II), but they were only 60% of the yields from other conditions used. Extended treatment in the sonication bath led to conversion of

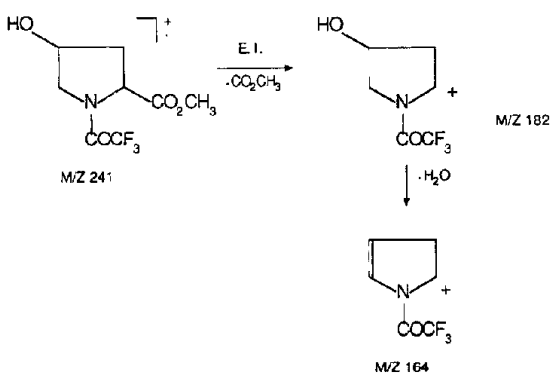


Fig. 3. Ion chemistry of N-trifluoroacetylmethylhydroxyproline.

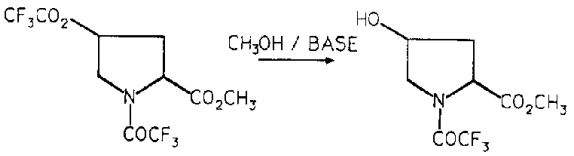


Fig. 4. Methanolysis of TAM-A to TAM-B.

TAM-B to unmeasured products. Heating at 80°C gave areas less than any other method; complete methanolysis of both TFA groups is the probable cause. Heating for 10–15 min at 60°C gave the optimum yield and this time interval was used for the rest of the work.

Because the dry IRA-93 resin (in bead form) is difficult to weigh out, a slurry of resin in methanol was used to add the catalyst to the TAM-A derivative. The yield of TAM-B, from approximately 0.5 μmol of TAM-A, was independent of the amount of beads added over a range of 1.1–40 mg (Table III). This was attributed to the fact that sufficient catalytic sites were available in even the smallest amount of beads (1.3 mequiv. of basic group per ml wet weight). Since the yield was the same throughout the range, it is clear that none of the derivative was absorbed. A 10-mg amount of beads was chosen for the remainder of the study because this amount was easily measured (see Experimental).

Low-molecular-weight carbohydrates released during hydrolysis of the collagen samples decompose in the injector, or on the column when using on-column injection. These compounds are easily removed from the acidified solution containing the hydrolysed collagen by a preliminary ion-exchange chromatography step. Recovery of hydroxyproline from three cation-exchange resins are shown in Table IV. These yields are independent of the volume of water used to wash the resin prior to amino acid elution.

TABLE I

COMPARISON OF DIFFERENT BASES FOR SOLVOLYSIS

100 equivalents added.

Base	TAM-B/ES ratio
IRA-93 resin	1.0
Triphenylamine	0.91
Quinoline	0.59
Triethylamine	–[a]

[a] No data available for this compound due to chromatographic interference from the quarternary salt.

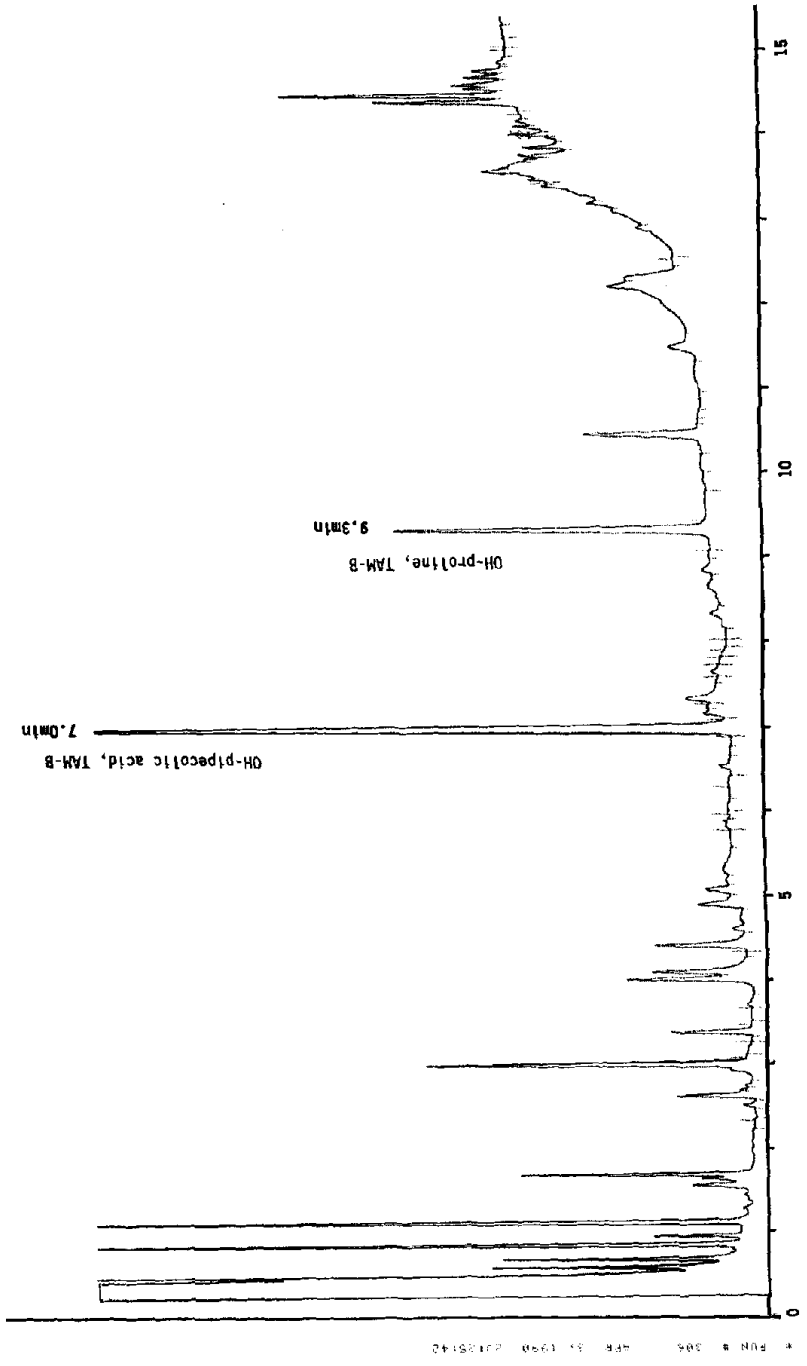


Fig. 5. Chromatographic profile of Type I rat skin collagen.

TABLE II

COMPARISON OF SOLVOLYSIS TIME AND TEMPERATURE CONDITIONS

Temperature condition	Time (min)	TAM-B/ES ratio
Room temperature	15	0.74
Sonication	15	0.59
Sonication	30	0.48
80°C	15	0.41
60°C	5	0.69
60°C	10	1.0
60°C	15	0.99
60°C	20	0.77
60°C	60	0.31

Quantitation

Measuring the amount of hydroxyproline in hydrolyzed rat skin collagen was one of the objectives of the analysis. Skin biopsies were taken over a period of several days from each rat exposed to ^{18}O-labeled oxygen gas for a short period of time. The collagen was extracted, separated into its Type I and III components and then hydrolysed by standard techniques [8].

5-Hydroxypipecolic acid was chosen as the I.S. since its structure is very similar to that of the analyte being measured. In addition, it fragments similarly, with corresponding ions offset by 14 mass units (Fig. 2B) and is well separated from the analyte on a DB-WAX column. Fig. 5 shows the chromatographic profile of a sample of hydrolysed Type I rat skin collagen with I.S. added.

Hydroxyproline concentration standards were prepared over a range of 0.022–2.13 mol of analyte per mol of I.S. and reported as mole ratios. The calibrants were measured by both GC–flame-ionization detection (FID) and GC–MS just

TABLE III

COMPARISON OF TAM-B YIELDS WITH INCREASING AMOUNTS OF IRA-93 RESIN

Resin weight (mg)	TAM-B/ES ratio
1.1	0.88
2.3	1.0
5.2	1.1
9.7	0.93
14.8	1.1
20.1	0.93
40.2	1.1

TABLE IV

ION-EXCHANGE RECOVERIES OF HYDROXYPROLINE AS COMPARED TO UNTREATED SAMPLES

Resin	Mesh	Recovery (%)
AG50W-X8	100–200	71.3
AG50W-X2	100–200	80.8
AG50W-X2	200–400	75.5

prior to and after collagen sample analysis. A graph of the area of the hydroxyproline peak normalized to hydroxypipecolic acid *versus* molar ratio is linear. Results of linear least-squares fitting are shown in Fig. 6. Regression equations and statistics are given in Table V.

In the case of GC–MS, the matter of ions monitored and data treatment must be considered. Samples of collagen studied by this method contain both natural abundance and 4-[^{18}O]hydroxyproline. In order to account for the contribution of all ions from the unlabeled and labeled species to the total analyte concentration, the individual areas of the seven monitored ions (an "ion packet") were summed and treated as one. Hydroxypipecolic acid was treated in a similar fashion, except the M − CO_2CH_3 − H_2O ion (*m/z* 178) was monitored instead of the M − CO_2CH_3 ion as it was for the hydroxyproline. This was done because *m/z* 178 is more abundant than *m/z* 196 (Fig. 2B).

Comparison of regression lines

Comparison of the regression lines for GC–FID and GC–MS shows that the r^2 values are equivalent and the data fit the line over a hundred-fold analyte

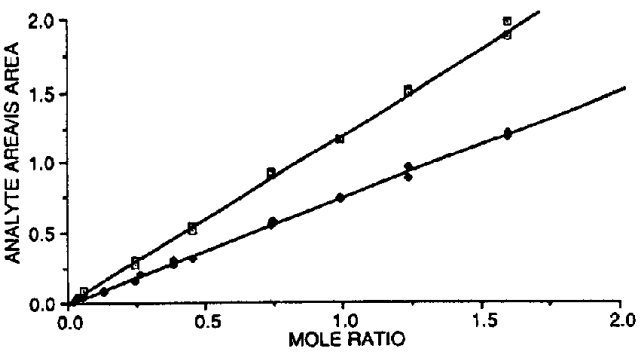


Fig. 6. Calibration graph for N-trifluoroacetylmethylhydroxyproline. (□) GC–MS; (◆) GC FID. Hydroxypipecolic acid was used as I.S.

TABLE V

RESULTS OF LINEAR REGRESSION OF N-TRIFLUOROACETYLMETHYLHYDROXYPROLINE WITH HYDROXYPIPECOLIC ACID AS AN INTERNAL STANDARD

Method	Stationary phase	Slope	Intercept	r^2	$S_{y/x}$[a]	Limit of detection (pmol)	Split ratio
GC–FID	DB-WAX	0.750	−0.0064	0.998	0.0184	36	6:1
GC–MS	DB-WAX	1.21	−0.0047	0.998	0.0294	33	Splitless

[a] Standard deviation of y-residual.

concentration range. The limit of detection [11] of both methods is also the same. In all cases the tissue samples analysed contained hydroxyproline well above the limit of detection.

Inter-assay coefficients of variation (C.V.) of hydroxyproline in a single hydrolyzed collagen sample, as measured by GC–FID, was 1.5% (molar ratio = 0.525 ± 0.008, n = 4). Similar results were obtained by GC–MS (C.V. = 1.8%). For a separate collagen hydrolysate the intra-assay C.V. for replicate derivatizations [4] was 1.2% (molar ratio = 0.682 ± 0.008, n = 8, duplicate analyses of each derivative).

For further comparative purposes, the quantity of Type I collagen hydroxyproline from five different rat skin biopsies was determined by both GC–MS and CG–FID in duplicate. The results are given in Table VI.

^{18}O *Incorporation*

Incorporation of $^{18}O_2$ into hydroxyproline from the collagen of two Wistar rats (Charles River Breeding Labs., Kingston, NY, U.S.A.) was measured. The chromatographic area of m/z 184 of the TAM-B derivative of hydroxyproline

TABLE VI

GC–MS AND GC–FID DETERMINATIONS OF COLLAGEN HYDROXYPROLINE QUANTITIES

Sample No.	Hydroxyproline per collagen sample (μmol)	
	MS	FID
1	0.591	0.610
2	0.429	0.446
3	0.646	0.689
4	0.267	0.314
5	0.143	0.147

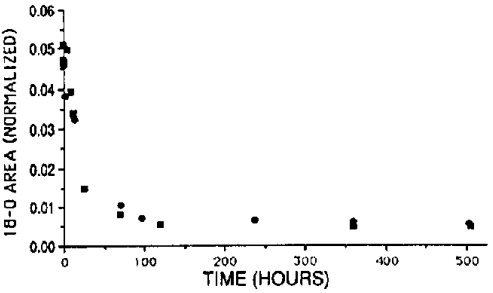


Fig. 7. ^{18}O Incorporation in Type I collagen.

was normalized to the sum of the areas of ions 180 to 186. Unlabeled hydroxyproline from hydrolysed rat tail collagen was measured over a forty-fold range of concentrations and the value for the normalized abundance ratio was 0.0055 ± 0.0005 (n = 16). For an ^{18}O-enriched sample measured in duplicate for three consecutive days, the normalized ratio was 0.0385 ± 0.0002 (C.V. = 0.54%).

The relative levels of incorporation of ^{18}O into hydroxyproline as a function of time are shown in Fig. 7 (Type I collagen) and Fig. 8 (Type III collagen). That the analysis is accurately measuring differences in incorporation is indicated by the initial difference observed between Type I and III collagens. Although only two rats were examined, values for the normalized abundance ratios at specific time points are nearly identical.

CONCLUSION

The lower cost and wide availability of benchtop mass spectrometers with EI capability make the method accessible to a large number of investigators. The method developed offers investigators interested in non-muscle protein turnover a less problematic and more versatile procedure for the derivatization and analysis of ^{18}O-labeled hydroxyproline. Large-scale and routine derivatizations are

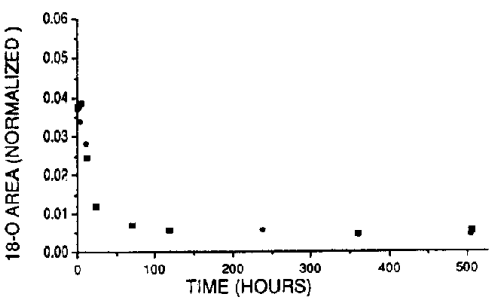


Fig. 8. ^{18}O Incorporation in Type III collagen.

facilitated since diazomethane is avoided. More parameters are available for control of chromatographic separations since any of the commonly used trifluoroacetylalkyl esters can be prepared.

Collagen turnover calculations involve not only ^{18}O levels, but also estimations of pool size (total hydroxyproline in collagen). The method described can measure both concentration of hydroxyproline in hydrolysed tissue sample and relative levels of [^{18}O]hydroxyproline.

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