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Review

Derivatization of posttranslationally modified amino acids

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Abstract

After a brief overview of posttranslational modifications of protein amino acids, the use of various derivatizing reagents for amino acid analysis is discussed. Derivatization and chromatographic separation of hydroxyproline, methylhistidine, and phosphorylated amino acids are discussed in detail to illustrate some of the strategies that can be applied to the analysis of posttranslationally modified amino acids.

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List of abbreviations

AQC	6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate
CBQCA	3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde
DABSYL	4-(Dimethylamino)azobenzene-4′-sulfonyl
DANSYL	1-Dimethylaminonaphthalene-5-sulfonyl
FDAA	1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide
FDNDEA	N,N-Diethyl-2,4-dinitro-5-fluoroaniline
FITC	Fluorescein isothiocyanate
FMOC-Cl	9-Fluorenylmethyl chloroformate
GC	Gas chromatography
HPCE	High-performance capillary electrophoresis
HPLC	High-performance liquid chromatography
HYP	Hydroxyproline
1MH	1-Methylhistidine
3MH	3-Methylhistidine
MPA	3-Mercaptopropionic acid
NBD-Cl	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole
NBD-F	7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole
NDA	Naphthalene-2,3-dicarboxaldehyde
OPA	*o*-Phthalaldehyde
PITC	Phenyl isothiocyanate

1. Introduction

Proteins are synthesized from a basic set of twenty different amino acids in a sequence specified by DNA encoded genetic information. After synthesis, the protein molecule is subjected to extensive modifications. Almost all available functional groups of the protein molecule, including the amino and carboxyl termini and the side chains of individual amino acids, can be covalently modified. As a result of these post-translational modifications, the number of different amino acids of which mature proteins are composed is far greater than the basic set of twenty primary amino acids. Most modifications are conducted by very selective enzymatic processes, and are thus restricted to specific amino acid residues. As a consequence, the abundance of a particular modified amino acid may be very low, *e.g.* one residue per protein molecule, making high demands upon the sensitivity of the analytical methods used.

The amino acid composition of proteins is usually determined after acid hydrolysis, *e.g.* 24 h at 110°C in 6 *M* HCl. Many modified amino acids cannot withstand these harsh conditions and require special hydrolysis conditions in order to be recovered in sufficient yield for subsequent analysis, *e.g.* mild acid hydrolysis, base hydrolysis, or enzymatic digestion. Optimal conditions have to be determined separately for each individual modified amino acid.

Of special relevance to the biomedical sciences is the analysis of modified amino acids in biological fluids. These amino acids, released after *in vivo* degradation of modified proteins, may be useful markers of certain biological processes.

Traditionally, amino acid analysis are performed by ion-exchange chromatography and post-column derivatization. In this way not only the twenty "primary" amino acids can be determined but also a large number of non-protein amino acids and posttranslationally modified amino acids. In recent years there has been a shift towards pre-column derivatization and separation of the derivatives by reversed-phase HPLC. The process of derivatization serves a dual purpose. In the first place, it increases detectability of the amino acids by converting them into adducts that can be detected with high sensitivity using UV absorption, fluorescence detection or electrochemical detection. At the same time, the hydrophobicity of the very polar amino acids is increased, allowing separation by reversed-phase mode. Separations by reversed-phase HPLC are usually much faster than separations by ion-exchange chromatography. Many reagents for derivatization of amino acids have been developed. Most of these reagents were developed for the analysis of amino acids in protein hydrolysates. They react with the amino

group, and as modifications of amino acids are usually limited to the side chain, they can also be used for the derivatization of posttranslationally modified amino acids. Some reagents can selectively label a particular modified amino acid using the proper reaction conditions. In this way the particular chemistry of the modified amino acid is exploited to enhance selectivity of the derivatization reaction. Another approach is to use a combination of two reagents in order to increase selectivity. In this paper some of these derivatization strategies and separation of the resulting adducts are reviewed.

2. Posttranslational modification of amino acids

The polypeptide chain resulting from the process of protein synthesis is often regarded as a final product. In fact, at this stage the protein molecule is but a half-finished product, that needs extensive modifications before it is ready to perform its biological task. Most proteins are processed by limited proteolysis of the polypeptide chain. Furthermore, the protein must adopt a specific three-dimensional structure. In many proteins this structure is stabilized by the formation of disulfide bonds between cysteine residues. In addition, amino acid side chains are extensively modified by various enzymatic processes. Side chain modifications include phosphorylation, hydroxylation, methylation, and glycosylation. Some amino acids may undergo multiple successive posttranslational modifications. For instance the hydroxyl group of hydroxylated proline and lysine represents a new site for further modifications by glycosylating enzymes. Several hundreds of modified amino acids have been identified to date and the number is still growing. A non-exhaustive list of side chain modifications is shown in Table 1.

After posttranslational modification of the protein, the finished product is ready to perform its biological function. However, during the limited lifetime of the protein molecule, further modifications may occur. These include very specific reactions, *e.g.* reversible phosphorylation as a means of enzyme activity regulation. But protein molecules also undergo less specific reactions, *e.g.* non-enzymatic glycosylation and oxidative damage, that in the long run may be detrimental to their functional properties.

After *in vivo* degradation of proteins, the twenty common amino acids can be reutilized for the synthesis of new protein molecules. Modified amino acids can not be recycled in this way. Most of these amino acids are either catabolized by the liver or excreted. Trimethyllysine, liber-

Table 1
Posttranslational modifications of amino acids

Modification	Amino acids involved
Carboxylation	Glutamic acid
Hydroxylation	Lysine, phenylalanine, proline
Acetylation	Amino terminus, lysine, serine
Phosphorylation	Arginine, aspartic acid, histidine, lysine, serine, threonine, tyrosine
Methylation	Aspartic acid, arginine, glutamic acid, histidine, lysine
Glycosylation	Amino terminus, asparagine, cysteine, serine, threonine
Deamidation	Asparagine, glutamine
Myristoylation	Cysteine
Sulfation	Tyrosine
Disulfide bond formation	Cysteine
Formylation	Amino terminus
Iodination	Histidine, tyrosine
ADP-ribosylation	Arginine
Pyroglutamate formation	Amino terminus
Phosphatidylinositol derivatization	Carboxyl terminus

ated after proteolysis of some proteins, is a rare exception to this rule, as it is used as a precursor in the biosynthesis of carnitine. A number of modified amino acids serve as useful markers for specific biochemical processes. Analysis of these amino acids in biological fluids is used as a diagnostic tool in medicine.

As this review is not intended to give an extensive overview of the phenomenon of posttranslational modification, only some of the more common forms of modification are briefly discussed in the next paragraphs. More detailed information on this subject can be found elsewhere [1,2].

2.1. Phosphorylation

Phosphorylation is one of the most ubiquitous and best studied forms of posttranslational modification [3–5]. Serine, threonine, and tyrosine are the amino acids most frequently forming phosphoester bonds. In contrast, in phosphorylated histidine, lysine, and arginine, the phosphorus atom is directly bound to a nitrogen atom of the parent amino acid.

Most phosphorylation reactions are reversible and regulate protein activity. Several biological processes are regulated by the coordinated activities of protein kinases, which catalyze phosphorylation, and protein phosphatases, which catalyze dephosphorylation. Protein phosphorylation and dephosphorylation play an important role in intracellular signal transduction. Many cellular receptors for growth factors and hormones possess protein kinase activity. Upon binding of the ligand, the receptor protein is autophosphorylated and becomes active as a kinase on intracellular proteins, thereby triggering a series of biochemical events.

2.2. Methylation

Methylation involves the amino acids arginine, lysine, histidine and the side-chain carboxyl groups of glutamate and aspartate. Methylation is usually catalyzed by methyltransferases, using S-adenosylmethionine as donor of the methyl group. Some proteins require methylation at specific residues in order to function properly, *e.g.* histones, the muscle proteins actin and myosin, alcohol dehydrogenase, and some ribosomal proteins.

The level of 3-methylhistidine (3MH) in urine is considered a valuable parameter for the estimation of muscle protein turnover. Methods to determine 3MH in biological fluids are discussed in section 4.2.

2.3. Hydroxylation

Lysine and proline are the two amino acids most frequently modified by hydroxylation. Collagen has a remarkable high content of hydroxyproline (HYP). The enzymatic reaction that carries out the hydroxylation of proline requires the presence of ascorbic acid. Levels of HYP and HYP containing peptides in urine reflect collagen turnover. Analysis of HYP is discussed in detail in section 4.1.

Collagen also contains posttranslationally hydroxylated lysine. Part of the hydroxylated lysine is further modified by enzymatic addition of sugar molecules to its hydroxyl group. Collagen aldehydes are generated from specific lysine and hydroxylysine side chains by lysyl oxidase. By inter- and intra-molecular reactions of these aldehydes, crosslinks are formed [6]. Two of these collagen crosslinks, *i.e.* pyridinoline and deoxypyridinoline, are excreted in urine and are regarded as useful markers of collagen breakdown [7].

2.4. Glycosylation

The carbohydrate groups of glycosylated proteins play an important role as recognition determinants in intercellular interactions, protein targeting and host–pathogen interactions [8]. Most glycosylation reactions are enzyme catalyzed. In O-linked glycosylation, sugars are bound to either serine or threonine OH groups. A second class of glycoproteins consists of proteins that have sugars linked to the amide nitrogen of asparagine residues (N-linked glycosylation).

Non-enzymatic reaction of reducing sugars

with the amino groups of proteins may lead to deterioration of the biological function of the protein, and may be involved in aging and in complications of diabetes [3,9]. A minor hemoglobin variant, called HbA1c, is formed by non-enzymatic attachment of glucose to the N-terminal valine of the hemoglobin molecule. As levels of HbA1c are related to the average level of glucose over the two-month period prior to sampling, measurement of HbA1c is used to monitor glycemic control in diabetes patients.

Collagen contains two glycosides of hydroxylysine: glucosyl–galactosyl–hydroxylysine and galactosyl–hydroxylysine. Both glycosides are almost completely excreted in urine during collagen degradation, and serve as useful markers for bone resorption in several clinical conditions [10,11].

3. Derivatization and chromatography of amino acids

3.1. General considerations

Although there is a trend towards increased use of amino acids analysis after pre-column derivatization, classical amino acid analysis by separation of underivatized amino acids by ion-exchange chromatography in combination with post-column detection, is still used. One of the reasons is that dedicated amino acid analyzers based on this principle are commercially available. Another, not less important, reason is that the resolving power of modern ion-exchange resins is very high, allowing the separation of many physiological amino acids including post-translationally modified ones [12,13].

All amino acids have at least two functional groups that are susceptible to derivatization, *i.e.* the carboxyl group and the amino group. Most derivatization reagents developed for HPLC use the amino group as target. Derivatization of the carboxyl group used for analysis by GC is not discussed in this paper. In addition, some amino acids have side chains with functional groups other than amino or carboxyl groups, that can be derivatized. An example is the sulfhydryl group present in the side chains of some amino acids, *e.g.* cysteine and homocysteine. Several methods to analyze these amino acids after pre-column derivatization with specific sulfhydryl reagents have been described [14]. Reagents used include monobromobimane [15–18], ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate [19–22], and 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole [20,23–25].

3.2. Reagents for pre-column derivatization

Table 2 lists some of the reagents developed for the derivatization of amino groups, together with data on reactivity and detection mode. Detection wavelengths enumerated in this table are only indicative. The optimal wavelength for absorbance detection may vary, depending on the particular amino acid derivatized and on solvent composition. Furthermore, many fluorescent compounds can be excited at more than one wavelength. The choice then usually depends on the available equipment. Deuterium lamps have a high output below 300 nm, whereas xenon lamps perform better above this wavelength. OPA derivatives for example are usually excited at 340 nm, but using a deuterium light source and excitation at 230 nm, some gain in sensitivity can be obtained.

The simple fact that there exist so many reagents indicates that derivatization of amino groups is not without problems. The ideal reagent for pre-column derivatization of amino acids should meet the following requirements:

(a) rapid and complete reaction with both primary and secondary amino acids;

(b) mild reaction conditions;

(c) each amino acid should yield a single derivative;

(d) stable derivatives;

(e) equal detector response for all derivatives;

(f) the reagent itself or its degradation products do not interfere with the chromatographic separation;

(g) the derivatization reaction is not disturbed by sample matrix components.

In fact none of the reagents developed sofar, satisfies all these criteria, although some of them

Table 2
Reagents for amino acid derivatization

Reagent	Reactivity with amines[a]	Detection mode(s)[b]	Detection wavelength(s)[c]
PITC	P,S	A	254
FMOC-Cl	P,S	F	260–320
OPA	P	F,E	340–455
NDA	P	F,E	420–490
DANSYL chloride	P,S	F	340–520
DABSYL chloride	P,S	A	450
NBD-Cl	P,S	F,E	470–530
NBD-F	P,S	F,E	470–530
AQC	P,S	F	250–395
FDNDEA	P,S	A	360
FDAA	P,S	A	340
Naphthylisocyanate	P,S	F	238–385
FITC	P,S	F	490–520
CBQCA	P	F	450–550
Fluorescamine	P,S	F	365–460

[a]Reactivity with primary amines (P) or secondary amines (S).
[b]A = absorbance, F = fluorometric, E = electrochemical.
[c]Appropriate detection wavelength(s) for absorbance or fluorometric detection (excitation and emission).

come close. Some shortcomings may be overcome by simple measures, *e.g.* instability of the derivatives does not have to be very problematic if the entire procedure can be automated. Some of the commonly used derivatization reagents are discussed in the next few paragraphs. A number of these reagents were also reviewed by Deyl *et al.* [26].

3.2.1. Phenylisothiocyanate

Separation of phenylthiocarbamyl derivatives, obtained after reaction of both primary and secondary amino acids with phenylisothiocyanate (PITC), by reversed-phase HPLC is well documented [27–37]. PITC, also known as the Edman reagent, was first used by Koop *et al.* to quantitate free amino acids in carboxypeptidase Y digests of cytochrome P-450 [36]. Detailed methods for derivatization and reversed-phase HPLC separation were published by Heinrikson and Meredith [33] and Bidlingmeyer *et al.* [37]. The reader is also referred to the excellent review of Cohen and Strydom on this subject [34].

A major advantage of the method is that all amino acids can be quantitated with approximately equal sensitivity. Although quantitation is performed using UV detection, sensitivity is excellent, allowing detection at the pmol level. The fact that pretested columns, guaranteed to give baseline separation of either protein hydrolysate or physiological amino acids, are marketed by Waters (Milford, MA, USA) has certainly contributed to the popularity of the method.

A practical drawback of PITC is that excess reagent has to be removed after derivatization. In most published methods removal of excess PITC is performed by drying the samples under vacuum, which makes it difficult to automate the derivatization procedure. However, it is also possible to remove the excess reagent by extraction with pentane [35]. This method has been used to develop a fully automated PITC derivatization [29].

3.2.2. Fluorenylmethyl chloroformate

9-Fluorenylmethyl chloroformate (FMOC-Cl) was originally used as a protective reagent for the amino group during peptide synthesis. Later

it was shown to be an excellent reagent for the labelling of both primary and secondary amino acids [38–43]. Reaction is complete within 1 min at room temperature. Excess of the fluorescent reagent has to be removed by extraction with pentane or pentane/ethylacetate before chromatography [39,42,43]. This step carries the risk of selective loss of hydrophobic amino acid derivatives. Alternatively, excess of reagent can be derivatized with the hydrophobic 1-aminoadamantane [41]. The derivatives show excellent stability, with the exception of the double-labelled histidine, which is slowly converted into the mono-substituted derivative [41,42]. Tyrosine also yields a mixture of the mono-substituted and di-substituted derivatives. It has been shown that at a relatively low pH during derivatization, tyrosine is mono-substituted [42]. Haynes *et al.* have shown that it is possible to convert di-substituted histidine and tyrosine to the mono-substituted derivatives by incubation with hydroxylamine [40]. This step simultaneously converts unreacted FMOC-Cl to FMOC-hydroxylamine. Using this technique these authors developed a fully automated amino acid analysis system based on FMOC chemistry [38].

FMOC derivatization has been successfully applied to the analysis of phosphorylated amino acids in protein hydrolysates [44], as will be discussed in section 4.3.

3.2.3. *o*-Phthalaldehyde

o-Phthalaldehyde (OPA) is an often used reagent for the derivatization of amino acids [45–55]. In the presence of a thiol compound it reacts rapidly at room temperature, forming highly fluorescent isoindole derivatives. The lack of intrinsic fluorescence of the reagent is an attractive feature that has contributed to its popularity. The amino acid derivatives are not very stable, but by automation of the derivatization procedure accurate results can be obtained. It has been shown that degradation of the isoindole derivatives is catalyzed by excess OPA [56]. Therefore, the concentration of OPA during derivatization is a critical parameter [55]. The reagent itself rapidly deteriorates during storage, probably by oxidation [57]. This process can be retarded by storing the reagent in tightly capped vials and addition of a suitable chelator [57]. Stability of the isoindole derivatives also depends on the nature of the sulfhydryl compound used. 2-Mercaptoethanol is most widely used [45–47,49,50], but it has been shown that other thiol compounds like 3-mercaptopropionic acid (MPA) or N-acetylcysteine yield more stable derivatives [48,58]. Many chromatographic separations of OPA-MPA derivatized amino acids have been published [48,51–55].

In addition to the stability problems discussed, the reagent has some more drawbacks. The double labelled derivatives of ornithine and lysine have a rather low fluorescence, probably due to internal quenching. Cysteine also has a very low fluorescence intensity, although this problem can be solved by alkylating its sulfhydryl group by treatment with a reducing agent followed by reaction with iodoacetic acid [52].

Electrochemical detection of OPA derivatized amino acids has also been described [50,59]. An advantage of electrochemical detection in comparison with fluorescence detection is that some amino acids with low fluorescence activity like cysteine and lysine are electroactive. Also small derivatized peptides that are hardly fluorescent do have electrochemical activity [59]. By varying the electrode potential particular amino acids (*e.g.* basic amino acids) can be selectively detected [59].

Another disadvantage of OPA is that it does not react with secondary amino acids. Some investigators have employed a double derivatization strategy to allow detection of both primary and secondary amino acids [60,61]. After derivatization of primary amino acids with OPA, secondary amino acids are labelled with FMOC-Cl. If MPA is used as thiol compound, the resulting isoindole derivatives of the primary amino acids are less hydrophobic than the FMOC derivatives of the secondary amino acids. Therefore, using reversed-phase HPLC, the OPA adducts elute first, followed by the FMOC adducts, and both can be detected at their optimal excitation and emission wavelengths using a programmable fluorescence detector.

3.2.4. Naphthalene-2,3-dicarboxaldehyde

Naphthalene-2,3-dicarboxaldehyde (NDA) reacts with primary amines in the presence of cyanide, forming N-substituted 1-cyanobenz-[*f*]isoindole derivatives [62,63]. These derivatives have an improved stability compared to the corresponding OPA derivatives. The intensely fluorescent derivatives can be detected at 490 nm using excitation at 420 nm or 246 nm. An interesting property of the derivatives is that quantum efficiencies increase with an increase in solvent water content. This property makes this reagent very suitable for separation by reversed-phase HPLC [62]. Analytes with two or more derivatizable groups yield products that are self-quenching, as is observed for OPA derivatives. This shortcoming of NDA can be circumvented by using electrochemical detection. Lunte *et al.* used electrochemical detection to analyze desmosine and isodesmosine, two cross-linking amino acids of elastin that contain four primary amino groups [64].

NDA has also been used to separate labelled amino acids by HPCE. In combination with laser-induced fluorescence detection, detection limits of approximately 1 amol were obtained [65].

3.2.5. Dansyl chloride

1-Dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) reacts with primary and secondary amino acids. This reagent has been frequently used for the identification of amino acids after separation by TLC, in peptide sequence studies. However, dansyl amino acids can also be separated by reversed-phase HPLC and detected fluorometrically at the pmol level [66–68]. Tapuhi *et al.* have thoroughly investigated the influence of several parameters on derivatization yield and stability of the dansyl amino acids [66]. Efficiency of amino acid labelling critically depends on the ratio of dansyl chloride to amino acid. Excess reagent probably promotes degradation of dansyl amino acids [66]. Therefore, it is important to neutralize excess reagent after derivatization is complete. Methylamine [67] or ethylamine [66] can be used for this purpose. The time needed to obtain complete derivatization at room temperature is approximately 30 min, although the time needed to obtain maximum yield varies between amino acids. Another problematic aspect of dansyl derivatization is that conversion of lysine, histidine and tyrosine into di-dansyl derivatives is pH dependent [66]. Furthermore, the derivatized amino acids are sensitive to light. A completely automated amino acid analysis system base on dansyl derivatization has been described [68]. Dansyl derivatization has been used for the determination of desmosine and isodesmosine, the major cross-linking amino acids of elastin [69].

Moro *et al.* described the analysis of two hydroxylysyl glycosides in urine after derivatization with dansyl chloride [70,71]. Both galactosyl–O–hydroxylysine and glucosyl–galactosyl–O–hydroxylysine eluted as double peaks, suggesting that the derivatization conditions used resulted in a mixture of mono- and di-dansyl derivatives.

3.2.6. Dabsyl chloride

4-(Dimethylamino)azobenzene-4′-sulfonyl chloride (dabsyl-chloride) reacts with both primary and secondary amino acids. Dabsyl derivatives can be separated by reversed-phase HPLC using photometric detection [72–77]. Reaction conditions were investigated by Jansen *et al.* [73]. Highest yields were obtained using a temperature of 70°C for 15 to 30 min, although the authors observed higher yields for some amino acids, if derivatization was performed at a lower temperature. pH of the incubation medium was not very critical. It was essential to neutralize excess reagent after the incubation by addition of ammonia. The derivatives showed maximum absorbance at 448–468 nm. Dabsylated amino acid were found to be stable in solution for up to one month, even when stored at room temperature in daylight [77]. Stocchi *et al.* reported the separation of 35 dabsylated amino acids with a run time of only 25 min [72].

Casari *et al.* analyzed urinary galactosyl–hydroxylysine and glucosyl–galactosyl–hydroxylysine as their respective dabsyl derivatives [78]. Both compounds were converted into di-dabsyl

derivatives within 15 min at 70°C. After 20 min, recovery decreased, probably by detachment of the dabsyl label from the reaction products.

Analysis of amino sugars obtained after hydrolysis of synthetic glycopeptides was performed by Gorbics *et al.* [79]. HPLC conditions were optimized to separate the dabsyl derivatives of N-acetylglucosamine and N-acetylgalactosamine from the dabsyl amino acids.

3.2.7. 7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole

7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) was introduced by Imai and Watanabe as a fluorogenic reagent for both primary and secondary amines [80]. It is more reactive than the analogous 7-chloro compound (NBD-Cl) [81]. Derivatization is complete after 1 min at 60°C. The fluorescent derivatives can be detected at the fmol level using detection at 530 nm with excitation at 470 nm. Reversed-phase HPLC has been used for the separation of NBD derivatives of protein hydrolysate amino acids [82] and serum free amino acids [83]. A major drawback of the reagent is the light-sensitivity of the derivatives. Due to the rapid reaction kinetics and the fact that the reagent itself is non-fluorescent, it is also suitable for post-column derivatization of amino acids, as a sensitive alternative for ninhydrin [84].

3.2.8. Other reagents

Many other reagents for the labelling of the amino acid amino group have been developed. Activated carbamate reagents react rapidly with both primary and secondary amino acids [85–88]. The recently developed 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) looks promising [86]. It forms stable fluorescent urea derivatives that can be detected at the fmol level. The fluorescence emission maximum of 6-aminoquinoline, the major byproduct of the reaction, is shifted relative to the AQC amino acid derivatives, thus allowing selective detection of the AQC amino acids without disturbance by reagent related peaks. Tyrosine is the only amino acid to produce an unstable di-derivatized product. However heating at 50°C for 10 min suffices to convert all tyrosine to the stable mono-derivatized form.

Pre-column derivatization of primary and secondary amino acids with N,N-diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA) has been described by Fermo *et al.* [89,90]. Complete derivatization required heating at 100°C for 15 min. Under these conditions lysine was completely converted into the N,N-bis-derivative and tyrosine into the N,O-bis-derivative. Using spectrophotometric detection at 360 nm, the detection limit was in the low pmol range. FDNDEA derivatives were found to be very stable, even when stored in solution at room temperature in daylight. The closely related reagent 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) was synthesized by Marfey for the analysis of enantiomeric mixtures of amino acids [91]. FDAA was used by Kochhar and Christen for the analysis of amino acids [92]. Derivatization was performed at 40°C for 60 min. FDAA amino acid derivatives were detected at 340 or 414 nm. The authors mentioned that the molar ratio of FDAA to total amino acids should no exceed 3:1, because at a higher ratio chromatograms were disturbed by large peaks of excess hydrolysed reagent and reagent related compounds. FDAA derivatives were also found to be very stable.

1-Naphthylisocyanate was reported to give stable amino acid derivatives by Neidle *et al.* [93]. Reaction is completed within 1 min and excess reagent is removed by extraction with cyclohexane. The resulting naphthylcarbamoyl amino acids are highly fluorescent, with excitation maxima at 238 and 305 nm and an emission maximum at 385 nm. Alternatively, the derivatives can be detected photometrically at 222 nm.

Fluorescein isothiocyanate (FITC) labelling of amino acids has been used in combination with HPCE separation and laser-induced fluorescence detection to analyze amino acids with very high sensitivity [94,95].

The reagent 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA) was designed for the ultrasensitive determination of primary amines by HPCE in combination with laser-induced fluorescence detection [96,97]. The excitation wavelength of the fluorescent isoindole deriva-

tives conveniently matches the 442-nm output of a helium/cadmium laser. CBQCA was also shown to react readily with small peptides [96] and amino sugars [97]. See also Deyl and Struzinsky [98] for detailed references on the subject of HPCE separation of derivatized amino acids and peptides.

4. Applications

4.1. Hydroxyproline

Hydroxyproline (HYP) is formed by posttranslational enzymatic hydroxylation of proline. Two positional isomers of HYP exist, differing in the location of the hydroxyl group. 4-HYP is the most abundant form of HYP, occurring in all types of collagen. Formation of 4-HYP is catalyzed by prolyl-4-hydroxylase, an enzyme that requires ascorbic acid for maximal activity. Collagen is the main reservoir of 4-HYP, but minor amounts are also present in some other proteins, *e.g.* acetylcholinesterase, elastin, and complement factor C1q [99,100]. The positional isomer 3-HYP is present in small amounts in basement membrane collagen. HYP released upon degradation of collagen is partly metabolized, the remainder being excreted. The N-terminal collagen peptides, that are cleaved off during maturation of newly formed collagen, also contribute to excretion of HYP [101]. More than 95% of HYP is excreted in the form of small peptides, of which the dipeptide Pro-HYP is the major one. Urinary HYP levels mirror collagen metabolism. As bone is the main collagen reservoir of the body [102], HYP excretion reflects changes in bone metabolism during growth, menopause, and aging [103]. Measurement of HYP in urine is useful in several clinical conditions, *e.g.* osteoporosis [104], Paget's disease, secondary bone cancers [105], and some hereditary disorders.

Most of the older colorimetric methods for the determination of HYP are based on the oxidation of HYP to pyrrole followed by coupling with *p*-dimethylaminobenzaldehyde [106–110]. However, some compounds present in urine are known to interfere with these assays.

Although some publications describing the analysis of HYP by GC have appeared [111–113], most modern methods use HPLC as separation tool. Most of these methods use derivatization reagents and procedures that are also employed for the analysis of common amino acids. However, in methods designed for the analysis of a single amino acid, a different separation strategy is appropriate. The only requirement of the separation, is baseline resolution of the target amino acid from other components. The other amino acids do not have to be resolved from each other. This usually leads to separation methods with a short analysis time, compared with the time required for a complete amino acid analysis. A typical example of a strategy to analyze HYP in a short time using a conventional amino acid analysis method was described by Macek and Adam [114]. They used post-column ninhydrin detection in combination with reversed-phase HPLC. The mobile phase contained propanol as modifier and sodium dodecyl sulfate as ion-pairing agent. Under these conditions HYP eluted prior to all other amino acids. After elution of HYP, the other amino acids were rapidly removed from the column by backflushing. In this way total analysis time was only 10 min.

PITC has been used successfully using either isocratic [115], or gradient elution [116,117]. In none of these methods an internal standard was used.

Dabsyl chloride has been used by several groups. Pang *et al.* used isocratic elution with N-methyltaurine as internal standard [118]. The same internal standard was used by Casini *et al.* who employed gradient elution, resulting in a total analysis time of 20 min [119]. Turpeinen and Pomoell used cysteic acid as internal standard and separated the dabsyl derivatives by gradient elution [120,121]. Both plasma and hydrolysed urine samples were analyzed in this way. In the case of urine, glutamine could also be used as internal standard, because endogenous glutamine present in urine is converted to glutamic acid during hydrolysis [120].

FDNDEA was used by Paroni *et al.* for the determination of HYP in urine samples, with

cysteic acid as internal standard [122]. Separation was performed isocratically with detection at 360 nm. After elution of HYP, more hydrophobic amino acid derivatives were eluted by a step gradient, resulting in a run time of 18 min. The authors mentioned that complete and reproducible derivatization using this reagent requires careful control of pH. Using hydrolysed urine samples, neutralization or removal of hydrochloric acid is therefore a critical step.

NBD-chloride is a popular reagent for the derivatization of HYP. The derivatives are separated by TLC [123] or HPLC [124–129]. An advantage of this reagent is that reaction rates of secondary amino acids are one order of magnitude higher than those of primary amino acids [124]. By carefully choosing the reaction conditions, selective derivatization of secondary amino acids can thus be achieved. The fluorescence quantum efficiencies of the NBD derivatives decrease as the water content of the solvent increases [124]. This is a disadvantage when using reversed-phase conditions for HPLC separation. In fact NBD derivatives can be monitored by UV detection and fluorescence detection with approximately equal sensitivity. It has been shown that there is an approximate 25-nm difference between the absorbance maxima of primary and secondary amino acid derivatives. This fact has been exploited to further increase the selectivity of HYP detection [127], by using a detection wavelength of 495 nm. The problem of low fluorescence in aqueous mobile phase was solved by Welch *et al.* by using electrochemical detection [128]. It has been noted that the NBD derivative of HYP is sensitive to light and subject to hydrolysis under alkaline conditions [124]. When stored at 0°C, shielded from light, and at a pH between 3 and 9, derivatives were reported to be stable over a 24-h period [128].

Another powerful strategy used to increase selectivity of the analysis of HYP, involves the use of two separate derivatization reactions. In the first step primary amino acids are derivatized with a reagent that does not react with secondary amino acids. Next, the remaining secondary amino acids are labelled with a second reagent. Spectral properties of both kinds of derivatives should allow separate detection of primary and secondary amino acids. In this way the secondary amino acids including HYP can be selectively monitored. As the number of secondary amino acids in plasma and urine is rather small, the task of separating these compounds by HPLC is less demanding than a separation of both primary and secondary amino acids. Several combinations of reagents have been used for this purpose. Tsuchiya *et al.* used formaldehyde to block the amino group of primary amino acids and then derivatized the secondary amino acids with Dansyl chloride [130]. Using gradient elution both proline and HYP were determined in plasma without interference from other amino acids.

OPA is another reagent used for the selective derivatization of primary amino acids. It has been used in combination with NBD-chloride, using TLC [131] or reversed-phase HPLC [132,133] to separate the derivatives. Using excitation at 470 nm, the fluorescent NBD derivatives are detected at 530 nm, without disturbance from the OPA derivatives, that are not excited at this wavelength. Using isocratic elution, Palmerini *et al.* separated the derivatives of proline, HYP, and the internal standard 3,4-dehydroproline within 14 min, using either deproteinized plasma or hydrolysed urine samples [132]. These authors also used this method to analyze unhydrolysed urine samples [133]. Using gradient elution a large number of small peptides containing proline or HYP were separated.

OPA can also be used in conjunction with FMOC-Cl as first described by Einarsson [134]. First, the primary amino acids are derivatized with OPA. Before addition of the FMOC reagent, the excess of mercaptoethanol present in the OPA reagent is neutralized by addition of iodoacetic acid. All three reaction steps involved proceed almost instantaneously at room temperature. After the reaction with FMOC-Cl is completed, the excess reagent is removed by extraction with diethyl ether or pentane. Using isocratic elution and fluorescence detection with excitation at 260 nm and emission at 310 nm, the FMOC derivatives of secondary amino acids are separated without interference by OPA adducts of primary amino acids [134–136]. An advantage

of this method is that the FMOC derivatives are very stable and allow detection at the fmol level. The OPA-FMOC derivatization strategy is used in our laboratory for the routine determination of HYP in hydrolysed urine samples [136]. Figs. 1A and B show representative chromatograms of a HYP standard solution and hydrolysed urine, respectively. The internal standard 3,4-dehydroproline is added to the samples before the hydrolysis step. In this way losses due to evaporation during the hydrolysis are compensated for. Because pH is a critical parameter during derivatization, it is important to carefully neutralize the hydrolysates before derivatization. We circumvent this critical step by derivatizing a very small aliquot of the hydrolysates. Usually 10 μl of hydrolysate is added to 1.0 ml of a 0.8 *M* borate buffer. After derivatization with OPA, reaction with iodoacetic acid, and FMOC derivatization, samples are extracted with diethyl ether. Before chromatography, samples are diluted twofold with mobile phase. Although the 10-μl volume injected corresponds to only 0.014 μl of urine, peaks of HYP and internal standard are easily detected. The standard in Fig. 1A corresponds to 1.4 pmol of HYP injected on column. Fig. 1C shows a chromatogram of the same urine sample as shown in Fig. 1B. OPA derivatization of primary amino acids was omitted in this case, so that both primary and secondary amino acids were converted to FMOC derivatives. Comparison of Figs. 1B and 1C reveals the tremendous increase in selectivity that is obtained by this double derivatization strategy.

4.2. Methylhistidine

Actin and myosin are the two most abundant proteins in the contractile elements of muscle. After protein synthesis, specific histidine residues of both proteins are methylated to 3-methylhistidine (3MH) [137]. After degradation of these proteins, 3MH released cannot be reutilized for protein synthesis as a specific transfer-RNA for 3MH is lacking. Furthermore, it has been shown that orally or parenterally administered radiolabelled 3MH is quantitatively

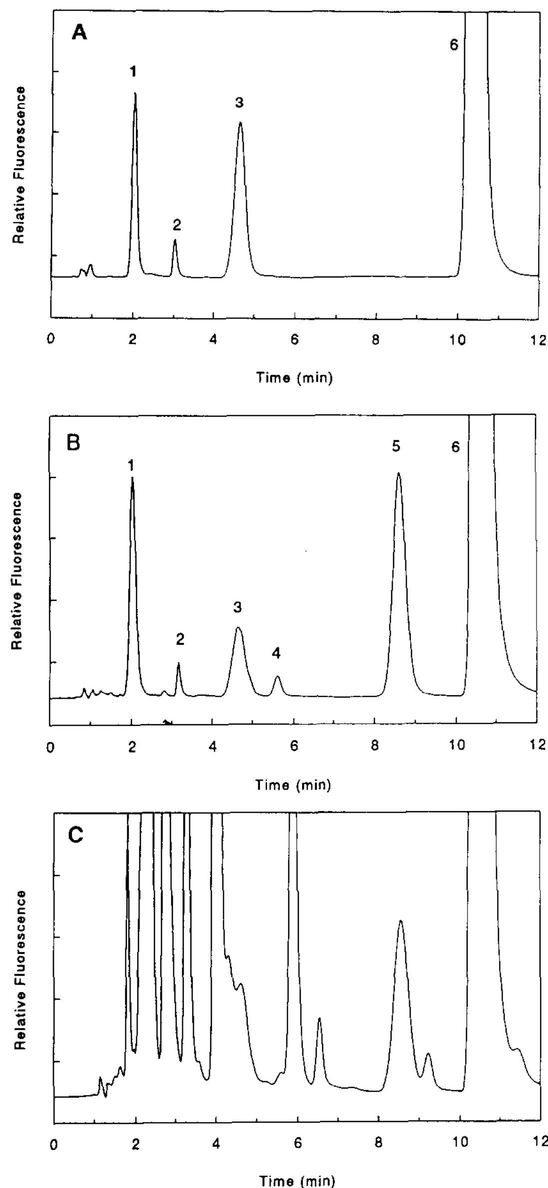

Fig. 1. Separation of FMOC derivatives of secondary amino acids after derivatization of primary amino acids with OPA. (A) Hydroxyproline standard solution (1.35 pmol injected). (B) Hydrolysed urine sample. (C) Same urine sample as shown in B, but OPA derivatization step omitted. Column: C_{18} (10 cm × 4.6 mm I.D., 3-μm particles) Mobile phase: 50 m*M* acetate (pH 4.3)–acetonitrile (66:34, v/v). Fluorometric detection (excitation: 260 nm, emission: 320 nm). Peaks: 1 = hydroxyproline; 2 = reagent related peak; 3 = dehydroproline (internal standard); 4 = N-methylglycine; 5 = proline; 6 = FMOC-OH. Derivatization conditions as described in ref. [136].

recovered in urine and faeces [137]. Therefore, it can be concluded that 3MH is hardly metabolized. Urinary 3MH originates mainly from skeletal muscle, with minor contributions from the skin and gastrointestinal tract. Therefore, urinary 3MH is regarded as a valid marker for myofibrillar protein catabolism [138]. Measurement of 3MH excretion is a useful parameter in some clinical conditions, *e.g.* sepsis, skeletal trauma and severe malnutrition [139,140]. Whereas measurement of 3MH in urine reflects whole body muscle protein turnover, it is also possible to study the protein metabolism of a single organ by measuring arterio–venous differences in 3MH plasma concentration. Therefore, urine and plasma are the two biological fluids most frequently studied. Each of these fluids poses its own problems from an analytical point of view. Due to the efficient excretion of 3MH, plasma levels are much lower than urine levels. On the other hand deproteinized plasma is a much "cleaner" matrix then urine. Therefore, the determination of 3MH in plasma presents a challenge in terms of sensitivity of the analytical method, whereas urine samples demand a high degree of selectivity. Another aspect of urine samples, of importance in the analysis of urine of experimental animals, is that in some species, *e.g.* rat, 3MH is excreted as its N-acetyl derivative. This means that 3MH in such samples can be measured only after acid hydrolysis of the urine. In human urine the N-acetyl derivative is only a minor fraction of total 3MH. Therefore, hydrolysis of human urine samples is not necessary.

Several analytical approaches to measure 3MH in biological fluids have been used, including thin-layer chromatography, GC, and HPLC. A number of papers describing the determination of 3MH by GC have been published [141–147]. In most cases derivatization steps include esterification of the carboxyl group followed by N-acylation of the amino group. In general, derivatization reactions required for GC are rather time consuming.

A large number of methods of 3MH analysis by liquid chromatography have been described in the literature. Some methods are based on ion-exchange chromatography. Detection is based on UV absorption at 210 nm [148], or post-column derivatization with ninhydrin [149–151]. Although ion-exchange chromatography often requires run times of several hours, the resolving power of this separation technique is very high. Zarkadas *et al.* described the separation of a large number of modified basic amino acids, including 1MH, 3MH, 5-hydroxylysine, several methylated lysine species, and the lysine-derived crosslinks of elastin, desmosine and isodesmosine [149].

OPA is also used for post-column derivatization of 3MH. Derivatives are detected fluorometrically with high sensitivity [152–155].

Ward described the use of a combined OPA-ninhydrin reagent [156]. This reagent was shown to react readily with 3MH under alkaline conditions yielding an adduct with maximal absorbance at 490 nm [156,157]. The reagent is semi-specific for 3MH. Glutamine, histidine, arginine, and ammonia were the only other amino compounds reacting with the OPA-ninhydrin reagent. All other amino acids tested, including 1MH, gave colour yields of less than 5 percent of 3MH. This reagent has been used for post-column derivatization in combination with ion-exchange chromatography [156,158] as well as for pre-column derivatization in combination with reversed-phase HPLC [159].

Several methods using pre-column derivatization with OPA have been described [160–163]. All methods separate the fluorescent derivatives by reversed-phase chromatography using either isocratic [163] or gradient elution [160–162]. Reaction of OPA with amino acids requires the presence of a thiol compound. Usually 2-mercaptoethanol is used for this purpose, but some authors prefer the use of MPA [161,162], because it yields more stable isoindole derivatives. Figs. 2A and B show chromatograms of OPA-MPA derivatives of a standard amino acid mixture and a plasma sample, respectively. Complete separation was achieved in 12 min, and total analysis time, including automated derivatization and reequilibration of the column amounted to 17 min [55]. A detail of the chromatogram of the plasma sample is shown in Fig.

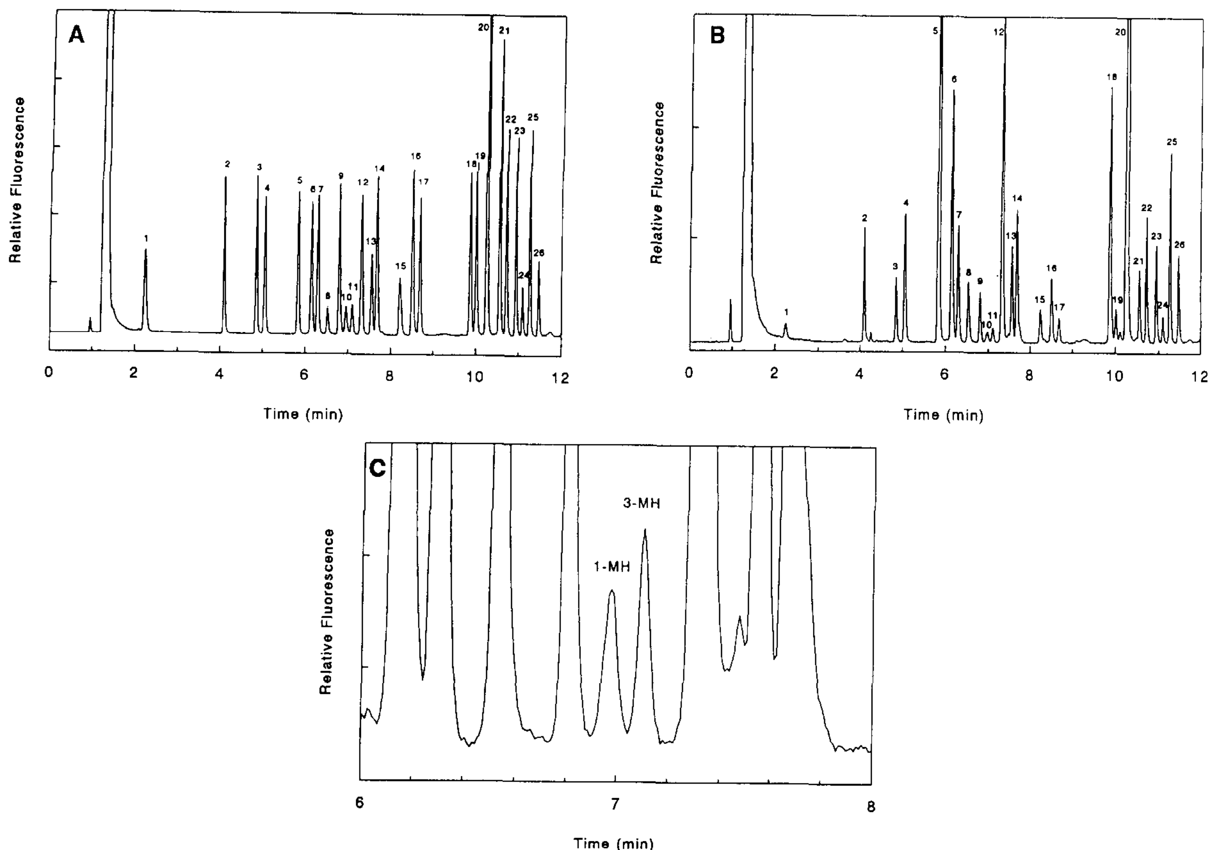

Fig. 2. Chromatographic profile of amino acid OPA derivatives. (A) Amino acid standard mixture. (B) Plasma after deproteinization with sulfosalicylic acid. (C) Detail of trace B, illustrating separation of methylhistidines. Column: C_{18} (10 cm × 4.6 mm I.D., 3-μm particles). Buffer used for the preparation of the mobile phases consisted of 9 mmol/l potassium dihydrogenphosphate and 0.05% (v/v) triethylamine, adjusted to pH 6.9 with KOH. Mobile phase A was prepared by mixing 1000 ml buffer, 1000 ml water and 4 ml tetrahydrofuran. Mobile phase B consisted of buffer–methanol–acetonitrile (50:35:15, v/v/v). Gradient conditions: 2–25% B from 0–3.5 min, 25–44% B from 3.5–5.2 min, 44–52% B from 5.2–6.9 min, 52–100% B from 6.9–10.0 min, then 100–2% B from 10.0–11.0 min. Flow-rate was 1.5 ml/min. Fluorometric detection (excitation: 230 nm, emission: 389 nm cut-off filter). Automated derivatization as described in ref. [55]. Peaks: 1 = aspartic acid; 2 = glutamic acid; 3 = asparagine; 4 = serine; 5 = glutamine; 6 = glycine; 7 = threonine; 8 = histidine; 9 = citrulline; 10 = 1-methylhistidine; 11 = 3-methylhistidine; 12 = alanine; 13 = taurine; 14 = arginine; 15 = reagent related peak; 16 = tyrosine; 17 = α-aminobutyric acid; 18 = valine; 19 = methionine; 20 = norvaline (internal standard); 21 = tryptophan; 22 = phenylalanine; 23 = isoleucine; 24 = ornithine; 25 = leucine; 26 = lysine.

2C to demonstrate the resolution between 1MH, 3MH and surrounding amino acids. The method can only be used for the analysis of plasma samples, as in urine samples 3MH is not completely resolved from other components.

Other reagents used for the pre-column derivatization of 3MH include NBD-Cl [164], PITC [165], and FMOC-Cl [166]. An example of the analysis of FMOC derivatives of 3MH using isocratic elution is shown in Fig. 3.

A derivatization reagent with very interesting properties is fluorescamine. Fluorescamine is a non-fluorescent reagent that reacts with primary amines, forming intensely fluorescent products. The reaction proceeds efficiently at room temperature in aqueous solutions at alkaline pH.

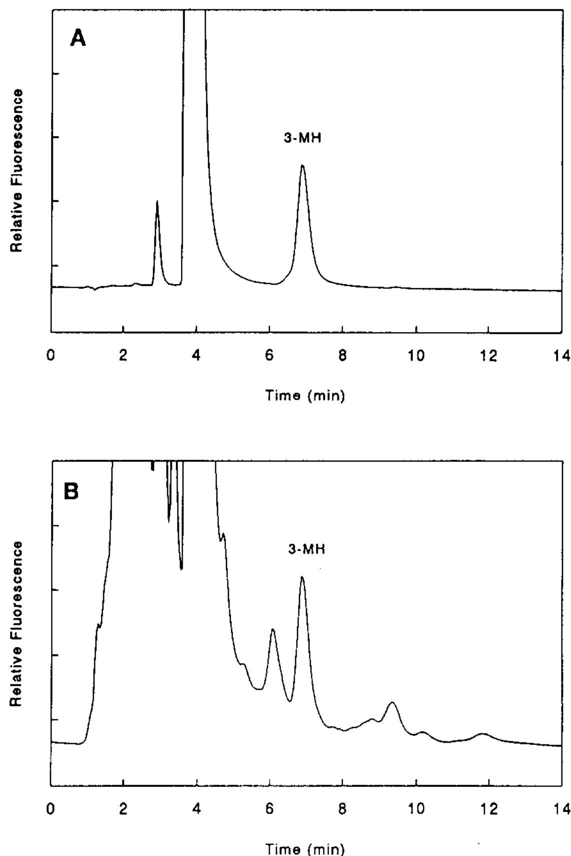

Fig. 3. Chromatographic separation of FMOC derivative of 3-methylhistidine. (A) 3-methylhistidine standard solution. (B) urine sample. Column: Spherisorb ODS (20 cm × 3.0 mm I.D., 5-μm particles). Mobile phase: 30 m*M* glycine (pH 3.0)–acetonitrile (50:50, v/v). Fluorometric detection (excitation: 260 nm, emission: 320 nm). Derivatization and separation conditions as described in ref. [166].

Some fluorescamine derivatives have unusual properties [167–169]. It has been shown that upon heating in acid, fluorescamine derivatives of histidine, histamine, 3MH, and peptides containing N-terminal histidine become intensely fluorescent, whereas the fluorescence of other fluorescamine labelled amino compounds disappears [167]. It was shown that an unsubstituted α-amino group and an unsubstituted nitrogen in the 1-position of the imidazole ring are structural requirements for this reaction. Therefore, 1MH, containing a methylated nitrogen at the 1-position, does not show any fluorescence after heating in acid. Several methods for the analysis of 3MH in urine and plasma employing this derivatization scheme have been published [170–172]. After reaction with fluorescamine at alkaline pH for a few minutes at room temperature, perchloric acid is added to the samples, and samples are heated at 80°C for 60 min. Derivatives can be separated by isocratic reversed-phase HPLC with either UV detection at 254 or 365 nm, or fluorescence detection at 460 nm using an excitation wavelength of 365 nm [172]. Due to the selectivity of the derivatization reaction, very clean chromatograms are obtained, with 3MH and histidine as major peaks. As can be seen in Fig. 4, resolution of the fluorescamine derivatives of 3MH and histidine is very good, both in urine and deproteinized plasma samples. Using a 10-cm column and a relatively high flow-rate the total analysis time is 5 min, using isocratic elution. Although the run time can be decreased by increasing the percentage of acetonitrile in the mobile phase, this results in a dramatic decrease in resolution. At 27% acetonitrile, 3MH and histidine are eluted as a single peak (Fig. 4D). Therefore, as a method used in daily practice over a period of several years should be robust, we prefer to use 23% acetonitrile. In this way acceptable chromatograms are obtained even if column efficiency decreases after the analysis of several hundreds of samples. The derivatization is performed in an autosampler vial with teflon-lined screwcap, to avoid losses due to evaporation during the incubation at 80°C. After the incubation, vials are cooled and transferred to the autosampler. Although no internal standard is used in the procedure, the between-run coefficient of variation is only 4.2%. The effect of heating the samples in the presence of perchloric acid is demonstrated in Fig. 5.

4.3 Phosphorylated amino acids

As mentioned in section 2.1. a large number of cellular processes are regulated by reversible protein phosphorylation. Serine, threonine and to a lesser extent tyrosine are the amino acids

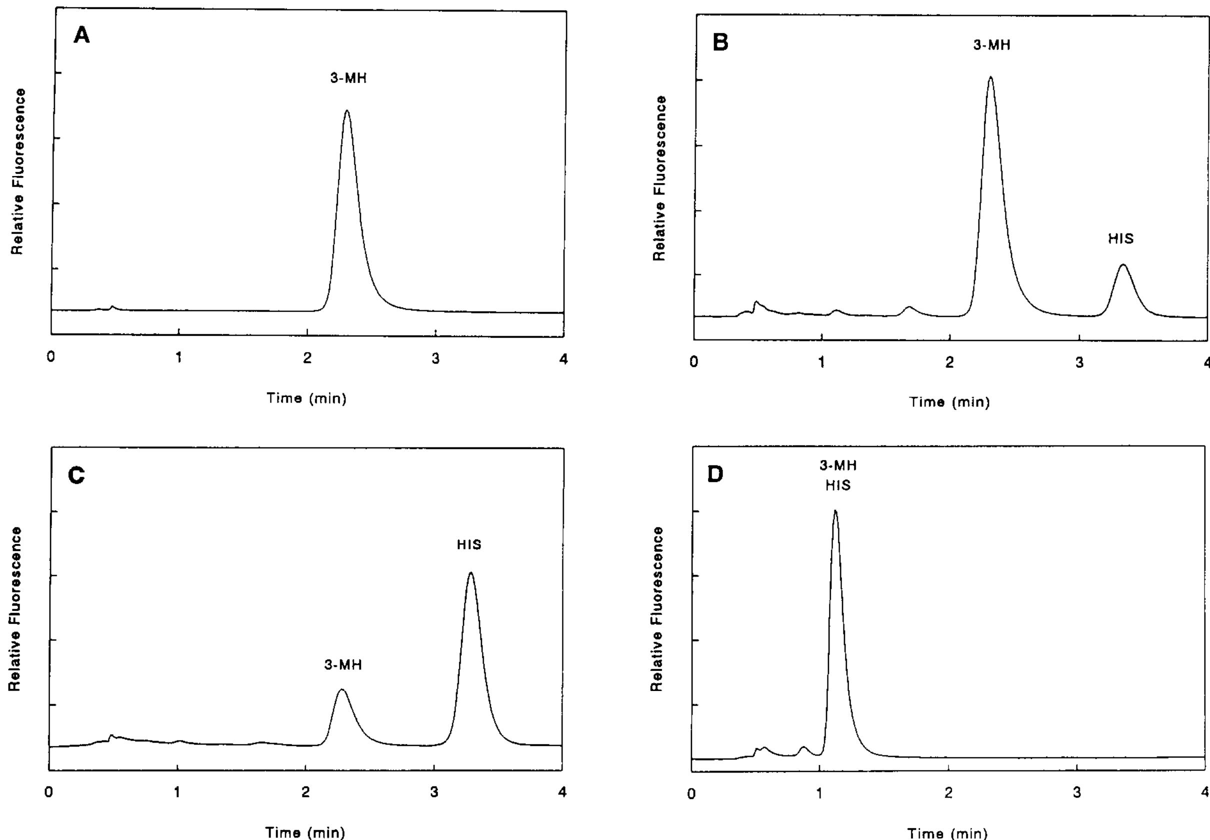

Fig. 4. Separation of fluorescamine derivatives of 3-methylhistidine. (A) 3-Methylhistidine standard solution (16.6 pmol injected). (B) Urine sample. (C) Plasma sample, derivatized after deproteinization with perchloric acid. (D) Separation of urine sample, using mobile phase containing 27% acetonitrile. Column: ODS Hypersil (10 cm × 3.0 mm I.D., 5-μm particles). Mobile phase: 20 m*M* sodium phosphate (pH 7.2)–acetonitrile (77:23, v/v). Flow-rate: 1.0 ml/min. Fluorometric detection (excitation: 367 nm, emission: 460 nm). Derivatization conditions: 0.2 ml 1:10 diluted standard or sample was mixed with 1.0 ml of 0.25 *M* borate (pH 9.5) and 1.0 ml of fluorescamine in acetonitrile (1 mg/ml); after 5 min at room temperature, 0.2 ml 70% perchloric acid was added and samples were heated at 80°C for 60 min; after cooling 10 μl were injected.

most frequently phosphorylated by formation of an phosphoester bond. Other, less frequently phosphorylated amino acids, include histidine, lysine and arginine. In these amino acids phosphorus is bound to a nitrogen atom by a phosphoramidate bond.

The phosphoesters of serine, threonine and tyrosine have a reasonable chemical stability under acid conditions, and usually limited acid hydrolysis is used for their analysis. A typical hydrolysis procedure involves heating at 110°C for 3 h in 6 *M* HCl. Phosphothreonine is the most resistant to acid hydrolysis and phosphotyrosine the least. Therefore, for the measurement of phosphotyrosine shorter hydrolysis times are usually recommended. An important problem with the use of short hydrolysis protocols is that due to incomplete hydrolysis the reaction mixture will contain a considerable amount of small peptides, that may interfere with the chromatographic analysis. Recoveries are to some extent dependent on the primary structure of the polypeptide surrounding the phosphorylation site. Malencik *et al.* have reported improved yields by performing a tryptic digestion prior to partial acid hydrolysis [173].

Alternatively, phosphoproteins can be analyzed after base hydrolysis with potassium hy-

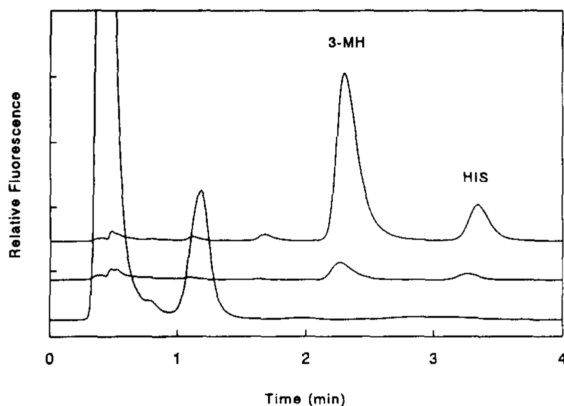

Fig. 5. Effect of addition of perchloric acid and heating on fluorescamine derivatives of 3-methylhistidine and histidine. Upper trace: urine sample; normal derivatization procedure as in Fig. 4. Middle trace: after incubation of the urine sample with fluorescamine, perchloric acid was added, but the sample was not heated. Lower trace: urine sample was injected immediately after incubation with fluorescamine, without acid treatment. See legend of Fig. 4 for chromatographic conditions.

droxide. Phosphotyrosine is almost completely recovered after 24 h hydrolysis with 2 *M* KOH at 105°C [174] or after 1 h with 5 *M* KOH at 155°C [44]. Phosphoserine and phosphothreonine are less resistant to base hydrolysis. Another option to liberate phosphoamino acids from phosphorylated proteins is enzymatic digestion with a combination of non-specific proteases and aminopeptidase. High recoveries of several phosphorylated and sulfated amino acids have been reported using this approach [174].

A contaminant which can cause problems in the analysis of phosphoamino acids is serine O-sulfate. Serine O-sulfate may be generated during acid hydrolysis of proteins in the presence of sulfate. Especially when the protein sample has been in contact with sodium dodecyl sulfate, one should be alert for the presence of serine O-sulfate.

In contrast to the amino acids containing a phosphorylated hydroxyl group, phosphorylated basic amino acids are extremely labile under acidic conditions. In these amino acids, *e.g.* N-phospholysine, N-phosphoarginine or N-phosphohistidine, phosphorus is covalently linked to a nitrogen atom by a phosphoramidate bond. These amino acids can only be recovered after base hydrolysis or enzymatic digestion, and chromatographic separation should be performed at near neutral or slightly alkaline pH [175].

Since protein hydrolysates usually contain low levels of phosphoamino acids in the presence of a very large excess of nonphosphorylated amino acids, it may be desirable to perform a selective enrichment step before the actual chromatographic analysis. Enrichment can be achieved by chromatography of the hydrolysate on a small column of Dowex 50W-X8. Phosphoamino acids are rapidly eluted with 0.1 *M* formic acid, whereas unphosphorylated amino acids are almost completely retained by the column [176].

Separation of underivatized phosphoamino acids can be performed by ion-exchange chromatography using detection by ultraviolet absorption at 210 nm [177]. Morrice and Aitken used indirect UV detection at 243 nm using ion-pairing reversed-phase chromatography with tetrabutylammonium hydroxide as ion-pairing agent and phthalic acid as UV active agent in the mobile phase [178]. Using post-column OPA derivatization in combination with fluorescence detection, detection limits of 10 to 50 pmol can be attained [174,175,179,180].

Several reagents for the precolumn derivatization of phosphoamino acids have been successfully employed. Calomagno *et al.* described the complete separation of the OPA derivatives of phosphoserine, phosphotyrosine, phosphothreonine, phosphoarginine, 1-phosphohistidine, and 3-phosphohistidine [181]. Isocratic reversed-phase separation was performed on a polymer-based column at pH 7.2. 1-Phosphohistidine was not retained under these conditions and the derivatives of aspartate and glutamate interfered with the analysis of phosphoserine and phosphotyrosine, respectively.

Simultaneous analysis of both phosphorylated and nonphosphorylated amino acids as their respective DABSYL derivatives has been described by Chang [182] and Malencik *et al.* [173]. The latter group used a mobile phase of pH 8.1 in combination with highly substituted C_{18} columns (carbon loads of 22 and 31%). In contrast to columns with a lower carbon loading, these

columns could be operated at this high pH without rapid deterioration. In this way excellent resolution of the dabsyl derivatives of phosphoserine, phosphothreonine, phosphotyrosine, and 17 other amino acids present in protein hydrolysates was obtained, with pmol sensitivity (Fig. 6).

Murthy and Iqbal described the analysis of phosphoamino acids as their PITC derivatives by reversed-phase HPLC [183]. The authors em-

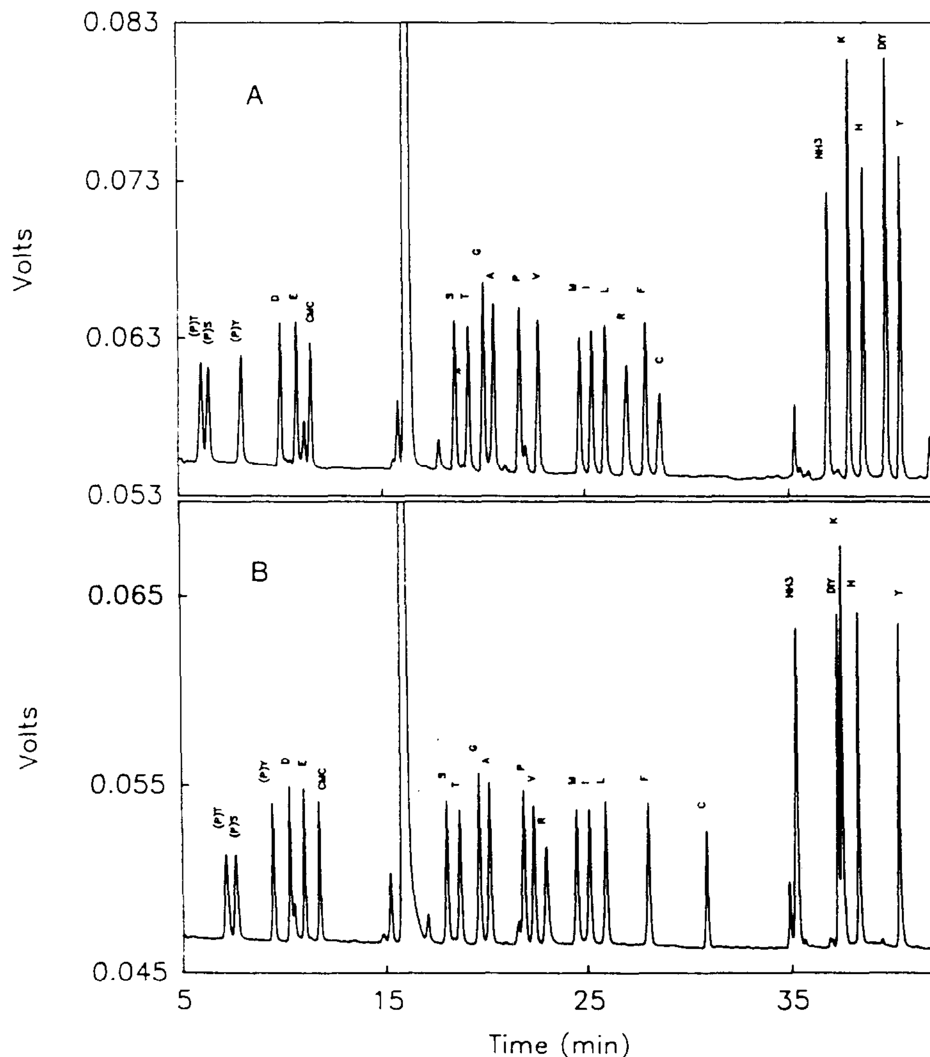

Fig. 6. Separation of dabsyl amino acid standards (60 pmol each except for 30 pmol cystine and 120 pmol phosphotyrosine) by reversed-phase HPLC after a 2-h hydrolysis at 110°C. (A) Results obtained with a Phenomenex Ultracarb 30 column (250 × 4.6 mm I.D.). (B) Results obtained with a Phenomenex Ultracarb 20 column (250 × 4.6 mm I.D.). Solvents: A, sodium bicarbonate (20 mM, pH 8.1) containing 4% dimethylformamide. B, acetonitrile–2-propanol (90:10, v/v). Gradient conditions: 18–25% solvent B from 0–6 min, 25–40% solvent B from 6–26 min, 40–70% solvent B from 26–41 min, 70% solvent B from 41–43 min, then 70–18% solvent B from 43–44 min. Flow-rate was 1 ml/min. Column temperature was 40°C. Detection at 460 nm. Standard one-letter abbreviations for amino acids except for (P)T = phosphothreonine, (P)S = phosphoserine, (P)Y = phosphotyrosine, CMC = carboxymethylcysteine, and DIY = dityrosine. (from ref. [173] with permission).

ployed the Waters Pico-Tag HPLC amino acid analysis system and adapted the separation conditions to allow the resolution of pmol levels of phosphoamino acids in the presence of a large excess of nonphosphorylated amino acids. Chromatography was carried out at pH 7.4 and 30°C using a complex gradient. Eluent A consisted of 157 m*M* sodium acetate containing 2% acetonitrile and eluent B was 60% acetonitrile in water. Fig. 7 illustrates the separation of phosphoserine, phosphothreonine and phosphotyrosine (10 pmol each) in the presence of a large excess of aspartate, glutamate, and other nonphosphorylated amino acids (882 pmol each).

Niebalski and Ringer separated FMOC derivatives of phosphoamino acids by anion-exchange chromatography using isocratic elution [44,176]. Phosphotyrosine, phosphothreonine, and phosphoserine were eluted with baseline resolution and detected fluorometrically with pmol sensitivity. Nonphosphorylated amino acid derivatives were hardly retained under these conditions, and eluted as a single peak, well resolved from the derivatives of the phosphoamino acids (Fig. 8).

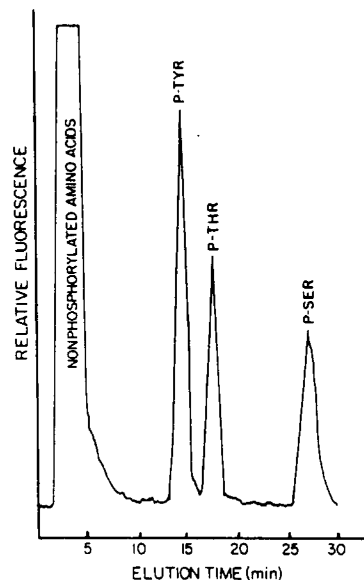

Fig. 8. HPLC separation of FMOC derivatives of phosphoserine, phosphothreonine, and phosphotyrosine (80 pmol each) on a Partisil 10 SAX column at a flow-rate of 1.5 ml/min with a buffer containing 55% methanol, 1% tetrahydrofuran, and 10 m*M* potassium phosphate, pH 3.9. (from ref. [44] with permission).

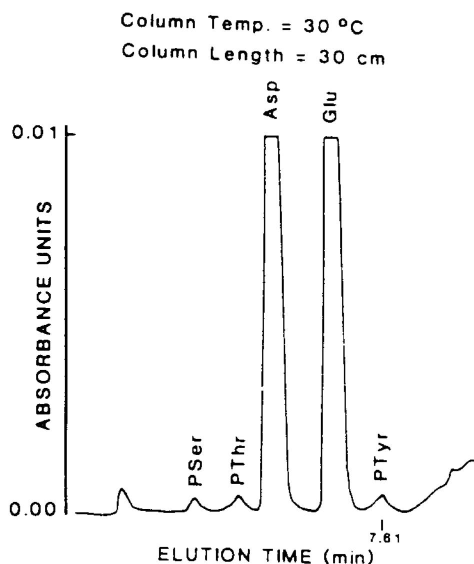

Fig. 7. HPLC separation of PITC derivatives of 10 pmol of each phosphoamino acid in the presence of 882 pmol of each nonphosphorylated amino acid. Column length, 30 cm; temperature, 30°C. Detection at 254 nm; Retention times: PSer, 3.90 min; PThr, 4.76 min; Asp, 5.40 min; Glu, 6.58 min; PTyr, 7.61 min. (from ref. [183] with permission).

5. Conclusions

From an analytical point of view, posttranslationally modified amino acids are not very different from unmodified amino acids. Most of the analytical methods developed for the determination of amino acids in protein hydrolysates and biological fluids, can also be applied to the determination of modified amino acids. All amino acids, whether modified or not, have an amino group that is used as a handle in most analytical procedures. In the unprotonated form, the amino group can easily react with a large number of reagents. Many reagents have been developed for this purpose, yielding amino acid derivatives with properties that make them easily

detectable by UV absorbance, fluorescence detection or electrochemical detection.

By the process of derivatization, the polar amino acids are converted into more hydrophobic products, that can be separated by reversed-phase HPLC. Although classical amino acid analysis, based on the separation of underivatized amino acids by ion-exchange chromatography, followed by post-column derivatization prior to detection, is unsurpassed in terms of resolving power, reversed-phase HPLC of pre-column derivatized amino acids has gained popularity due to its very high sensitivity and the relatively short analysis times that can be attained.

The main problem in amino acid analysis is to obtain adequate resolution of a large number of amino acids, *i.e.* 20 in the case of protein hydrolysates and 40 or more in the case of free amino acids in biological fluids. Regarding the large number of parameters that can be varied, *e.g.* type of buffer, ionic strength, pH, organic solvent, additional modifiers, gradient shape *etc.*, complete optimization may be considered a formidable task.

When trying to set up an analytical procedure for the determination of a particular modified amino acid, several options are available.

(1) One can choose one of the amino acid derivatization reagents and then attempt to separate the derivative from other amino acid derivatives present in the particular sample matrix. The only requirement is that the amino acid of interest is separated from other amino acids and from reagent peaks. This task is much easier to accomplish than a complete separation of all components. Often this can be achieved by isocratic elution. After elution of the target analyte, other derivatives can be rapidly eluted using a step gradient or by backflushing. Alternatively, a simple linear gradient can be used.

(2) Another possibility is to increase selectivity by using a combination of derivatization reactions. An example was given in the section on the analysis of the secondary amino acid HYP. In this example, primary amino acids were first reacted with OPA, followed by derivatization of secondary amino acids by either NBD or FMOC. In this way secondary amino acids can be selectively detected, which makes the development of a chromatographic separation a much easier task.

(3) A third option is to exploit the chemical properties of the particular modified amino acid. An example of this strategy was given in the section on 3MH. Fluorescamine rapidly reacts with all amines in alkaline medium. Upon heating in acid, most derivatives decompose, except for amines containing an imidazole ring, like histidine and 3MH. The latter derivatives are rearranged into stable, highly fluorescent products. After derivatization of urine or deproteinized plasma, 3MH and histidine are the major fluorescent products, that are easily separated in a short time using isocratic conditions.

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