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Determination of collagen and protein turnover by high-performance liquid chromatography

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of tissue collagen and protein synthesis rates in vivo, together with an index of collagen degradation. The technique utilises post-column reaction with 7-chloro-4-nitrobenzofurazan (NBDCl), which shows higher reactivity towards the secondary amino acids, proline and hydroxyproline, and also exploits differences in absorbance and fluorescence spectra to avoid interference by primary amino acids, including cysteine which reacts rapidly with NBDCl. The relative benefits of using fluorescence or absorbance detection are discussed. A detailed description is given of the steps involved in sample preparation and data for five normal tissues in the mouse are presented using fluorescence detection.

INTRODUCTION

Collagen metabolism and its regulation are of interest in a number of normal and pathological situations. For example, in late radiation damage in the lung there may be increased synthesis and breakdown of the extracellular matrix [1], and methodologies have been developed to study these changes. Collagen metabolism is most conveniently studied by the determination of the incorporation of radiolabelled proline into protein and its subsequent hydroxylation to hydroxyproline, an amino acid virtually unique to collagen. Many previous studies relied upon colorimetric assays [2] to detect proline and hydroxyproline but there are now a number of high-performance liquid chromatographic

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(HPLC) methods published using both pre- and post-column derivatisation techniques to give chromo- or fluorophores suitable for detection.

Post-column techniques include that of Roth [3] who used 7-chloro-4-nitrobenzofurazan (NBDCl) after separation by ion-exchange chromatography, but each analysis required 70 min and only referred to plasma. Macek et al. [4] used ninhydrin, which is not specific for secondary amines, and therefore a complex separation of all the amino acids is required, again giving a long analysis time. Pre-column techniques include those of Ahnoff et al. [5] and Sessa et al. [6] using NBDCl, and that of Takahashi and Lee [7] using dansyl chloride. The latter is also non-specific for secondary amino acids giving a 1-h analysis time. NBDCl has a higher reactivity towards secondary compared to primary amino acids by an order of magnitude [5]. However, exploiting this differential requires careful timing of the derivatisation step, making multiple analyses time-consuming. In addition, in studying collagen metabolism using radioactive proline, although tissue cold proline and hydroxyproline concentrations are high and simple to detect, specific activity (dpm/μg), particularly of hydroxyproline, is low, so that as much as possible of the sample must be collected for scintillation counting. The tissue-free pool (TFP) hydroxyproline concentration, which can provide important information about turnover, is also very low. Pre-column derivatisation results in sample dilution which may then require further concentration. Also, derivatisation reactions are rarely 100% complete, especially if differential reactivity is being used; thus Sessa et al. [6] and Takahashi and Lee [7] both quote 90% conversion. This does not present a problem for quantitation purposes, assuming derivatisation efficiency is the same in a complex biological matrix compared to a standard, but there is a potential problem in the specific activity determination where the proline is much higher (by a factor of ~ 100) than the hydroxyproline. Underivatised labelled proline may co-elute with derivatised hydroxyproline (particularly if the column is being heavily loaded) causing peak broadening, leading to an overestimate of hydroxyproline specific activity.

We have therefore chosen to use post-column derivatisation with NBDCl for the determination of proline and hydroxyproline specific activity, making use of both the differential reactivity towards secondary amines and previously unpublished spectral differences. This allows the calculation of protein and collagen synthesis rates and also gives sufficient resolution and sensitivity to determine the low concentration of hydroxyproline in the TFP required for assessing collagen degradation. Primary amines and thiol-containing amino acids such as cysteine (which reacts very rapidly with NBDCl) then cause negligible interference.

EXPERIMENTAL

Materials

Reagents were obtained as follows: NBDCl from Lancaster Synthesis (Morecambe, U.K.); L-[5-^{3}H]proline from Amersham International (Amersham,

U.K.); sodium lauryl sulphate (SDS) (Primar grade) from FSA (Loughborough, U.K.); HPLC solvents from Rathburn (Walkerburn, U.K.); all other chemicals were from BDH (Poole, U.K.). Bond Elut columns were from Jones Chromatography (Hengoed, U.K.).

Tissue labelling

Tissue labelling techniques were as described by Murray and Parkins [1]. Briefly, female CBA HtfBSVS mice aged twenty weeks were injected intravenously with a saline solution containing [5-^{3}H]proline (30 MBq/kg) accompanied by a flooding dose of 7 mmol/kg unlabelled proline. At 30 min the animals were killed and the tissues removed and frozen immediately.

Determination of proline and hydroxyproline specific activity

Tissue samples were weighed and homogenised in 1 ml of ethanol [8] and left at 4 °C overnight. After centrifugation, the supernatant was removed, the pellet washed twice with 0.5 ml of ethanol, respun, and the supernatant combined to give the TFP. These and the ethanol-insoluble pellet were dried under nitrogen at 40 °C. A 1- or 0.5-ml volume of 6 *M* hydrochloric acid (for tissue and TFP, respectively) was added, the samples were lightly capped and heated to 105 °C in a dry block, driving off dissolved oxygen. After 30 min the samples were tightly capped and further heated for 18 h to hydrolyse the protein in the pellets and any ethanol-soluble peptides in the TFPs. Water (1 ml) was then added to all the tubes and the samples were placed in a Savant sample concentrator (Stratech, London, U.K.) connected to a vacuum pump via a glass trap containing potassium hydroxide maintained at −70 °C, to prevent ingress of hydrochloric acid into the vacuum pump. After ~1 h samples were transferred to a vacuum desiccator connected to the vacuum pump and dried overnight. The initial stage in the Savant prevented sample bumping in the desiccator. Samples could not be taken to dryness in the Savant because of the corrosive nature of the hydrochloric acid. Samples were resuspended in 1 ml of water and passed through a C_{18} Bond Elut column [9] (100 mg for TFP, 200 mg for tissues) previously conditioned with methanol followed by water. The sample tubes were rinsed with 3 ml of water, the combined eluent containing the amino acids was again taken to dryness for 48 h as above, and the samples were then reconstituted in 250 μl of HPLC eluent (see below) prior to analysis. Any precipitate was removed by centrifugation. The amino acids were unretained on the Bond Elut, but the column retained both a large amount of particulate matter, but also more hydrophobic contaminants in the sample.

High-performance liquid chromatography

A Waters 840 [Millipore (U.K.), Harrow, U.K.] was used for HPLC control and data handling. A gradient method was employed for the separation of hydroxyproline and proline, using a 5-μm Spherisorb 50DS2 column (12.5

cm × 0.46 cm I.D., Hichrom, Reading, U.K.). The solvents were: A, 5 g/l SDS, 1 g/l trichloroacetic acid, 20 m*M* H_3PO_4; B, tetrahydrofuran–acetonitrile (30:70). The flow-rate was 2 ml/min, and the linear gradients used were: 0–4 min, 5% B; 4–8 min, 5–33% B; 8–9 min 33–70% B; 9–10 min, 70% B; 10–11 min 70–5% B. The column was maintained at 20 °C. The post-column derivatising reagents were 0.4 *M* sodium borate, pH 11.0, and 2 m*M* NBDCl in methanol, separately pumped at 0.5 ml/min using a Kratos PCRS (Applied Biosystems, Warrington, U.K.). The reaction coil was 1 ml in volume, maintained at 70 °C. After leaving the reactor, the eluent was cooled to 20 °C. Detection was by absorbance at 510 nm using a Waters 490 detector and by fluorescence using a Perkin Elmer LS1 fluorimeter (Beaconsfield, U.K.) with 510 nm excitation (interference filter) and 540 nm emission (monochromator). Fractions were collected using a LKB Helirac (LKB, Milton Keynes, U.K.) in 25-ml glass vials (Kochlight, Hatfield, U.K.) containing 100 µl of 6 *M* hydrochloric acid, which prevents further colour development and high quenching. A 15-ml volume of PCS scintillant (Amersham International) containing 0.7% acetic

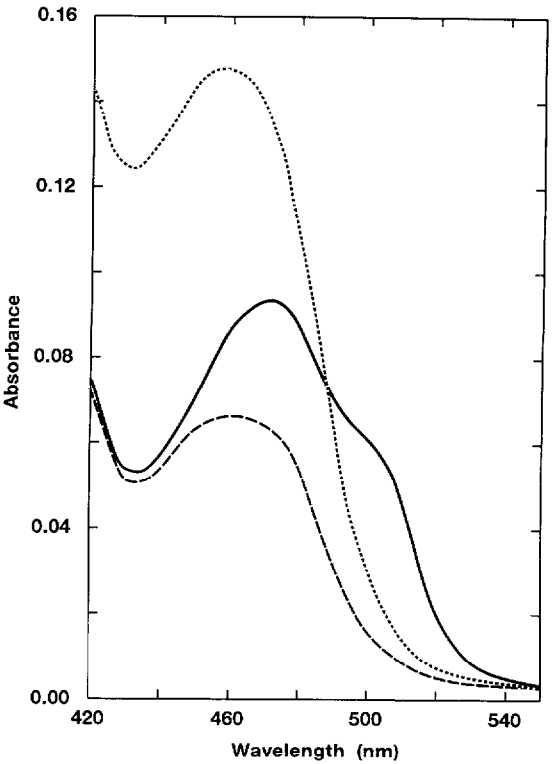

Fig. 1. Absorption spectrum of the NBDCl adducts of 100 nmol proline (—), 1000 nmol glycine (– – –) and 40 nmol cysteine (----)

acid was added, and the samples were counted in a Packard 2000 CA (Canberra Packard, Pangbourne, U.K.).

Collagen and protein metabolism

Fractional synthesis rates (FSR; the fraction of the tissue protein or collagen turned over in 24 h) were determined from the sample activities using the expression below [10]:

$$\text{Collagen FSR} = \frac{\text{specific activity tissue } [^3\text{H}]\text{hydroxyproline}}{\text{specific activity TFP } [^3\text{H}]\text{proline} \times \text{time}}$$

$$\text{Protein FSR} = \frac{\text{specific activity tissue } [^3\text{H}]\text{proline}}{\text{specific activity TFP } [^3\text{H}]\text{proline} \times \text{time}}$$

An index of collagen breakdown was calculated as described by Laurent and McNaulty [11]:

$$\frac{\text{TFP hydroxyproline}}{\text{TFP hydroxyproline} + \text{tissue hydroxyproline}} \times 100$$

Similarly, an index to the degradation of newly synthesized collagen was calculated as described by Laurent [10]:

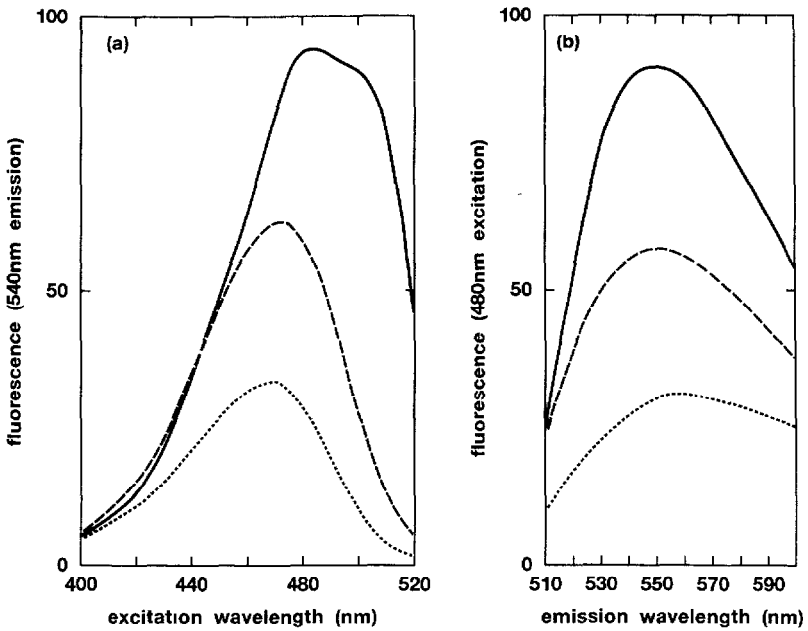

Fig. 2. Fluorescence spectra of the NBDCl adducts of 100 nmol proline (—), 1000 nmol glycine (– – –) and 40 nmol cysteine (----). (a) Excitation; (b) emission.

$$\frac{\text{TFP hydroxyproline total dpm}}{\text{TFP hydroxyproline total dpm} + \text{tissue total dpm}} \times 100$$

RESULTS

In order to assess the optimum wavelength for absorbance and fluorescence detection, under the solvent conditions used, the fractions corresponding to the peaks from 100 nmol proline, 1000 nmol glycine and 40 nmol cysteine were collected and their absorbance and fluorescence spectra determined immediately (Figs. 1 and 2). Chromatograms are shown for absorbance (Fig. 3) and fluorescence (Fig. 4) detection of a 10-μl injection of a standard mixture of 5 μmol/ml hydroxyproline, 10 μmol/ml proline and 10 μmol/ml glycine (a) and

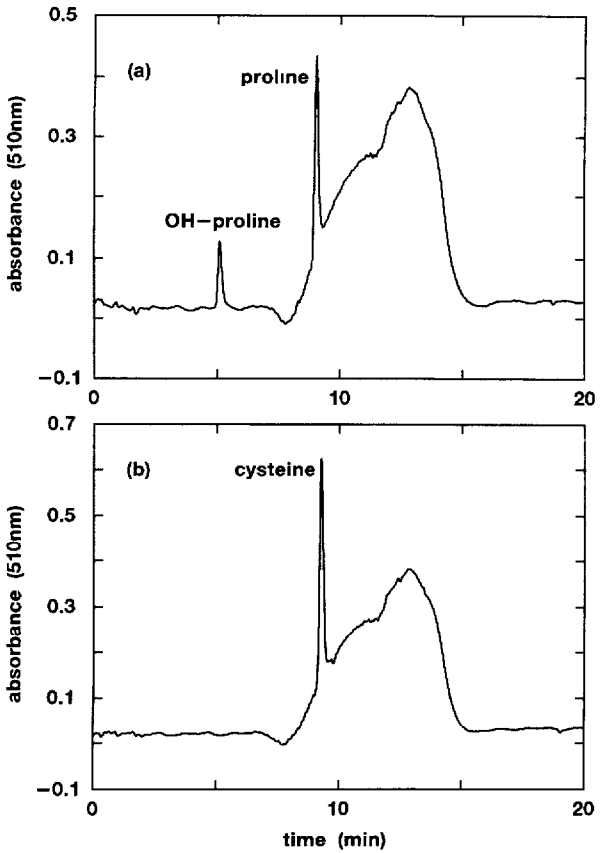

Fig. 3. Standard chromatograms with absorbance detection of (a) 50 nmol hydroxyproline, 100 nmol glycine and 100 nmol proline and (b) 50 nmol cysteine.

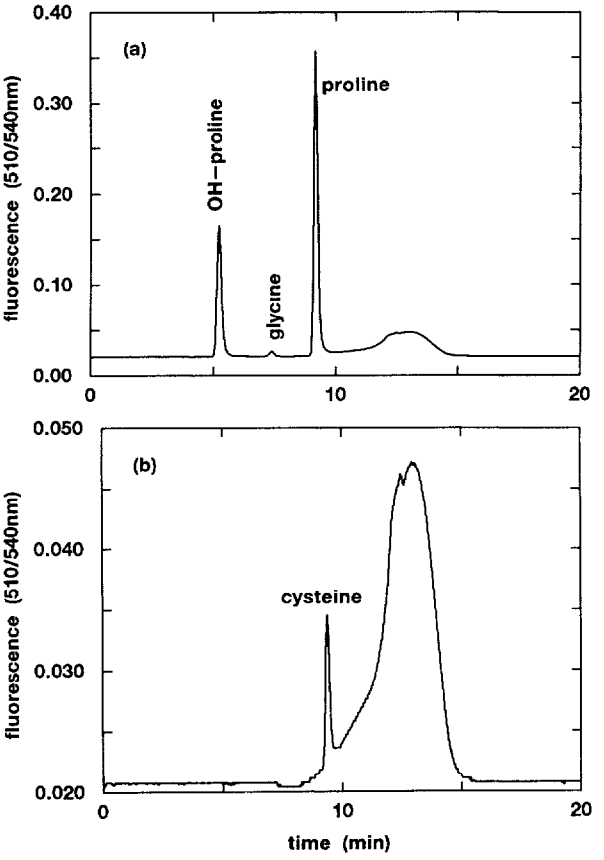

Fig. 4. Standard chromatograms with fluorescence detection of (a) 50 nmol hydroxyproline, 100 nmol glycine and 100 nmol proline and (b) 50 nmol cysteine.

TABLE I

RELATIVE PEAK AREAS OF THE NBDCl ADDUCTS OF PROLINE, GLYCINE AND CYSTEINE

Amino acid	Relative area	
	Absorbance	Fluorescence
Proline	100	100
Glycine	0.5[a]	1.9
Cysteine	317	6.9

[a]Using acetonitrile as organic modifier.

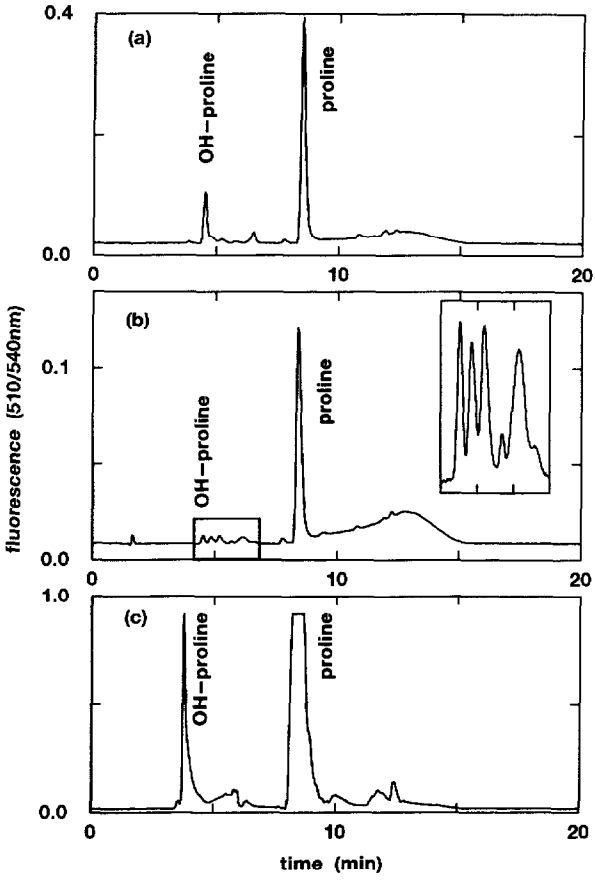

Fig. 5. Chromatograms of tissue and TFP samples with fluorescence detection. (a) Lung tissue, 10 μl injection for quantitation; (b) lung TFP, 50 μl injection for quantitation and fraction collection; (c) lung tissue, 100 μl injection for fraction collection

TABLE II

FRACTIONAL SYNTHESIS AND DEGRADATION RATES OF SOME CONTROL MOUSE TISSUES

All values are means ± S.E. (n = 5–6).

Sample	Collagen fractional synthesis rate (% per day)	Protein fractional synthesis rate (% per day)	Collagen degradation index
Small intestine	61 ± 10	238 ± 36	1.81 ± 0.13
Lung	13 ± 2.0	55 ± 4	0.34 ± 0.06
Heart	9.6 ± 2.7	30 ± 7	0.89 ± 0 17
Muscle	11 ± 2.0	21 ± 5	2.51 ± 0.45
Colon	5.9 ± 1.0	60 ± 9	1.07 ± 0.07

TABLE III

PRECISION OF ANALYSIS

All values are means ± S.E ($n=5$).

Amino acid	Amount (μmol per sample)	Radioactivity (dpm per sample)	Specific activity (dpm/μmol)
Tissue			
Hydroxyproline	0.959 ±0.003	70± 4	80± 4
Proline	0.530 ±0 001	34960±170	1230±13
TFP			
Hydroxyproline	0.0036±0.0001	150± 14	
Proline	0.530 ±0.001	34960±170	66000±210
Collagen fractional synthesis rate (% per day)			5.8 ±0.3
Protein fractional synthesis rate (% per day)			89.3 ±1.0
Total collagen degradation index			0.37±0.01
Newly synthesised collagen degradation index			65.7 ±1.8

5 μmol/ml cysteine (b). Glycine is a major constituent amino acid of collagen [12] while cysteine has a highly NBDCl-reactive thiol group. There is a good separation between the two peaks of interest of ~4 min which is important to ensure that there is no cross-contamination between the fractions because of the low specific activity of the hydroxyproline relative to the proline. As expected, the response for a primary amino acid such as glycine is very small, while cysteine shows a big response on absorbance, but a very small response on fluorescence. The relative areas, normalised to a constant amount injected, are shown in Table I.

Linear calibration curves of nanomoles injected against area (covering the range used for quantitation) for proline and hydroxyproline with fluorescence detection were obtained, with correlation coefficients >0.999. The regression equations were: $y=3.24\cdot10^4x+4.3\cdot10^4$ for proline; $y=3.77\cdot10^4x+1.1\cdot10^4$ for tissue hydroxyproline; and $y=19.2\cdot10^4x-0.27\cdot10^4$ for TFP hydroxyproline (detector gain×5). Linearity based on area was maintained up to 300 nmol (hydroxy)proline, limited presumably by the NBDCl. However, there was considerable peak broadening with a loss of resolution and also some changes in retention time for tissue samples with the large injected volumes needed for the determination of the specific activity of hydroxyproline. Because the specific activity of the hydroxyproline is low, two injections for tissue samples were used: one of 10 μl (corresponding to ~50 nmol hydroxyproline and ~100 nmol proline) for quantitation and a second of 100 μl for fraction collection. For the TFPs, where only the concentration of hydroxyproline and the relatively high proline specific activity was required, and which cause less peak

broadening, a single injection of 50 μl (up to 2.5 nmol hydroxyproline and ~100 nmol proline) was used for both counting and quantitation. We have applied the technique to a number of normal tissues in the mouse and some typical chromatograms for the lung are shown in Fig. 5 for tissue, TFP and tissue collection. The expanded inset in Fig. 5b shows the separation of hydroxyproline from interfering peaks in the TFP. The collagen and protein synthesis rates obtained for these tissues together with the index of collagen degradation are presented in Table II. Replicate injections of a pooled sample of colon digests were also made to assess reproducibility, and the results are presented in Table III.

DISCUSSION

The eluent employed was based on that of Macek et al. [4], who used trichloroacetic acid with SDS as an ion-pairing agent. However, with the high column loading required for specific activity determinations, a better peak shape was achieved with 5 g/l SDS instead of 3 g/l. Acetonitrile gave better chromatography than either propanol or methanol alone, but tetrahydrofuran was also required to achieve resolution of hydroxyproline from interfering peaks, particularly in the TFPs. Maintaining the column at a sub-ambient temperature also improved the peak symmetry. The additional gradient after the elution of the proline was to ensure that no late-eluting activity remained on the column to increase the background of subsequent injections. Although this significantly increased the total run time, early experience suggested that the background count did increase for subsequent injections, perhaps due to retention of unhydrolysed small peptides.

Ahnoff et al. [5] showed that secondary amines react approximately ten times faster than primary amines, but additional differential can be obtained by making use of spectral differences (Fig. 1). The absolute absorbances are only approximate, since the reaction will continue after collection, although at a lower rate because of the reduced temperature, and spectra were run within 5 min of collection. A comparison of glycine and proline confirms the difference in reactivity between the two, in view of the ten-fold difference in amount injected, but in addition, there is both a ~10 nm increase in the peak absorbance with proline, but, more importantly, a shoulder on the side which increases significantly the absorbance above 500 nm (where the absorbance of glycine is negligible). For this reason, absorbance or fluorescence excitation monitoring was carried out at 510 nm. That this is advantageous is confirmed by the data shown in Table I, where relative areas for the same injected amounts are shown. They indicate a differential for secondary over primary amines of ~200 using absorbance detection or ~50 for fluorescence. The somewhat poorer differential for fluorescence detection may relate in part to the relative bandwiths of the two detectors. The fluorescence detector used in this work

has an interference filter with a bandwidth of 11 nm to select the excitation wavelength compared to 4 nm for the absorbance detector, and superior results may be obtained with a twin monochromator machine. This differential reactivity means that the HPLC method does not need to separate all the primary amino acids, allowing a short analysis time. The only primary amino acid which causes a problem is cysteine, since the –SH group reacts even more rapidly than proline and also elutes close to it. However, comparing the fluorescence emission spectrum shown in Fig. 2b with the absorbance spectrum (Fig. 1) shows that despite cysteine exhibiting some absorbance at 510 nm it does not fluoresce significantly when excited at 510 nm, and the emission is monitored at 540 nm. This is confirmed by the data in Table I, where cysteine gives a ca. three times larger area than proline using absorbance, but only ~7% of the

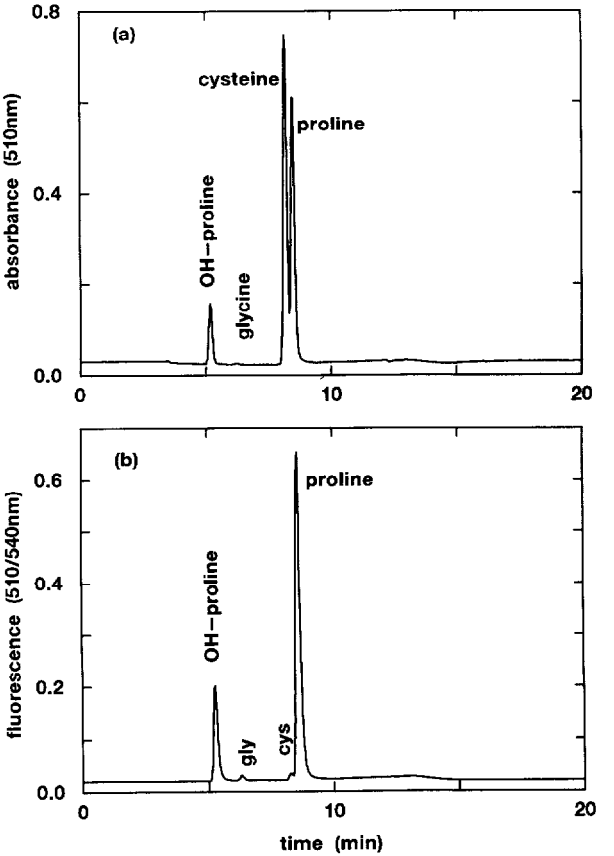

Fig. 6. Standard chromatograms of 50 nmol hydroxyproline, 50 nmol cysteine, 100 nmol proline and 100 nmol glycine, using the acetonitrile alone gradient (a) Absorbance detection; (b) fluorescence detection.

area with fluorescence detection. Thus, although cysteine is poorly resolved from proline, it does not significantly interfere using the latter detection mode.

The tetrahydrofuran incorporated into the eluent resulted in a very marked deterioration in the baseline of the absorbance detector, perhaps because of its possible reactivity with NBDCl. This was the reason why the value for glycine using absorbance detection in Table I was obtained using acetonitrile, since it could not be detected above background noise with the tetrahydrofuran present. Thus, for all the data in Table II, fluorescence detection was used exclusively. This mode is required anyway to give the high sensitivity needed to quantitate the TFP hydroxyproline for degradation rates.

If a fluorescence detector is not available, or degradation rates are not important, then absorbance detection, omitting the tetrahydrofuran from the eluent, could be employed. In this case, a simple gradient from 5 to 60% of 95% acetonitrile, 5% water, run over 10 min, can be used. This solvent system adequately resolves cysteine from proline, but would result in a minor overestimate in most cases of the hydroxyproline concentration in tissues because it would no longer be well resolved from the peaks eluting immediately after it. If cysteine (which is not a major component of collagen) caused a problem it could easily be removed by oxidation. A standard chromatogram, using this system, of a 10-μl injection of 5 μmol/ml hydroxyproline and cysteine and 10 μmol/ml glycine and proline is shown in Fig. 6, using both absorbance and fluorescence detection.

The data for collagen and protein synthesis rates shown in Table II agree well with the limited amount of published data for the mouse [1,13] although higher than those seen in the rat [8], while the data in Table III illustrate the reproducibility of the assay, showing that even the low levels of hydroxyproline in the TFP can be reliably quantitated. The largest source of error is in the counting of the hydroxyproline peak, and this error could be reduced by increasing the specific activity of the dosing solution or by the use of ^{14}C-labelled proline. However, Table III shows that with five repeat analyses the standard error of the mean for the collagen synthesis rate is only 5%, which is small relative to the inter-animal variation shown in Table II. The method avoids the potentially hazardous toluene extraction, and by automating the analysis results in considerable time savings. We believe the technique described here will prove to be a useful tool in the study of protein and collagen metabolism.

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REFERENCES

1 J.C. Murray and C.S. Parkins, Radiat. Res., 111 (1987) 498-510.
2 B Peterkovsky and D.J. Prockop, Anal. Biochem., 4 (1962) 400-406.

3 M. Roth, Clin. Chim. Acta, 83 (1978) 273–277.
4 J Macek, L. Mıterova and M. Adam, J. Chromatogr., 364 (1986) 253–257
5 M. Ahnoff, I. Grundevik, A. Arfwidsson, J. Fonselius and B.-A. Persson, Anal. Chem., 53 (1981) 485–489.
6 W.C. Sessa, Jr., R.L. Rodgers and C.O. Chichester, J. Chromatogr., 382 (1986) 258–263.
7 S. Takahashi and M.-J. Lee, Anal. Bıochem., 162 (1987) 553–561.
8 R.J. McNaulty and G.J. Laurent, Collagen Rel Res , 7 (1987) 93–104.
9 N P. Stimler, Anal. Biochem., 142 (1984) 103–108.
10 G.J. Laurent, Biochem. J., 206 (1982) 535–544
11 G.J. Laurent and R.J. McNaulty, Am. Rev. Respir Dis., 128 (1983) 82–88.
12 E.J. Miller and S. Gay, Methods Enzymol., 82 (1982) 3–32.
13 J C. Murray and C.S. Parkins, Radiother Oncol., 13 (1988) 137–143.