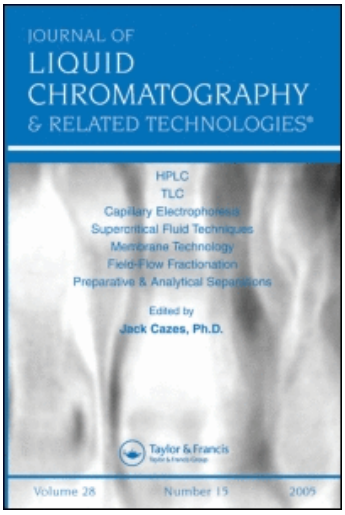

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TLC of Phenylthiohydantoins of Amino Acids: A Review

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TLC OF PHENYLTHIOHYDANTOINS OF AMINO ACIDS: A REVIEW

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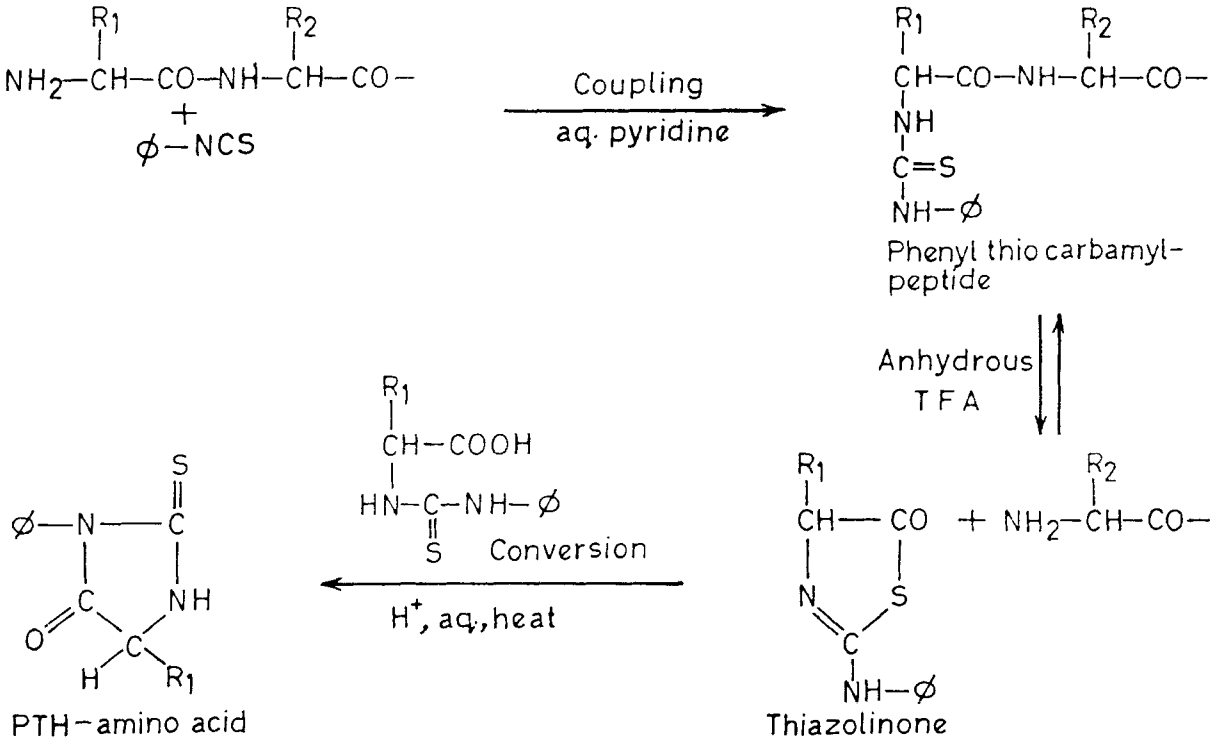
INTRODUCTION

Proteins are among the most important components of all living systems. Their function range from catalysts (enzymes) to regulators to structural components. The building blocks and language of proteins are about 20 amino acids (H_2N CHR COOH), linked together by peptide bonds (-CO-NH-) in chains that may consist of a few dozen to more than 1000 amino acids. The determination of primary structure of proteins, namely the sequence (arrangement) of the various amino acids along the chain is still a challenging task. Edman reaction lies virtually at the core of all modern sequencing

strategies [1,2]. The N-terminal polypeptide is first coupled to phenyl isothiocyanate to form the phenylthio carbamyl peptide; this derivative is then cleaved with anhydrous acid to expose a new N-terminus and to release the original N-terminal amino acid as a 5'-thiazolinone (Scheme-1). The excess reagents and by products are extracted by an organic solvent wash. The extract of thiazolinone amino acid (obtained either from liquid-phase or solid-phase degradation) is evaporated and converted to the phenylthiohydantion derivative by 5 N HCl/CH_3COOH (1:2 v/v) at 52°C for 50 min. The sample is extracted with ethyl acetate, dried and redissolved in a suitable volume of ethanol for TLC identification. Repetition of this process with identification of the released PTH-amino acids enables the determination of sequence of amino acids from the N-terminal end. For smaller peptides PITC may be used to remove the amino terminal amino acid while a chromophore or fluorophore such as dansyl chloride or DABITC, which reacts with the newly exposed amino terminus, is used to identify the new amino terminus. Both manual and automated methodologies are currently used for small and large polypeptides which rely upon identification of amino terminal amino acid as PTH derivative. A large number of papers have been and continue to be published on the analysis of the PTH derivatives of amino acids.



Scheme 1: Formation of phenyl thio hydantoin amino acid.

Though five different techniques may be considered to reflect the evolution and impact of instrumentation development on methods of PTH analysis, the simplest, cheapest and sufficiently sensitive method is undoubtedly TLC which was used by Edman and Begg [2] in their classical publication describing the automatic sequencer. This method is suitable for both manual and automated techniques but suffers from the disadvantage that the analyses are qualitative rather than quantitative and analysis is limited to the n-mol range. However, the method has advantage that several samples can be analysed at the same time. It is possible to semi quantify TLC analysis by elution of spots from the chromatographic plates. TLC analysis needs to be supplemented with a method such as UV or back hydrolysis, or HPLC for the PTH derivative of arginine and histidine. The third method involves GLC which was introduced into the field of sequence analysis in the mid-1960s. Rapid and quantitative determination of most, but not all of the PTH amino acids can be made by GLC and it is this failure to give a complete analysis which has proved its major drawback. Besides, derivatization, for example trimethylsilyation, is also required for improving the gas chromatographic behaviour of most of the PTH-amino acids [3]. The fourth

method uses HPLC which was introduced in mid 1970s which was found to be rapid, sensitive, quantitative and capable of identifying all the PTH amino acids at pmol level. The fifth analytical technique involves mass spectrometry [4] which is used by only a few laboratories in view of the requirement of complex and costly instrumentation. Besides, several other techniques have also been used for the determination of PTH-amino acids, for example Oscillopolarography [6], proton magnetic resonance spectroscopy [7], and electrokinetic chromatography [8] which is based on miscellar solubilisation and electrokinetic migration in an open tubular capillary under varying conditions of voltage and SDS concentration.

The choice of analytical method is clearly related to cost, the availability of instrumentation and to the sensitivity needed for the analysis. Nevertheless, TLC can be considered to have several advantages over othr methods of chromatography because it is sensitive, inexpensive and simpler; allows densitometric scanning for quantitation; sample and standards can be run in parallel under identical conditions on the same plate; allows formation of a derivative without resorting to extensive purification and recrystallization; the components of the mixture can be eluted and used subsequently and

allows easier detection of colourless compounds. The inert character of the thin layer material makes it ideally suited for use with stronger corrosive reagents and perform several kinds of reactions on the plate. The great advantages of TLC are the wide variety of development techniques allowing separation of complex mixtures and numerous detection procedures are possible because of the static nature of the detection process. Detection levels are comparable for HPTLC and TLC and typical coefficients of variation for HPTLC analysis are 2-5% for the entire procedure and $\leqslant$ 1% for standards. The inherent accuracy of TLC may be better than for the column methods because standards are run directly in parallel with samples [6]. Some aspects of TLC that still are not appreciated adequately by the majority of people using chromatography were outlined by R.E.Kaiser in an editorial in the March 1984 issue of HRC & CC. These include unrestricted access to the separation process, introducing magnetic, thermal, electrical and other physical forces to improve resolution; high sample throughout; truly multidimensional separations; and the use of controlled multiple gradients. The combination of robotics, with the continued development of theory,practice, and instrumentation will lead eventually to TLC systems that are unrivalled for speed,versatility,accuracy, precision and sensitivity.

In view of above advantages and high scope of TLC and importance of PTH amino acid in protein chemistry a thorough literature search towards the TLC studies of PTH-amino acids was made through chemical abstracts after 1972 and the general TLC reviews [6-8]. Almost all the references thus noted were obtained by xeroxing the papers from journals. Moreover, the current work on TLC of PTH-amino acids in the author's laboratory [9-15] prompted to undertake this review.

Various TLC systems with different kinds of adsorbents like alumina, silica gel, and polyamide etc. have been reported in the literature followed by new and improved methods.

TLC ON POLYAMIDE PLATES

Kulbe [16] reported a successful and rapid method for two dimensional separation of 24 PTH-aminoacids on polyamide glass plates available commercially for ready to use. The solvent systems used were as follows:

Toluene-n-pentane-glacial acetic acid(60:30:35,v/v);
Ethylenechloride-glacial acetic acid(90:16, v/v);
Toluene-n-pentane-glacial acetic acid(60:30:20,v/v);
Carbontetrachloride-glacial acetic acid(80:30,v/v);
35% acetic acid; and 30% acetic acid. He was also able to separate Glu/Asp, Gly/N-phenyl-N-Phe, Ser/Thr and Leu/Ile which were earlier reported

inseparable by Pataki [17] and Cherbuliez et al. [18,19]. Though he could not separate PTH-Tyr/Cys his methods were better than those reported by Wang et al. [20] on similar plates where the polyamide plates were unstable in strong acids. However, the sensitivity of this method using commercially prepared polyamide coated glass plates and iodine-sodium azide spray reagent dictated a lower limit of detection of 5-10 n moles of the PTH derivative. Summers et al. [21] extended this technique to polyamide sheets with a fluorescent indicator to that used by Edman and Begg [2], and they found that it was possible to detect as little as 0.05 n moles of material, without resorting to a visualization spray of the chromatogram developed in toluene-n pentane-acetic acid (60:30:35). Further they used polyamide sheets coated on both sides for the comparison with standard mixtures. The method of Summers et al. [21] was used by Hopp [69] who separated PTH derivatives of Arg, His and S-S pyridyl ethyl cysteine from contaminants by TLC on polyamide sheets using 10% aqueous pyridine as solvent; development of chromatogram in 35% aqueous acetic acid for the second dimension containing a fluorescent indicator, resulted into the identification of these PTH aminoacids in sub nano mole amounts, and 16 of the 20 PTH-aminoacids were resolved. Karl [22] detected PTH-amino acids by HPTLC and on polyamide

sheets using a scanner to measure the diffused reflected light from the plate, and claimed his TLC results were comparable to HPTLC and were obtained in less time. Moreover the sensitivity obtained on polyamide was two-fold than that obtained by HPTLC. Kulbe [23,24] reported several sensitive and rapid multi sample identification of PTH and MTH aminoacids. The most significant of these was [25] separation of phenyl and methyl thiohydantoins of arginine, cysteic acid and histidine on 5x5 cm polyamide sheets using ascending TLC and about 1µl of the sample. The chromatograms were developed for 10 mins in the solvents ethyl acetate-n butanol-glacial acetic acid (35:10:1) and ethyl acetate-tert-butanol-acetic acid (35:10:1) containing 250 mg/l of the fluorescent indicator and visualization was made under short wave UV. The method [25] was important because histidine and arginine derivatives were easily detected. The quantitation of micro scale was possible which was otherwise very laborious and difficult when carried out by back hydrolysis to the free amino acids followed by their deteremination in aminoacid analyzer. Bose and Brewer [26] reported an improved system for the separation and identification of PTH-histidine and PTH-arginine by TLC on poly-amide sheets using the solvent system n-butylacetate-methanol-aceticacid (29:20:1) containing

30 mg of butyl-PBD fluorescent reagent per liter. The method was reported to be superior to that by Kulbe [23] and Summers [21]. GLC, electron impact and CI-Massspectroscopy have also been extremely useful for the identification of PTH derivatives [27-29] but are less successful than these TLC methods [25,26] particularly for PTH-Arg and His because these derivatives cause problems in separation and quantification by GC & MS being low volatile and undergoing thermal decomposition.

Nakamura et al. [30] carried out two dimensional TLC using glass plates coated with polyamide containing three fluorescent additives when all PTH-amino acids were seen as colored spots under UV lamp emitting light over the range 200-400 nm. The fluorescent materials had a characteristic color when suitably irradiated. About 0.1 n mole of PTH-amino acid could be detected and characteristic changes in the color of some PTH-amino acids were observed by heating the plate after spraying with an alkaline solution.

The typical results are given in Table-1. His method was superior to the earlier reports [31,32], where TLC of PTH-amino acids was carried out on silica gel plates and colored spots were located after ninhydrin spray, however, the PTH-derivatives of Val, Leu and Ile [33] and PTH-Tyr [32] did not yield

TABLE - 1

Characteristic colors of PTH aminoacids on polyamide FM plates containing mixed fluorescent additive[30]

PTH-amino acid	Color after second treatment	Color after Alkaline treatment
Valine	Red	Red
Proline	Red	Red
Alanine	Red	Red
Glycine*	Red	Brownish red
Serine	Red	Brownish red(blue)
Asparagine*	Red	Greenish brown(bluish green)**
Asparticacid	Red	Brownish red(dark brown)
Methionine*	Red	Brownish red
Leucine	Red	Brownish red
Isoleucine	Red	Red
Lysine	Red	Red
Tyrosine*	Red	Red(bluish green)**
Threonine	Red	Bluish green(blue)
Glutamine*	Red	Greenish brown(white yellow)
Glutamic acid	Red	Red
Phenylalanine*	Red	Greenish red(white blue)**
Tryptophan*	Red	Greenish red(white blue)**
Histidine*	Red	Blue(light blue)**
Arginine*	Red	Purple(blue)**
Cysteicacid	Red	Brownish red(dark brown)

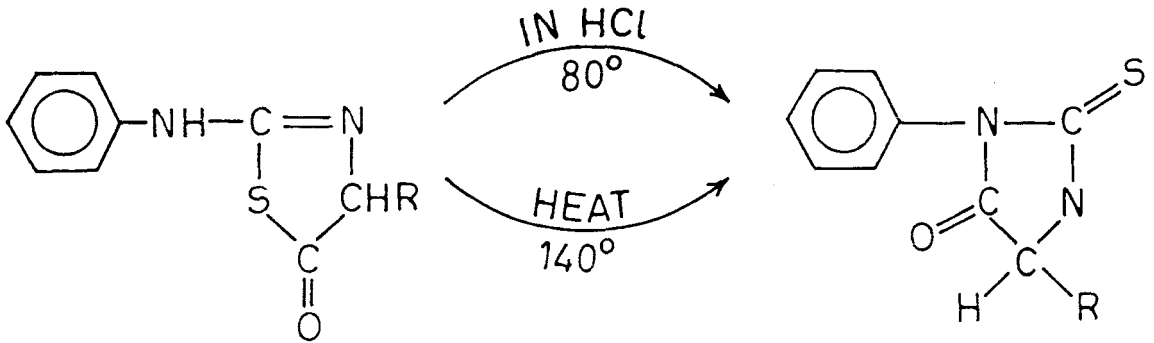
*Spots appear yellow, except glycine(pink)
**Fluorescent
Solvents: Toluene-n-pentane-aceticacid(6:3:2) and aceticacid-water(1:3) for 1st and 2nd dimension respectively.
Alkaline treatment: Spray 0.05M NaOH in methanol-water(1:1), heating at 150° for 30 mins, UV.

a color product in a ninhydrin reaction. Though in the method of Nakamura all PTH-aminoacids gave colored spots without any decomposition, nevertheless, distinction between PTH- Leu and Ile remained an unsolved problem. TLC separation of PTH-derivatives of Met, Glu, Lys, Ile and S-(carboxy methyl) cysteine on polyamide films using 30% HOAc and toluene-heptane-HOAc, 6:3:3.5 [34], and that of PTH-Leu and Ile in the solvent system 10% HCOOH-EtOH, 10:9 [35] have also been reported.

TLC ON SILICA PLATES

After an earlier review in 1972 by Rosmus and Deyl [36] and Neiderwieser [37], Inglis and Nicholls [38] reported TLC of PTH-amino acids in preference to coated glass plates on an aluminium plate coated with silica gel containing a fluorescent dye and stored over P_2O_5 to remove excess moisture, using ethylene chloride-acetic acid (60:7). The results showed that PTH-Ile/Phe were resolved from PTH-Leu and PTH-Val respectively, while PTH-Asp, Glu and Tyr showed increased separation in comparison to the system reported by Edman [39]. Further, Inglis [38] reported that the use of aluminium plates had advantages over glass plates as the former were more resistant to abrasion, the PTH mixtures exhibited more reproducible R_f values across the plate, and

the color reactions were more permanent. Inglis and coworkers [40] later described a simple procedure using silica gel plates for converting 2-amino-5-thiazolinones (ATO) from the protein sequenator to the more stable PTH amino acids without using the conventional HCl conversion procedure. The method was based on an early observation of Edman [41] that the conversion of ATO's to PTHs can be accomplished by heat alone as shown in scheme-2. Inglis [40] applied solutions of ATOs, from sequenator onto an aluminium backed TLC plate containing the fluorescent indicator Kieselgel 60 F_{254}, followed by 1µl heptafluoro butyric acid. The plates were heated for 10 min at 140°, and were developed [38] with reference PTH standard mixtures. After chromatography, measurements of the yields of derivatives on the plate were made with a variable wavelength thin layer chromatographic scanner, which was shown to be a useful adjunct for identification. Inglis [42] further modified the above procedure to overcome the limitations of the heat conversion method. The extract from the sequenator was dried in a stream of nitrogen and dissolved in 100µl of 1,2 dichloro ethane plus 1µl of pentafluoro propionic acid containing 5 mg/ml dithiothreitol. An appropriate aliquot was slowly loaded onto an aluminium backed silicagel plate using a multispotter with heating



ATO PTH

Scheme 2: Heat conversion of ATO to PTH.

at approximately 70°. After loading, the TLC plate was covered with a 20x20x 0.4 cm glass plate and heated at 140° for 10 mins. The presence of dithiothreitol significantly decreased the possibility of desulphuration of thiocarbonyl groups by oxidation either during loading or during heating at 140°. Later, Inglis [43] reported that the sensitivity was increased using the larger plates and exposing the chromatograms to iodine vapour, which followed quick photography under UV light, in comparison to the TLC on silica gel containing fluorescent dye [38].

A rapid color coded system was described by Walz and Reuterby [33] for the complete resolution of all common PTH-aminoacids by using the information of Inagami and Murakami [32]. In this system, only the PTH derivatives of dehydrothreonine, valine, leucine and isoleucine failed to give a characteristic color and were analysed by GC. They used plastic backed precoated silica gel TLC plates without fluorescent indicator which were developed in heptane-ethylenedichloride-propionic acid (45:25:30) and Xylene-methanol(80:10). The chromatograms were sprayed with iodine-azide and 1.7% ninhydrin in MeOH-collidine-AcOH (15:2:5) and were heated at 90° for 20 min. Certain characteristic color changes were detected

by blowing a saturated ammonia atmosphere over the ninhydrin plate. The typical results are given in Table-2. The colors produced easily permitted the identification of those aminoacids which had nearly identical R_f values; for example lysine and serine degradation product; alanine, methionine, and phenylalanine; and tyrosine and threonine. The method can be considered as significant because it gave consistently positive identifications of PTH-Ser, Lys, Glu, and Asp acids and their respective amides which could not be identified by GC. Moreover the sensitivity of the technique was the greatest for those PTH-derivatives which failed to be resolved by GC. The PTHs can also be determined [44] during their identification on TLC, by spraying a mixture of ninhydrin, collidine, and ethanol which formed red dehydration products. Kubota et al. [45] described a method for identification and quantitation of PTH-aminoacids by TLC on silica gel plates, with a scanning, two wavelength densitometer. This method was found to be satisfactory to the determination of amino terminal residues of bovine ∝-chymotrypsin. The application of the linear detector system for the quantitative determination of PTH-amino acids on the thin layer chromatogram was demonstrated by Zwolinski and Treiber [46]. According to them this method was rapid and more sensitive than the Edman

TABLE - 2

Characteristic colors of PTH-amino acids following ninhydrin spray [33]

PTH derivative	Color properties	NH_4OH color change
Proline	UV, colorless	light blue after heating
Alanine	Purple	deeper color
Glycine	Orange	
Serine	UV, purple	
Serine break-down	faint orange	weak red
Asparagine	yellow	move intense
Carboxymethyl-cysteine	UV, purple	
Methionine-sulfone	light tan	
Methionine	faint tan	
Lysine	very faint pink	weak blue after heating
Tyrosine	UV, yellow before spray	intense yellow
Threonine	Colorless	Light tan
Glutamine	dark green	dark blue
Phenylalanine	UV, Colorless	faint yellow
Tryptophan	UV, yellow before spray	deep yellow
Asparticacid	UV, pink	darker
Glutamicacid	grey	dark blue

procedure, and as little as 0.5 n mole of PTH-aminoacid could be detected quantitatively. Horn et al. [47] studied the chromatographic behaviour of both PTH and MTH aminoacids in similar conditions using the same sequential development technique with the same two solvents. They carried out TLC on silica gel

plates with fluorescent indicator. The chromatograms were developed in (I), $CHCl_3$-EtOH (98:2) followed by (II) $CHCl_3$-EtOH-MeOH (89.25:0.75:10) in the same direction and were visualized under short wavelength light. All but 2 of 20 common PTH-aminoacids were resolved by this method. Residues which ran at, or near, the baseline into the solvent I were adequately separated in solvent II. Only PTH-Asp and PTH-cysteic acid were co-chromatographed. The system provided better resolutions in comparison to HPLC as one pair and one triplet of MTH-amino acids not resolved by HPLC were definitely resolved. Datta and Datta [48] described a method for the identification of para phenyl azophenol PTH-derivatives of amino acids by ascending TLC on silica gel. The developing solvents were acetonitrile-0.2M NH_4OAc-Me_2CO (75:15:19) or acetonitrile - 0.1 M NH_4OAc-Me_2CO(55:35:10), they achieved the separations of threonine from aspartate, serine from histidine, and valine from phenylalanine. Further they [49] described some spray reagents for the detection of p-phenyl azo PTHs of aminoacids on silica gel plates. The spray reagents contained phenyl naphthylamine or ninhydrin with or without copper nitrate or cyclohexylamine or isatin and zinc acetate.

Tarr [50] reported that modifications of the classical Edman cycle permitted the complete sequential

degradation, without prior derivatization of all small and medium sized peptides with a free N-terminal. The derivatives were identified with a fast and sensitive thin layer chromatography. Sequences upto 40 residues appeared possible with most long peptides. Further Klemn [51] described a method for N-terminal sequencing with TLC identification of phenylthiohydantions. He also described the mechanism for PITC coupling to peptides, cleavage and conversion reaction. Solal and Bernard [52] described an adaptation of previous methods [36,39] involving 1/3 size silica gel sheets, whcih was very sensitive (at 10^{-10} M level) and the chromatography was complete in 15 mins. It was probably the first attempt of micro TLC on PTH-aminoacids. The ascending chromatography in one solvent was followed by another ascending chromatography in the same direction in a different solvent and the chromatograms were sprayed with iodine-azide solution, the typical results are given in Table-3. The quality of the separation was comparable to GC and conventional TLC procedures but the micro TLC was found to be more rapid as it could identify eight PTHs in half an hour contrary to GC which identified only one in equivalent time. However, the application of both GC and micro TLC was recommended for quantitation. Some effective solvents were reported by Munier and Drapier[53,54]

TABLE - 3

Results of miniature TLC using two different solvents for the same direction[52]

Procedure	Solvent components	Proportions	Conventional technique on 20x20 cm plates		Microscale technique on 6.3x6.3 cm plates	
			Distance of solvent front(cm)	Time of migration (min)	Distance of solvent front(cm)	Time of migration (min)
Standard technique for screening	Chloroform Methanol	90 10	8	60-90	2.7	3-5
For Asn,Gln, Glu,Asp,His, Arg	Chloroform Methanol	80 20	8	90-120	2.7	6-7
For Val,Phe, Leu,Ile,Trp, Tyr.	n-heptane Pyridine	70 30	15	90-120	5.0	10

for the rapid identification of PTH-aminoacids by one dimensional TLC on silicagel plates or cellulose. The best systems were $CHCl_3$-n-butyl acetate (90:10), diisopropyl ether-EtOH (95:5), and dichloromethane-EtOH-HOAc (90:8:2). Kinoshita et al. [55,56] have reported the application of thiamino hydrochloride solution and N-chloro-5-dimethyl amino naphthalene-1-sulphonamide (NCDA) as the locating reagents for PTH-amino acids on conventional TLC. NCDA was non-fluorescent but developed intense fluorescence on reaction with PTHs and was effective in the determination of PTH-amino acids present at 40-60 p mole level while with thiaminohydrochloride the plate was illuminated at 365 nm and the fluorescence intensity was measured for the detection. As little as 60 p mole of PTH-Lys was detected by UV (366 nm) irradiation on a fluorescence containing silica gel plate after development with ethanol-acetic acid (7:3), [57].

Thin layer chromatographic resolution and identification of PTH-aminoacids on silica or cellulose or polyamide layers as discussed above indicated difficulties in achieving discrimination between derivatives of Leu and Ile [16], and resolution of complex mixtures without two dimensional chromatography [58]. Besides difficultieis to resolve

combiantions of PTH-Phe/Val/Met/Thr [38,59]; and PTH-Leu/Ile [30,38,59] had also been observed. Recently Bhushan and coworkers[9-15] in an attempt to resolve these difficulties have successfully reported for the first time the application of silicagel layers impregnated with various metal salts including transition metals and other reagents like (+) - tartaric acid and (+)-ascorbic acid for the resolution of multicomponent mixtures of PTHaminoacids and their enantiomeric mixtures. The methods reported by them were very good because the spots were very compact; the methods were applicable for the indentification of unknown PTH-derivatives besides resolution of multicomponent mixtures; they provided a convenient method over iodine-azide method wherein the latter bleached spots were observed on light brown background of the paper or thin (silica gel) layer chromatogram and caused greater difficulty in demarcating the exact spots and measurements of accurate R_f; the PTH-aminoacids remained unaltered chemically and could possibly be used subsequently after elution.

Resolution of 10 component mixture of PTH-amino acids on thin silica layers impregnated with 3 different concentrations of Zn^{++}, Cd^{++}, Hg^{++} using chloroform-H_2O-EtOAc (28:1:1); CCl_4-HOAc (19:1) and $CHCl_3$-MeOH-C_6H_6 (14:1:5) [10]; resolution of 15

component mixture on silica plates impregnated with different concentrations of Fe^{++}, CO^{++} and Zn^{++} in $CHCl_3$-C_6H_6-EtOAc (25:5:3) [11] and different concentrations of Cr^{+++}, Mn^{++}, Fe^{++}, Co^{++}, Ni^{++}, Cu^{++}, Zn^{++}, Cd^{++} and Hg^{++} in $CHCl_3$-EtOAc (29:3) [12] have been reported. It was found that impregnation of metal ions influenced the chromatographic behaviour of PTH-aminoacids most likely by the complex formation due to electron pairdonation from N, O, or S atoms of PTH-aminoacids to the 'd' orbitals of transition metal ions and it was the solubility/adsorption of such a complex which was responsible for different R_f and hence resolution, for example, the complexes of PTH-amino acids with Co^{++} and Zn^{++} had a higher solubility than those of Fe^{++} in the solvent system $CHCl_3$-C_6H_6-EtOAc (25:5:3) as was evident from their increased and decreased R_f values respectively in comparison to control plates [11] is shown in Table-4. The solvent system $CHCl_3$-EtOAc (29:3) for plates impregnated with Fe^{++}, Ni^{++} (0.2%) provided resolutions for PTH-derivatives of Leu, Ile, Thr and Val; Leu, Ile, Gly; and Thr, Met [12].

The study of the effect of different anions and cations on the thin layer chromatographic behaviour of PTH-amino acids by the same group [14,15] resulted into resolution of several difficult combinations

TABLE - 4

hR_f Values of PTH-amino acids on Fe^{++}, Co^{++} & Zn^{++} impregnated silica plates [11]

PTH-amino acid	Plain	Fe^{++} 0.3%%	Co^{++} 0.1%	Zn^{++} 0.2%
Alanine	40	34	47	41
Aspartic acid	0	0	0	0
Glycine	26	23	28	27
Glutamicacid	0	0	0	0
Isoleucine	74	68	84	84
Leucine	74	70	87	78
Lysine	11	4	15	5
Methionine	49	40	58	53
Proline	89	86	80	97
Serine	5	3	6	7
Tyrosine	72	73	90	80
Tryptophan	85	77	94	87
Threonine	62	55	82	72
Valine	65	57	73	67

Solvent: Chloroform-benzene-ethylacetate(25:5:3), developing time-35 minutes, for 10 cm.

[30,38,59,66] and development of several new solvent systems viz: n-heptane-butylacetate (15:5); n-heptane-propionic acid (20:4); C_6H_6-EtOAc (15:3); $CHCl_3$-n-butylacetate (10:5), and $CHCl_3$-EtOAc (25:2).

The anions such as Cl^-, CH_3COO^-, and SO_4^{--} influenced markedly the resolution pattern since the silica gel slurries in aqueous solutions of these metal

ions were at pH 5.5 to 6.0 when PTH-aminoacid being a weak base combined with a H^+ and finally made ion pairs with available anions and it was the solubility/ adsorption of these ion pairs which effected the chromatographic behaviour [14,15] rather than that of a complex as mentioned above [11,12].

The resolution of 9 racemic PTH-amino acids viz: PTH-Met, Phe, Try, Val, Ile, Thr, Tyr, Ala and Ser is one of the most significant reports on thin layer chromatographic resolution of not only mixtures of PTH-aminoacids but also of their optical isomers,

TABLE - 5

hR_f of pure and resolved enantiomers of PTH amino acids on(+)-tartaric acid impregnated plates [13]

DL-mixture of PTH-amino acid	hR_f pure L	hR_f from mixture	
		D	L
Met	83	16	83
Phe	85	15	85
Try	95	-	95
Val	80	21	80
Ile	92	12	92
Tyr	95	16	95
Thr	85	30	85
Ala	55	12	55
Ser	84	10	84

Solvent: Chloroform-ethylacetate-water (28:1:1)
Developing time-35 minutes; Solvent front 10 cm.
Room temperature: 25±1°C

by Bhushan and coworker [13], which in itself is a challenging problem. The silica gel was impregnated with (+)-tartaric acid or (+)-ascorbic acid and the chromatograms were developed in $CHCl_3$-EtOAc-H_2O (28:1:1) and n-butylacetate-$CHCl_3$ (1:5) respectively [13]. The enantiomers of PTH-aminoacids resulted into (+ -) and (+ +) diastereomeric salts with tartaric acid or ascorbic acid, having different solubilities and giving good resolution. The typical results are given in Table-5. This report provided a simple and sensitive method for the resolution of optical isomers of PTH-aminoacids without their prior treatment or involving sophisticated instrumentation.

HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY

Improvements in all practical aspects of TLC process culminated in a performance breakthrough in thin layer chromatography which resulted in an increase in separation efficiency, sample detectability limits and reduced analysis time [60-64]. The specific advance in instrumentation was termed as HPTLC. That HPTLC could be used with advantage for the separation of PTH-aminoacids was recognised by Bucher and Yang [65,66]. But they could not achieve separation of all 20 common

TABLE - 6

Optimum experimental conditions for the separation of the PTH-amino acids by continuous multiple development HPTLC [64]

Development step	Mobile phase composition	Plate length (cm)	Time (min)	PTH-amino acid derivative identified
1.	CH_2Cl_2	3.5	5	Pro
2.	CH_2Cl_2-$(CH_3)_2CHOH$(99:1)	7.5	10	Pro,Leu,Ile,Val,Phe.
3.	CH_2Cl_2-$(CH_3)_2CHOH$(99:1)	7.5	10	Pro,Leu,Ile,Val,Phe,Met, Ala/Trp,Gly,Lys,Tyr,Thr.
4.	CH_2Cl_2-$(CH_3)_2CHOH$(97:3)	7.5	10	Pro,Met,Lys,Tyr,Thr, Ser,Glu.
5.	$C_2H_5OOCCH_3$-CH_3CN-CH_3COOH(74.3:20:0.7)	7.5	10	Asn,Glu/Gln ,Asp,Cm-Cys, His,Arg.

PTH-aminoacids. Schuette and Pooles [64] used a continuous multiple development on a silica gel chromato-plate and were able to separate 18 samples and standards simultaneously using 5 development steps with 4 changes in mobile phase and scanning densitometry. Their typical results are given in Table-6. Butler et al. [67] studied the separation of PTH Leu/Ile/Pro by HPTLC using multiple wavelength detection. Fater [68] carried out separation and quantitation of PTH-aminoacids by over pressured layer-chromatography using chloroform-ethanol-acetic acid (90:10:2) for the resolution of polar PTH-derivatives and dichloromethane-ethyl-acetate (90:10) for the resolution of non-polar PTH-aminoacids and claimed his method to be superior to those of HPTLC methods [65-67] particularly in having relatively increased migration distance resulting in the resolution of complex mixtures containing a large number of derivatives.

CONCLUSION

The application of TLC supplemented with different kinds of instrumentation is getting more popular, however, the simple TLC remains widely used for the analysis of PTH-aminoacids in conjunction with Edman method of protein sequence analysis and leaves much scope for the resolution of multicomponent as well as racemic mixtures.

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