Journal of Chromatography, 379 (1986) 177—250
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2994

REVIEW

PROFILING OF AMINO ACIDS IN BODY FLUIDS AND TISSUES BY MEANS OF LIQUID CHROMATOGRAPHY

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(Received January 15th, 1986)

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0378-4347/86/$03.50

1. INTRODUCTION

Amino acid analysis in general has received much attention because of its importance in biological sciences, and virtually all chromatographic techniques have been applied for this purpose. In principle, separations are based either on the charge or on hydrophobicity differences. Because of the vast number of possible applications of amino acid analysis, it is clear that none of the available chromatographic techniques will fit all demands. Thus, e.g., the phenylthiohydantoin procedure is intimately related to sequence analysis. Any technique one may choose exhibits particular advantages and disadvantages.

In this review we shall limit ourselves to the specific demands of profiling and, in view of the personal experience of the authors, it is limited to liquid chromatography. Such separations can be carried out either in columns or in a planar arrangement. Because of the necessity for screening very large numbers of samples in a clinical laboratory, planar separations have retained a firm position for acquiring preliminary information about a particular case. Only when the possibility of an amino acid disorder has been indicated in the preliminary step are the samples subjected to a more thorough analysis by one of the column techniques that ensure better quantitation of the results.

Also here we consider only marginally the problem of the separation of

amino acid enantiomers; the importance of these amino acid derivatives in amino acid profiling in the clinical routine has not yet been fully exploited, although it may be predicted that it will attain considerable importance in the future. In this respect the reader searching for more detailed information is directed to papers as Nimura et al. [1], Buck and Krummen [2] and Weinstein et al. [3] and other references cited therein. It is clear that the first stage of applications of this type will refer to peptide drugs and their metabolism, as this is particularly where the enantiomeric purity of constituents is of great interest.

Another area omitted from this review is that of measuring ^{14}C-labelled amino acids (for introductory information, see Potashner et al. [4]).

Profiling of free amino acids is of great importance today and has found broad application in clinical practice. Interest in the determination of free amino acids in biological fluids is as old as the knowledge of amino acids themselves [5—7]. It is rarely realized today that the rediscovery of column liquid chromatography by Martin and Synge in 1941 [8] emerged from a need for amino acid separation. The method was soon improved by the introduction of the paper chromatography of amino acids by Consden et al. [9] in 1944. Apparently because of its simplicity, the latter method was soon applied to the analysis of free amino acids in blood and urine [10]. In parallel, these methods were used at the University of Texas by a group headed by Kirby-Berry [11]. The first comprehensive reviews on this topic published in book form were written by Hais [12] and Smith and Seakins [13]. Progress in these early days of chromatography was fast. In the 1950s automated sample collectors were introduced into column liquid chromatography, making the separation of compounds easier. This period culminated in the first fully automated amino acid analyser constructed by Spackman et al. [14]. Subsequently, paper chromatography was step-by-step overtaken by thin-layer chromatography and ion exchangers dominated the amino acid analysis area in the 1960s and 1970s.

It was in the early 1950s when the stage of development of amino acid analysis allowed the first hereditary disorders of amino acid metabolism to be diagnosed [15—21]. In addition to diagnosis, the analysis of free amino acids allows prolonged dietary treatment of these disorder to be followed and offers the possibility of genetic prevention in both the pre-marital and prenatal stages [22,23]. In addition, there are demands put on amino acid analysis in the production of both low-protein and high-protein dietetic products, in the control of parenteral nutrition, in determining the degree of malnutrition and in complementary assays for determining the body radiation dose and in the investigations of liver, kidney and other metabolic disorders [24,25].

The main diagnostic application of free amino acid profiling is to capillary or venous blood, serum, plasma, urine and amniotic fluid [26—29]. Other biological fluids, such as breast milk [30], sweat [31], sperm [32], faeces [33], saliva [34], tears [35], synovial fluid, cerebrospinal fluid, exudates and transudates [36] are analysed much less frequently. As far as tissues and cell populations are concerned, free amino acids are determined not only in fibroblast cultures [37] but also in homogenates of liver cells, brain cells [38], erythrocytes [39], leucocytes [40], in hair [41], nails, teeth, bones, muscles [42] and other tissues [43—46].

The content of free amino acids in peripheral blood is relatively constant and reflects the metabolic equilibrium that results from the continuous turnover of tissue proteins. The main components of blood amino acids are glycine, taurine, glutamine, valine, leucine and lysine [47,48]. The determination of amino acids in urine is difficult owing to the presence of numerous compounds possessing the primary amino group. The main urinary amino acids are histidine, glycine, taurine, glutamine, alanine, lysine, methylhistidines and serine [49,50]*.

In this review we attempt to summarize the literature relevant to amino acid profiling mainly with regard to hereditary disorders of amino acid metabolism. It should be emphasized that most of these discoveries and even current applications were achieved by means of classical column liquid chromatography using cation exchangers and low-pressure apparatus. There are several reasons: first it reflects the delay with which the modern chromatographic techniques have penetrated some areas of application; second, although tremendous attempts have been made to increase the column overpressure and to shorten the analysis time, and modern amino acid analysers are able indeed to reduce this period to between 20 min and 1 h when working in the picomole range, with respect to resolution none of the modern systems is better than that published by Spackman and co-workers [14,51] in 1958; and third, whereas the classical type of analysers of amino acids exploiting the cation-exchange principle were single-purpose apparatus, the penetration of new techniques is connected with standard high-performance liquid chromatographs that have to be combined with various additional equipment such as a fluorescence detector, a post-column reactor and an oxidation system for *o*-phthalaldehyde derivatives. This places some demands on the user whose main interest is not in the way the amino acids are separated, but in diagnosing a patient's condition. This is a conflicting situation as the producers of the equipment for modern chromatography seek to develop primarily the most versatile apparatus suitable for diverse types of separations with minimal modifications.

For a chromatographer it may be surprising to find that low-speed chromatographs and paper chromatography are routinely used in this area today. However, this has to be accepted and if this review shows some potential for modernization in this respect, then we shall certainly be pleased.

We focus attention mainly on the analysis of complex amino acid mixtures; the separation of the so-called marker amino acids (see Section 2) is limited to references only. This is because we feel that at present the analysis of marker amino acids is more related to the structural analysis of proteins. In other words, the literature surveyed here has been strictly selected, as a summary of all the literature available is beyond the space available. In this respect the reader is directed to several recent reviews in which the different methodologies for amino acid analysis are overviewed in terms of chromatographic parameters, resolution, efficiency, detection sensitivity, accuracy, reproducibility and areas of application [52—54].

After the general introduction, we offer a brief survey of liquid chromatographic methods available, followed by their applicability in a clinico-chemical laboratory.

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*All abbreviations, unless explained in the figure legends, are specified in Section 5.

2. GENERAL STRATEGY

It has been mentioned already that the choice between the planar and column arrangements is given by the need either to analyse many samples side-by-side in a preliminary screening or to obtain a more detailed profile of a single sample.

The methods of planar chromatography have remained inexpensive and offer the handling of a much larger number of samples than even the most sophisticated modern high-performance liquid chromatographic (HPLC) procedures, although of course the latter offer quantitative results more readily. Also, the differences in analysis costs favour planar chromatography unless sophisticated densitometric devices are used for quantitative evaluation. There is a bewildering choice of methods for amino acid investigations by planar techniques. Most of these are minor variations on the few original methods and for further information the reader is directed to the excellent review by Ersser and Smith [55]. The solvent systems, methods of detection and analytical procedures summarized in their review are still the most reliable ones even today. It is worth mentioning that care must be taken when comparing ones own results with those published in the literature, particularly with regard to the evaluation of amino acid disorders, as both the sample pre-treatment and the chromatographic procedure used may result in differences.

In column liquid procedures the type of chromatography used depends on whether one is separating underivatized amino acids or amino acid derivatives. Although perhaps it is unnecessary, let us mention that most underivatized amino acids (with the exception of the aromatic types and few others) cannot be detected, e.g., by UV absorption unless very short wavelengths are used. Therefore, they nearly always have to be converted into suitable derivatives, either before or after their chromatographic separation.

Another factor in the selection of a particular chromatographic method for amino acid analysis is the question of whether one is seeking the profile of all amino acids or rather searching for a particular category of amino acids (e.g., branched-chain amino acids) or even a single amino acid. In other words, if we are following an alteration in a metabolic pathway then, depending on our knowledge of the disorder, we must study the whole amino acid profile or a considerable part of it. If, however, we are looking for metabolic alterations to a particular protein then it would be of advantage to have a marker amino acid that is specific for the protein in question. Thus one may follow collagen metabolism by assaying urinary free and peptide-bound hydroxyproline; 1-methylhistidine reflects the metabolism of muscle proteins, γ-carboxyglutamic acid reflects the alterations in blood coagulation system or, if assayed in hard tissues, it appears to be related to the calcification processes (arising from thrombin in the former instance and from osteaocalcin in the latter). In practice, however, in many instances this is not considered beforehand and even experienced laboratory workers may be tempted to use any amino acid analyser available. This holds not only for the amino acid analysis as such, but for the sample preparation step, where the method of sample hydrolysis may be of considerable importance. The liberation of the γ-carboxyl group from γ-carboxyglutamic acid in acidic media and subsequent conversion of this amino acid into glutamic acid may serve as a good example.

In general, when approaching the problem of amino acid separations one has basically two possibilities: either to separate underivatized amino acids by cation-exchange chromatography, i.e., in the more or less classical manner with an additional post-column derivatization reaction, or to convert the amino acids into suitable derivatives before separation and to run the separation in the reversed-phase mode using commercial HPLC equipment. The choice is considerably influenced by the equipment available and, if the separations are carried out with adequate precautions, both can offer excellent results. In any event, it can be stated that modern HPLC procedures for amino acid analysis have established themselves firmly (in addition to the conventional methods) in the area of clinical biochemistry [56–62]. Let us comment here that the chemical nature of the derivatives used for pre- and post-column derivatization is usually the same.

2.1. Separations avoiding pre-column derivatization

The separation of underivatized amino acids is carried out on cation exchangers with a gradient of acidic buffers: the more acidic amino acids (possessing, e.g., an additional carboxyl group) elute first and those with more than one primary amino group or possessing a guanidyl residue elute at the end of the chromatogram. After separation, these are converted into coloured ninhydrin derivatives for spectrophotometric detection, or into *o*-phthalaldehyde derivatives for fluorescence detection. The latest trend in this field is to use 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole to obtain highly fluorescent derivatives (the detection limit being 5–10 pmol depending on the nature of the amino acid).

New achievements in this area lie mainly in the application of advanced post-column derivatization reagents and in the way in which the post-column reaction is effected, mainly to prevent undesirable post-column peak spreading.

Finally, it is also possible to detect amino acids at extremely short wavelengths, e.g., around 200 nm, but, as many compounds absorb in this area, the risk of artefacts and the demands put on the solvent purity and their choice are high.

Other progress in the ion-exchange chromatography of amino acids is seen with computerized systems, particularly with regard to the method of gradient formation. However, as on many occasions stepwise gradients are sufficient, computerization of the whole system is fairly simple. On the other hand, automated evaluation (and mainly quantitation) of the numerous peaks emerging from the column makes the life of the bench worker much easier than 20 years ago when all this was done by tedious counting of the dots from which the chromatographic profile was constructed. Computerization is not limited, however, to cation-exchange procedures and is a normal part of systems working in the reversed-phase mode.

In conclusion, let us emphasize that the methodology of the cation-exchange chromatography of amino acids is so well worked out that not much further progress can be expected in the future. For this reason, in the methodological part of this review this area is only briefly surveyed to show the large number of compounds that can be described as amino acids and that can be separated side-by-side in a single chromatogram.

On the other hand, there is a possibility of separating underivatized amino acids using the ion-pair reversed-phase technique. This procedure is fairly new and, if convenient ion-pairing agents can be found that can retain the individual members of the amino acid family more than the presently used sodium lauryl sulphate, the technique certainly has good prospects [63].

2.2. Separations of derivatized amino acids

If the choice is to separate some amino acid derivatives, no matter which ones, reversed-phase chromatography is the preferred separation procedure. Octadecylsilane hydrophobized sorbents are obviously the most popular. Nevertheless, with these the practical problem is that, e.g., C_{18} silica gels differ in their properties not only with respect to different producers but sometimes even in different batches obtained from the same producer. Other reversed phases, e.g., C_8, are used much less frequently.

A wide variety of solvent systems are currently available, mostly containing methanol, acetonitrile and less frequently tetrahydrofuran as the organic component of the mobile phase. The choice of the organic component is influenced by its selectivity for separating certain pairs of amino acids and by how their presence influences the back-pressure of the column.

2.3. Identification of individual amino acids and liquid chromatographic—mass spectrometric applications

In classical methods amino acids were identified by their position on the chromatogram. For routine screening and diagnostic purposes this approach, no matter how obsolete and occasionally unreliable, suffices even today. On the other hand, amino acids have frequently been used in establishing combined liquid chromatography—mass spectrometry (LC—MS) as a technique, particularly for the evaluation of different types of interfaces. So far, there have been only a few reports dealing with the on-line LC—MS identification of amino acids; they are limited to the LC—MS identification of tryptophan [64], to the identification of the so-called branched amino acids (valine, leucine and isoleucine together with creatine) and some aromatic amino acids [65].

Although applicable in the clinical routine, these procedures have not yet penetrated this area. The main impact of LC—MS coupling lies at present in protein sequence analysis, and a description of these techniques is clearly beyond the scope of this review. Because of the main area of application, the fundamental papers on LC—MS coupling involve the use of either methylthiohydantoin or phenylthiohydantoin derivatives of amino acids. In the former instance atmospheric pressure ionization LC—MS has been applied, and in the latter thermospray ionization has been used. According to the results of Games and Ramsey [66] the thermospray ionization approach appears more suitable.

The moving belt system has been used with both conventional and microbore columns whereas the thermospray approach has been used with conventional columns only. The results are, however, limited to six aromatic amino acids. With phenylthiohydantoin amino acid derivatives, data are available for more members of the amino acid family, but the number is still too small for

practical purposes. The prospects here lie not only in discovering yet unknown amino acid derivatives (and metabolic deviations) but also in the high sensitivity, which for full-scan electron-impact spectra ranges from 2 nmol to 20 pmol.

3. PREPARATION OF THE SAMPLE

3.1. Sampling of the biological material for analysis

3.1.1. Blood

The content of some amino acids in red blood cells is much higher than in plasma or serum. As intracellularly located amino acids are of lower diagnostic value, plasma or serum is preferred for analysis [24,26,27]. In the laboratory routine, blood is taken after 10—12 h of fasting, mostly between 7 and 9 a.m. There appears to be no need to control the composition of the diet during the previous day. However, during the first orientation assay the diet should not contain an excess of protein, in order to avoid alimentary hyperaminoaciduria [47,67,68]. In contrast, in targeted, repeated examinations in some benign deviations the intake of proteins should be increased or even complemented by an increased intake of vitamins. In small children or in sucklings the fasting period is reduced to 6–8 h. The onset of hypoglycaemia is prevented by an intake of glucose.

Venous blood is sampled from the antecubital vein into a vial containing heparin. After mixing, the blood is briefly centrifuged (10 min at 28 000 *g*). The plasma is immediately removed, care being taken not to aspirate the layer of thrombocytes and leucocytes [37]. If this step is not carried out carefully, the plasma becomes contaminated with thrombocyte or leucocyte amino acids, in which the contents of, e.g., aspartic acid, taurine and glutamic acid are about 100-fold higher than those in plasma, which obviously leads to incorrect results [40,69,70]. Also, if the plasma is allowed to stand overlaying the layer of cells, increased values of some amino acids, particularly taurine, glutamic acid, aspartic acid and phosphoethanolamine, are observed [38,43]. After haemolysis plasma cannot be used for amino acid analysis. The amino acids eluted from erythrocytes lead to increased values of taurine (4×), phosphoethanolamine (20×), glutamate (20×) and aspartate (50×) [69,71,72]. The contents of glutathione and some polypeptides also become elevated [57]. Plasma stored in air contains a decreased amount of cysteine derivatives; similarly, the level of arginine decreases as a result of the action of erythrocyte arginase, which converts it into ornithine [26].

Deproteinization of plasma or serum should be carried out within 30 min after sample collection. Deproteinized supernatants should be analysed as soon as possible or stored at low temperatures (−20 to −40°C).

Capillary blood sampling from newborns is carried out routinely after 2–3 h of fasting, i.e., before the second suckling. Finger-tip blood is taken from the heel or finger of the newborn baby. The first drop of blood is wiped off with sterile gauze and from the subsequent drops of blood at least three spots are taken using, e.g., Schleicher & Schüll 2992 paper cards. The spots are dried and the card is sent to the screening laboratory. Care must be taken to achieve regular wetting of the circles on both sides of the card [73].

The choice of the anticoagulant is also important. Heparin appears to be the most popular, although an excess of it may cause haemolysis and consequently liberation of ninhydrin-positive compounds from red blood cells [69,74]. Problems may also arise with EDTA, which may contain haemolysis-causing contaminants.

There are some differences between the contents of free amino acids in samples of venous and capillary blood; elevated values of taurine, aspartic acid, serine and glutamic acid are explained by admixing of the tissue fluid with capillary plasma. Less pronounced elevation was observed with glycine, alanine, ethanolamine and ornithine [71,75].

A survey of factors that influence the determination of blood amino acids is given in Table 1.

Collection of blood samples in the early morning hours after fasting should eliminate post-absorption changes in the amino acid levels. In addition to absorption, hormonal and circulatory effects, the blood levels of amino acids are determined by circadial rhythms, the amino acid levels generally being higher in the evening and lower in the morning [44, 76]. This may be found in the levels of e.g. phenylalanine or tyrosine in healthy children. In phenylketonuria patients the trends are reversed [77]. Equally, prolonged fasting or stress may affect the circadial variations and levels of amino acids. The amplitude of the circadial variation is about 30% around the average daily value, the lowest being in the afternoon. The variations of individual amino acids may be more pronounced than the variation of the sum of free amino acids, and these values may differ considerably for the individual members of the amino acid family [76–78].

3.1.1.1. Storage of blood samples before analysis. The addition of preservatives to plasma or serum is generally avoided as these compounds may interfere with the analysis. For short-term storage of plasma or serum temperatures around 4°C suffice. However, even at this temperature minor changes in the amino acid levels occur [79]. Thus, e.g., the level of taurine decreases immediately after the sample has been collected, reaching a minimum after 30 min and increasing to the original level after a further 30 min. Serum hydrolases are believed to be the cause of the decreases in asparagine and glutamine levels, which are usually accompanied by an increase in free glutamic acid [26,29,69]. Cystine and cysteine exhibit a tendency to bind to disulphide protein bonds, which results in a decrease in these amino acids in their free form [69].

When stored at −15 to −20°C, plasma or serum does not exhibit detectable changes within 72 h. After storage for 1 week cysteine almost disappears and after 3 months a decrease in glutamine and tyrosine levels was observed, whereas the level of glutamic acid increased. There are also reports of an increase in aspartic acid as a result of an asparagine decrease and reports of a decrease in albumin-bound tryptophan [26,29,79]. In order to obtain correct levels of cysteine, the addition of antioxidants (e.g., thiodiglycol) seems inevitable. Storage at −60°C, as suggested by Dickinson et al. [74], results in no visible changes in the amino acid profile even after several months.

In the laboratory routine it is therefore advisable to remove proteins as soon as possible and to store the deproteinized supernatant at −20 to −35°C in a deep-freezer. The changes that may occur in the amino acid profile under these

TABLE 1

SURVEY OF ARTEFACTS THAT MAY ARISE DURING BLOOD AND URINE COLLECTION AND STORAGE

Sample	Artefact causing operation	Increased	Decreased	References
(A) Plasma, serum	Haemolysis	Aspartic acid, glycine, taurine, phosphoethanolamine, ornithine, polypeptides, glutathione	Arginine, cystine	26, 29, 38, 43, 69, 70—72, 75, 79
	Excessive meat or milk ingestion before collection	Methylhistidine, peptides, leucine, homocitrulline, taurine		47, 67, 68
	Poultry meat ingestion	Carnosine		26, 207
	Load with protein hydrolysates	Peptides, all amino acids		29, 67, 68, 79, 338, 339
	Delayed deproteinization	Taurine, glutamic acid, phosphoethanolamine	Cystine, homocysteine, disulphides, tryptophan	26, 27, 74, 339
	Contamination with sweat	All amino acids		340
	Prolonged storage at 4°C	Glutamic acid, aspartic acid	Glutamine, asparagine, tryptophan, cystine, cysteine, homocysteine disulphides	26, 29, 339
	Excessive amount of heparin	Taurine, glutamic acid, phosphoethanolamine		69, 74, 79
	Addition of EDTA (not pure)	Artefact resembling methionine and taurine in ion-exchange chromatography		Personal experience of one of the authors (J.H.)
	Contamination of plasma with thrombocytes or leucocytes	Aspartic acid, taurine, glutamic acid		67—72, 75, 79
	Haemocoagulation, fibrinolysis	Aspartic acid, taurine, glutamic acid, arginine, phenylalanine, polypeptides		26, 69

	Admixture of tissue fluid	All amino acids		29, 37
	Administration of antibiotics, mixtures of amino acids	Artefact in the area of methionine, leucine and tyrosine in ion-exchange chromatography		339, 341
(B) Urine	Contamination with stool	Glutamic acid, proline, tyrosine, methionine, valine, leucine, basic amino acids		33, 38, 69, 339
	Antibiotics (ampicillin, pombritin, cloxacillin)	Artefact in the area of leucine, tyrosine and methionine in ion-exchange chromatography		339, 341
	Prolonged storage at 4°C	Glutamic acid, aspartic acid	Asparagine, glutamine, cystine, homocysteine, disulphides	26, 29, 69, 339
	Administration of corticoids	Generalized hyperamino-aciduria		339, 342
	Administration of oestrogens, anabolics, thyreoidin		All amino acids	339, 342
	Administration of contraceptives		Tyrosine, phenylalanine	339, 343
	Administration of anticonvulsants	Generalized hyperamino-aciduria		339, 344
	Administration of antimetabolites	β-Alanine, β-aminoisobutyric acid, homocystine		194
	Administration of liver extracts, Lipovitan, Mucomyst	Artefacts in the area of methionine, tyrosine, cystine, disulphides in ion-exchange chromatography		339, 345
	Excessive protein load	Tyrosine, leucine, methionine, valine, glycine, basic amino acids, methyl-histidines, polypeptides		67, 68, 79, 338

(Continued on p. 188)

TABLE 1 (continued)

Sample	Artefact causing operation	Increased	Decreased	References
	Desalting on a cation exchanger	Glutamic acid	Taurine, cysteic acid, glutamine	26, 29, 346, 348
	Desalting on an anion exchanger	Glutamine	Arginine, lysine, glutamic acid, ethanolamine	26, 29, 346
	Repeated defrosting	Generalized hyperamino-aciduria		26, 29
(C) Capillary blood impregnated on filter-paper cards	Insufficient wetting on the test paper		All amino acids	339
	Excessive wetting of the test paper	Distorted separation of all amino acids		339
	High haematocrit	Distorted separation of all amino acids		339
	High fixation temperature		Phenylalanine, tyrosine, tryptophan, ethanolamine, phosphoethanolamine	29, 339
	Contamination with sweat	All amino acids		339, 340
	Contamination with detergents		All amino acids	339
	Contamination with stool	Proline, glutamic acid, alanine, tyrosine, valine, basic amino acids		29, 33, 339
	Excessive protein load	Tyrosine, valine, methionine, leucine, glutamic acid		67, 68, 338, 339
	Increased relative humidity		Phenylalanine, tyrosine	70
	Decreased relative humidity	Phenylalanine, tyrosine		70
	Aged test papers	All amino acids		339
	Drug administration	Antibiotics, anticonvulsants and corticoids lead to generalized amino acid increase	Oestrogens and anabolics lead to generalized amino acid decrease	Personal experience of one of the authors (J.H.)

conditions can be tolerated without affecting the validity of subsequent analyses.

3.1.2. Urine

For preliminary screening of urinary amino acids, samples of morning urine can be used. As the content of urinary amino acids is subject to variations during the day, 24-h urine must be used for quantitation [80]. In the samples it is necessary to determine the creatinine content and to determine the specific gravity in order to be able to determine the diuresis [29]. Aliquots are usually removed to be stored for control analyses. For urine collection, disposable devices such as Celoplast, U-Bags, Urinokol or Paediatric Urine Collectors may be used. The use of these and similar collecting devices is necessary in order to avoid contamination of urine with stool [33]. Such contamination causes falsely elevated levels of some amino acids owing to the high content of amino acids in stool. Increases in proline, alanine, glutamic acid, tyrosine, valine, methionine and leucine but not hydroxyproline are indicative of such contamination. Old urine samples and samples decomposed by bacterial action are not suitable for amino acid analysis as they usually contain a number of compounds that possess primary amino groups, diverse artifacts and peptidic fragments that affect the amino acid profile [29,35].

3.1.2.1. Conservation of urine before analysis. Changes in the urinary amino acid content are most frequently caused by the action of hydrolases that originate in damaged kidneys or the urinary tract or from bacterial infection. Also purely physical processes such as crystallization or chemical processes such as hydrolysis may affect the concentrations of individual amino acids. In order to eliminate these negative effects, preservatives are usually added to urine samples. The most popular are organic solvents such as toluene, chloroform or 10% thymol (in isopropanol). Alternatively, a few crystals of thymol may be added directly to the urine sample. More modern preservatives include merthiolate (sodium ethylmercurithiosalicylate). Mixtures of organic solvents, e.g., toluene and chloroform, can also be used [26,29,81]. Other biological fluids that may contain bacteria are preserved in a similar way [50]. During collection, the urine sample should be stored in a cold room at a temperature not exceeding 4°C. More conveniently, the samples are stored in a freezer at −25 to −30°C or more preferably deep-frozen at −68°C. The samples are defrosted just before analysis [26]. Repeated melting results in an increase in some amino acid levels on the account of the decomposition of some urinary peptides, decomposed epithelial cells and leucocytes [29,50].

Even if all precautions are taken when storing urine samples, some changes in the amino acid profiles (depending on the storage temperature) still occur; mainly glutamine and asparagine are converted into free amino acids and also the level of free tryptophan decreases.

3.2. Deproteinization of biological samples

Proteins present in plasma, urine or tissues influence considerably the determination of free amino acids. During analysis they may adhere to the start line and distort the separation in planar chromatography or they may adhere to ion

exchangers in columns, causing peak spreading and resulting in an increased column back-pressure [29,82]. Deproteinization of body fluids can be based on chemical or physical principles. Of the chemical methods, precipitation and elution procedures are most frequently used. Thermal fixation, ultrafiltration, dialysis and ultracentrifugation represent the physical approach to this problem. Depending on further work-up of the sample, any of these procedures has its advantages and disadvantages.

3.2.1. Chemical precipitation

Precipitation of proteins by chemical reagents is the oldest deproteinization technique. During the formation of the precipitate, part of the amino acids present in the sample are occluded in the precipitate and therefore it is necessary to wash the precipitated proteins several times, which is time consuming. Also, during precipitation the protein-bound amino acids (hydroxyproline, tryptophan) may be liberated. Therefore, there is no agreement between the results obtained after deproteinization by precipitation and by ultrafiltration.

Sulphosalicylic acid appears to be a very suitable deproteinization reagent. Its advantage is mainly that it needs not to be removed from the supernatant [26,14,83]. During the ion-exchange chromatography of amino acids it moves with the front of the starting buffer and does not interfere with the analysis. The filtrate obtained after sulphosalicylate deproteinization has a pH between 1.0 and 2.0, which is almost ideal for subsequent ion-exchange chromatography. Sulphosalicylate solutions of concentrations up to 20% may be used; when working with less concentrated solutions, the dilution of the sample may present some problems. Therefore, when only small plasma samples are available it is recommended that deproteinization be carried out with solid sulphosalicylic acid. It is preferable to use as little of the precipitating reagent as possible. This, according to Mondino [84], represents 20—50 mg of the substance per millilitre of plasma. When larger amounts are used, the ion-exchange separation of amino acids remaining in the supernatant is distorted; in particular, some critical pairs such as threonine—serine and aspartic acid– methionine sulphone are not resolved.

Although various procedures for deproteinization with sulphosalicylic acid have been described, in our experience deproteinization with 100 mg/ml of solid acid meets best the requirements of precise profiling [85]. For very accurate assays it is necessary to wash the precipitate at least twice with sodium citrate buffer (pH 2.2). Deproteinization with a two-fold amount of diethyl ether and subsequent evaporation of the solvents has been used in the past for the deproteinization of samples, particularly in combination with planar chromatography [86]. Its advantage is the easy evaporation involved. The concentration of ethanol must be at least 80% to ensure good deproteinization. Usually 4– 9 parts of 96% ethanol are mixed with one part of serum or plasma. The mixture is shaken and allowed to stand for at least 10 min, then centrifuged and the precipitate is washed with 0.2–0.3 ml of ethanol. The extract is evaporated to dryness and the residue is dissolved in isopropyl alcohol. A sample prepared in this way can be spotted directly on the starting line of a flat-bed chromatograph. Deproteinization in this way is, however, far from perfect and the losses are considerable, particularly of aromatic amino acids [87,88].

3.2.2. Deproteinization using ion exchangers

In this procedure amino acids are bound to a suitable ion exchanger (e.g., a cation exchanger in the H^+ form such as Chromobead A) in a beaker by shaking the solution with the ion-exchange resin. After about 1 h the mixture is centrifuged, the supernatant containing the proteins is discarded and the ion-exchanger-bound amino acids are released with 0.1 *M* hydrochloric acid. The released amino acids can be loaded directly into a column packed with an ion exchanger. It should be emphasized that taurine and cysteic acid are not released by 0.1 *M* hydrochloric acid and cannot be assayed in this way. The determination of cystine by this procedure gives almost double the yields given by precipitation procedures, probably as a result of absorption of this amino acid on the protein surface.

The extraction of amino acids with a citrate buffer with subsequent deproteinization is in principle analogous to the procedure just described [80]. Serum amino acids are bound to a suitable cation exchanger in the H^+ form and released again with citrate buffer (pH 2.87). By using this delicate procedure it is possible to analyse all of the nineteen most important amino acids, with the exception of taurine. Deproteinization of breast milk may serve as an example of the application of this procedure. The sample is mixed with citrate buffer (pH 1.5), allowed to stand in a cold room for 30 min and centrifuged. The supernatant is removed and the ion exchanger with bound amino acids is loaded on top of an ion-exchange column and eluted with 0.1 *M* hydrochloric acid. According to the comparison of Gerritsen et al. [89] of various deproteinization procedures, this technique shows the best reproducibility and the highest recoveries of amino acids. The only problems arise with tryptophan [89].

In precipitation procedures, losses due to the absorption of amino acids on the protein surface always occur. These losses are particularly important with those amino acids which are present in small amounts (aspartic acid, asparagine, citrulline, ornithine, cystine and tryptophan). Another source of errors may be contamination of the sample from external sources such as with sweat from the hands of the worker.

3.2.3. Physical methods of deproteinization

Of the physical procedures, ultrafiltration appears to be the most popular. Special membrane filters are used for this purpose and the ultrafiltrate is devoid of proteins as judged by, e.g., sulphosalicylate precipitation. The ultrafiltration procedure is slow and therefore it must be speeded up in several ways. The ultrafiltration methods, although simple, are not widely applied in practice as, for unknown reasons, they decrease the retention times during chromatographic separation on ion-exchange columns. This consequently leads to distorted separations of critical pairs of amino acids, particularly serine—threonine, asparagine—glutamine and tyrosine—phenylalanine.

3.2.3.1. Ultracentrifugation. Ultracentrifugation is another means of separating proteins from free amino acids. A thorough comparison of this methodology with precipitation methods was made by Gerritsen et al. [89]. Using α-amino-γ-guanidinobutyric acid as an internal standard, they were able to demonstrate that the recovery given by ultracentrifugation deproteinization is 100%, by precipitation with 1% picric acid 93–98% and by sulphosalicylate precipitation only 77—78%.

3.2.3.2. Deproteinization by thermal fixation on cellulose fibres. The application of filter-paper discs to the analysis of free amino acids as a screening method is due mainly to the possibility of immobilizing the protein fraction on cellulose fibres by increased temperature, acetic acid vapour or drying. For the purpose of the Guthrie and Sussie bacterial inhibition screening test, the use of elevated temperature is preferred as it helps to remove the contaminating microorganisms (even spores) from the material to be analysed [90]. Immobilization of proteins by heating cellulose fibres (paper) is irreversible. However, non-proteinous components are also immobilized to different extents according to their chemical nature. With respect to amino acids, their elution from filter-paper discs is also incomplete, e.g., being 75—80% for phenylalanine with capillary blood and serum. It was also demonstrated that phenylalanine is not destroyed, but is firmly bound to the immobilized protein fraction. Similar effects were seen with urea or uric acid. Another problem here is the anomalous behaviour of haemoglobin, which is incompletely fixed and moves with the solvent front during subsequent elution [91].

Deproteinization with acetic acid vapour appears to be the most delicate means of deproteinization, but it is routinely used only for galactose screening [92].

3.2.4. Deproteinization of urine samples

In normal situations, the low level (about 1%) of urinary protein does not interfere with amino acid profiling. If in pathological situations deproteinization of urine samples is needed, then the application of solid sulphosalicylic acid can be recommended. Usually 50—100 mg of solid substance are used per millilitre of urine. Other workers have used methanol, chloroform or solid picric acid for the same purpose [93].

3.2.5. Deproteinization of tissue samples

Tissue extracts are deproteinized either by the classical procedure of Moore and Stein [82] using 1% picric acid or 4—20% sulphosalicylic acid. The amount of the precipitation reagent should be increased as the content of proteins in tissues is usually high. In the deproteinization of fibroblast cultures in prenatal diagnostics, the application of sulphosalicylic acid at a concentration of 200 mg/ml can be recommended [37]. Liquor can be deproteinized by adding 10% sulphosalicylic acid in a ratio of 1 : 10. After centrifugation or filtration the sample is ready for amino acid analysis [94]. Sweat and liquor can be also deproteinized by adding 25 mg of solid picric acid to 1 ml of the sample [89].

3.3. Desalting

High concentrations of salts bias most of the separation procedures, particularly with large urine samples. Small urine samples can be handled directly, but the quality of separation is never good [95]. The removal of salts by electrolytic desalting is not perfect, as the recovery is only 50—96%, and arginine is converted into ornithine. Acidic compounds such as taurine, phosphoserine and phosphoethanolamine are completely destroyed. Even when used on the micro-scale, electrolytic desalting requires large volumes of urine

and the technique itself is fairly complicated, so that it is not suitable for routine examinations. Equally, extraction of amino acids by organic solvents cannot be recommended as the losses are too high [96].

The most common method of desalting is the application of ion exchangers; strong cation exchangers are used for this purpose [29,95]. The bound amino acids are eluted from the cation-exchange column with a strongly basic, preferably volatile buffer; 0.3—0.5 *M* ammonia solution is excellent for this purpose. When anion exchangers are used for desalting, elution of amino acids is performed with 1% acetic acid or 1 *M* hydrochloric acid. The application of less concentrated eluents should be avoided as they may cause partial fractionation of bound amino acids and consequently give erroneous results. When cation exchangers are used for desalting, highly acidic components such as taurine, cysteic acid, phosphoserine and phosphoethanolamine are not retained.

The column eluate is normally diluted and, in addition to amino acids, it contains a large amount of the eluting reagent. Therefore, the desalted samples are either freeze-dried or evaporated to dryness in vacuo [29]. The latter procedure is the method of choice in routine analyses. Evaporation should be carried out at 40—60°C as higher temperatures may cause losses of the more volatile components of amino acids such as ethanolamine, phosphoethanolamine and aromatic amino acids. Moreover, in the presence of urinary keto acids, transamination giving artefacts may occur at elevated temperatures [26,29].

During the chromatographic analysis of very small urine samples, desalting can be omitted. This is applied in practice in those situations when in screening procedures flat-bed separation is the method of amino acid separation. The urine sample is diluted according to the creatinine content with isopropyl alcohol—water to give a creatinine concentration of 0.5 mg/μl. When 1 μl of this sample is applied to the chromatograph, the procedure replaces desalting [97].

3.4. Special sample treatment before analysis

3.4.1. Oxidation

In those instances where the determination of sulphur-containing amino acids is needed, namely with cystine, cysteine and related disulphides, methionine, homocystine, homocysteine and related disulphides, cystathionine, cysteic acid, lanthonine and penicillamine and its derivatives, oxidation is performed before analysis. The oxidation products of these compounds differ in their retention times and their R_F values can be used for partial identification. These differences are used for the differentiation of valine and methionine in flat-bed separations. Oxidation of biological samples can be carried out either in a test-tube or directly on the starting line of a flat-bed chromatograph. Hydrogen peroxide (30%), either alone or in combination with a solution of ammonium molybdate or formic acid as catalysts, is used for this purpose [13, 26, 29, 98].

3.4.2. Hydrolysis

Routine hydrolysis, both acid and alkaline, is used mainly for differentiating urinary peptides from amino acids. Although in most instances acid hydrolysis is preferred, the choice is not arbitrary. The determination of γ-carboxy-

glutamic acid, related to calcification processes in tissues, which under the conditions of acid hydrolysis is converted into glutamic acid whereas it is preserved in alkaline media, may serve as a good example [99]. Further, hydrolysis is necessary in situations where protein-bound amino acids are to be determined or when acetylated amino acids such as N-acetyl-L-lysine or phenylacetylglutamine are to be assayed [100].

3.4.3. Extraction of lipidic components

Lipids and related compounds, if present at higher concentrations, give a considerable bias in the chromatographic separation of amino acids and in hyperlipaemic serum or in breast milk they have to be removed by extraction prior to analysis. Typically, the desalted sample is evaporated to dryness and the residue dissolved in light petroleum, diethyl ether or chloroform. In all these procedures considerable losses of amino acids occur owing to their solubility in non-polar solvents [101].

3.4.4. Removal of ammonia

In most chromatographic procedures applied to urine samples the high concentrations of ammonia distort part of the chromatogram. Typically in ion-exchange chromatography the separation of basic amino acids is poor in the presence of an excessive amount of ammonia. Removal of ammonia is effected routinely by adding 1 *M* sodium hydroxide solution to give a pH of 10—12 and placing the sample in a desiccator over concentrated sulphuric acid under low pressure [102]. Removal of ammonia with tetraphenylboron has many advantages.

4. LIQUID CHROMATOGRAPHY

4.1. Column liquid chromatography

As stressed already in Section 2, there are generally two ways to carry out amino acid analysis. One that involves specialized amino acid analysers uses pellicular cation exchangers and gradient buffer elution with different post-column derivatization procedures (*o*-phthalaldehyde, 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole and ninhydrin being the common ones). The other approach exploits advanced HPLC equipment, using reversed-phase systems in which pre-column derivatized amino acids are separated. The detection itself is either spectrophotometric or more recently fluorimetric, although other methods of detection can be also occasionally applied.

A typical setup of an advanced single-purpose amino acid analyser is shown in Fig. 1. It consists of a set of buffer solution reservoirs, a reservoir for the regeneration solution and a set of solenoid valves that are operated by a programmer. A buffer pump and autoloader ensure sample application and subsequent elution of separated amino acids. Separation is carried out in a thermostated ion-exchange column. From a separate ninhydrin reservoir the reagent is delivered to a mixing block through a separate ninhydrin pump; the eluate mixed with the reagent passes the reaction coil and the detector. The recorder and the programmer are attached to a data system unit, which is essentially

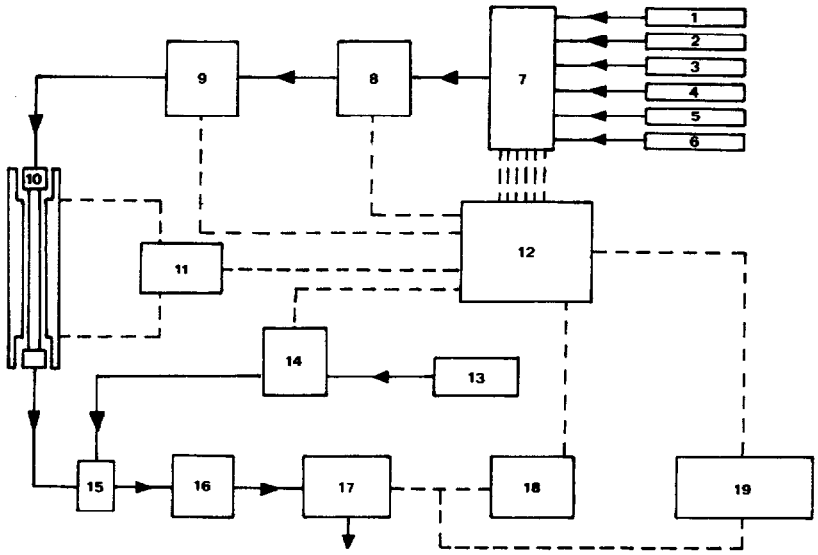


Fig. 1. Schematic representation of an advanced single-purpose amino acid analyser. 1—5 = Buffer solution reservoirs; 6 = reservoir for the regeneration solution; 7 = set of solenoid valves; 8 = buffer pump; 9 = autoloader; 10 = ion-exchange column; 11 = solid heating device (thermostated); 12 = programmer; 13 = reservoir for ninhydrin solution; 14 = ninhydrin pump; 15 = mixing block; 16 = reaction coil; 17 = photometer; 18 = recorder; 19 = Ultrodata system (integrator and computer).

a computing integrator that produces reports of retention times, peak areas, calibration factors and concentration units. The use of such specialized amino acid analysers has in practice the advantage that the purchaser acquires an instrument with an optimized performance. The disadvantages are the higher costs and the increasing likelihood that, owing to the integrated approach, it may not be possible to operate the individual units separately in the future.

Another approach may be to use some of the sophisticated HPLC equipment available today and to use it for the separation of amino acid derivatives. This again may be acquired as an integrated unit or assembled from modular units designed to work independently. The disadvantages with the integrated units are the same as with the single-purpose amino acid analysers, namely that one obtains an optimized instrument at a higher price and at the risk that its individual parts may not be suitable for separate operation.

No matter which approach is chosen, automated sample loading, automated processing of the chromatograph and automated processing of the results are highly desirable today in laboratories dealing with clinical amino acid analysis. A description of all this equipment is clearly beyond the scope of this review, and the reader is directed to more specialized compilations [103—105].

As far as the sorbents are concerned, in ion-exchange separations pellicular strongly acidic, presumably sulphonated polystyrene-based cation exchangers are used that are available under a number of commercial names already in pre-packed form, such as Ultropac 8, Kyowa gel and the Perkin-Elmer high-speed amino acid analysis column. In the separation of amino acid derivatives the choice, with few exceptions, is limited to reversed-phase C_{18}-based systems.

The detection modules are mostly spectrophotometric or fluorescence detectors; electrochemical detection is rare. In those situations where underivatized amino acids are separated, post-column reactors are inserted between the column effluent and the detector. These are predominantly coiled tubular reactors to which the desired reagent solution is supplied by a separate pump. Although considerable advances have been achieved in order to minimize post-column spreading [106], packed bed reactors are occasionally encountered. The borderline between the ion-exchange separation of underivatized and the reversed-phase separation of derivatized amino acids is occasionally crossed. As shown by Zakaria et al. [107], elevated levels of aromatic amino acids are observed in some neoplasias in blood and in the evaluation of hypertyrosinaemia and phenylketonuria; in these situations the changes in amino acid profiles are followed concomitantly with monitoring of elevated levels of nucleosides and bases (uridine, adenosine, inosine, guanosine, hypoxanthine and xanthine). Separation may be carried out in the reversed-phase mode (Partisil ODS, 5 cm × 3.9 mm I.D. column) with an ionic strength gradient. Clear separations of tyrosine and phenylalanine from the nucleosides and bases is obtained. Optical detection at 254 nm is applied. Aromatic amino acids can be assayed directly in blood or urine after careful deproteinization by reversed-phase chromatography [108] using methanol—50 m*M* KH_2PO_4 (pH 4) (13:87) and UV detection.

Of combinations with other chromatographic techniques one can mention first planar (thin-layer) chromatography which, particularly owing to its cheapness, has retained a firm position in preliminary screening procedures. Other approaches, such as LC—MS coupling, are certainly promising as they may reveal yet unknown amino acid derivatives or metabolites and lead to the discovery of an additional (already fairly large) number of amino acid metabolism disorders. However, as emphasized at the beginning of this review, these are not common situations in practice.

4.1.1. Pre-column derivatization

4.1.1.1. The o-phthalaldehyde procedure. Derivatization of amino acids with *o*-phthalaldehyde reagent offers the advantage over the other types of fluorescence derivatization reactions of the use of a non-fluorescent derivatization reagent. It is therefore well suited to the analysis of complex biological mixtures (for a review, see Lee and Drescher [109]). In principle, the *o*-phthalaldehyde reaction can be carried out either before or after the chromatographic separation. The main disadvantage of the procedure is, however, the inability of *o*-phthalaldehyde to form fluorescent derivatives with secondary amines unless the reaction is carried out in the presence of oxidative reagents.

It is worth mentioning that the derivatization procedure itself is very simple: 50 μl of plasma are typically used for analysis, deproteinized with methanol and, after centrifugation, reacted with four volumes of *o*-phthalaldehyde (OPA) reagent. After standing at room temperature for 2 min, the solution is ready for analysis. Cerebrospinal fluid and gastric juice have to be diluted 5—10-fold for optimal results.

In the pre-column derivatization procedure, separation is conveniently carried out in the reversed-phase mode (C_8 or C_{18} [110]) using either fluores-

cence or electrochemical detection. Pre-column derivatization of amino acids with *o*-phthalaldehyde appears to be a very reliable method, but its reproducibility and the separation are considerably dependent on the age of the column material. The columns usually do not withstand more than 200 runs even if all possible precautions are taken. The reaction product is only moderately stable, but nevertheless it still allows operation in the post-column derivatization mode. Wiedmeier et al. [111] stated that no decrease in fluorescence was observed 96 h after the derivatization reaction was completed. On the other hand, *o*-phthalaldehyde adducts with amino acids degrade at different rates, which precludes the use of an automatic sampler. Because of this, the use of an automated pre-column derivatization device is recommended.
A detailed study was made of the on-column stability of *o*-phthalaldehyde derivatives during reversed-phase separations [112].

As shown by Linderoth and Mopper [113], *o*-phthalaldehyde derivatives are well retained and resolved on reversed-phase columns. Several workers [114, 115] have applied this procedure to the determination of amino acid profiles in diverse biological samples. In all these situations fluorescence detection (excitation at 365 nm with a 470 nm cut-off filter) was applied. However, the isoindole group that arises during the reaction of the *o*-phthalaldehyde reagent with amino acids also exhibits electrochemical activity that can be used for electrochemical detection in the 0.4—0.7 V range. With most, but not all, amino acids this mode of detection offers a higher sensitivity than fluorescence measurement.

As mentioned, the separation is conveniently carried out in the reversed-phase mode. Isocratic elution with 0.1 *M* phosphate buffer—methanol (1:1) as the mobile phase offers a good resolution of α-aminobutyric acid, tryptophan, methionine, valine, phenylalanine, isoleucine and leucine, i.e., it resolves the more hydrophobic amino acids.

For more extensive profiling of amino acids, gradient elution is essential. Acetonitrile, methanol or tetrahydrofuran, or other organic modifiers, can be used for gradient formation. Using methanol, typically 0.05 *M* sodium phosphate buffer (pH 5.5)—methanol (80:20) for solvent A and a ratio of 20:80 for solvent B can be used. A low pH of the buffer is necessary in order to resolve histidine from glutamine and γ-aminobutyric acid from alanine. The solvent programme is 0—10% B for the first 10 min and 10—85% B for the next following 30 min, then the column is returned to the original state by running 85—0% B for 5 min followed by re-equilibration in A. For other gradient types see, e.g., Linderoth and Mopper [113]. The sensitivity is fairly high; good peaks are obtained with a standard amino acid mixture containing 400 pmol of each amino acid. In natural samples 1 μl of plasma is needed for a run in both the isocratic mode and the gradient system. This procedure has been applied successfully not only to the separation of plasma amino acids but also to the profiling of amino acids occurring in the brain or its different regions, and the profiling of amino acids in brain perfusates or gastric juice is also possible.

Typical results obtained with an acetonitrile gradient were described by Krishnamurti et al. [116]. The column used was μBondapak C_{18} (300 × 3.9 mm I.D.), eluted with a gradient of phosphate buffer (75—25% solvent A over 50 min); 15 m*M* phosphate—25.9 m*M* sodium (pH 7.2) served as the

solution A of the gradient system. Solution B contained almost equal amounts of the phosphate buffer (however, of about double the concentration in solvent A) and acetonitrile (buffer: acetonitrile ratio = 244:256). A slight variation of the acetonitrile gradient system was reported by Fernstrom and Fernstrom [117]. Here two buffers were used, buffer A consisting of 15 m*M* sodium phosphate (pH 7.2) and buffer B of 45% buffer A and 55% acetonitrile. The gradient is specified in Table 2. This gradient, however, is unable to resolve threonine and glycine, and if these two amino acids are to be separated a

TABLE 2

COMPOSITION OF THE BUFFER GRADIENT USED FOR THE SEPARATION OF AMINO ACIDS IN PHYSIOLOGICAL FLUIDS USING PRE-COLUMN DERIVATIZATION WITH *o*-PHTHALALDEHYDE

Reproduced from Fernstrom and Fernstrom [117] with permission.

Time (min)	Buffer A (%)	Buffer B (%)	Gradient
0 (initial)	90	10	Linear
50	40	60	Linear
65	10	90	Linear (reverse)
70	90	10	

TABLE 3

EFFECT OF TEMPERATURE ON THE RELATIVE RETENTION TIMES (t_R) OF PRE-COLUMN OPA AMINO ACID DERIVATIVES

Reproduced from Krishnamurti et al. [116] with permission.

Amino acid	t_R at different column temperatures					
	22—24°C			28°C*		
	Mean**	S.D.	C.V. (%)	Mean***	S.D.	C.V. (%)
Asp	0.070	0.006	9.2	0.062	0.001	2.2
Glu	0.103	0.007	6.3	0.080	0.002	2.9
His	0.251	0.004	1.4	0.209	0.011	5.4
Ser	0.328	0.002	0.6	0.255	0.015	5.9
Thr	0.371	0.004	1.2	0.313	0.017	5.3
Gly	0.430	0.001	0.3	0.367	0.018	4.9
Tyr	0.445	0.003	0.7	0.432	0.009	2.0
Ala	0.531	0.004	0.8	0.484	0.023	4.7
Arg	0.596	0.005	0.8	0.537	0.011	2.1
Lys	0.632	0.004	0.7	0.601	0.008	1.4
Val	0.786	0.006	0.7	0.742	0.028	3.7
Met	0.827	0.005	0.6	0.799	0.009	1.1
Ile	0.921	0.003	0.4	0.912	0.004	0.4
Leu	0.968	0.003	0.3	0.970	0.000	0.0
Phe§	1.000	0.000	0.0	1.000	0.000	0.0
Ethanolamine§§	1.402	0.006	0.4	1.359	0.010	0.7

*All the t_R values at 28°C were lower than those at 22—24°C ($P < 0.05$).
**Calculated from three measurements during 1 week at 22– 24°C.
***Calculated from 26 measurements over a 2-month period at 28°C.
§Phenylalanine taken as unity.
§§Ethanolamine used as an internal standard.

separate buffer system has to be applied. Separations were carried out at constant temperature (28°C) and the fluorescence detector was set to 338/425 nm.

An elevated temperature appears to be important for the separation of some critical pairs of amino acids, as follows from Table 3. This is particularly true for the separation of tryptophan and leucine. Increasing the temperature of separation from 22 to 28°C decreases significantly ($p < 0.05$) the retention of all amino acids. Under the conditions described cystine is not resolved as it has to be converted into a derivative of iodoacetic acid in order to form a fluorescent adduct. Secondary amino acids such as proline are known not to form *o*-phthalaldehyde derivatives at a sufficient speed. Citrulline, asparagine and 3-methylhistidine were incompletely resolved from glycine, histidine and tyrosine, respectively. The retention times of glutamine, 1-methylhistidine and tryptophan relative to phenylalanine were 0.27, 0.41 and 0.94.

The technique was used for the amino acid profiling of foetal and adult sheep plasma, liver, diabetic rat plasma as such and after insulin treatment, human and dog cerebrospinal fluid, muscle and animal feeds [116].

The variable stability of the fluorescence of *o*-phthalaldehyde derivatives has been the subject of several studies [117—119]. In order to minimize the variations, it has been suggested that the time between derivatization and injection be kept constant. Under such circumstances the molar responses of fluorescent *o*-phthalaldehyde derivatives summarized in Table 4 can be expected. Generally, the fluorescence intensities of the *o*-phthalaldehyde derivatives of glycine and lysine are relatively high, whereas those of aspartate, glutamate and threonine are low. There is a discrepancy between the data published by Krishnamurti et al. [116] and Hogan et al. [120]; the latter authors observed

TABLE 4

MOLAR RESPONSE OF FLUORESCENT OPA AMINO ACID DERIVATIVES

Reproduced from Krishnamurti et al. [116] with permission.

Amino acid	Response (pmol)	Response relative to glycine	Rank*
Asp	3.883	0.021	15
Glu	11.859	0.065	14
His	41.461	0.228	6
Ser	38.128	0.210	9
Thr	13.047	0.072	13
Gly	181.976	1.000	1
Tyr	42.005	0.231	5
Ala	42.231	0.232	4
Arg	28.546	0.157	10
Lys	71.795	0.395	2
Val	27.259	0.150	12
Met	49.354	0.271	3
Ile	39.723	0.218	8
Leu	40.487	0.223	7
Phe	27.562	0.152	11

*Ranking from highest (1) to lowest intensity (15).

TABLE 5

FLUORESCENT RESPONSE OF OPA AMINO ACID DERIVATIVES AND PRECISION OF DETERMINATION

Reproduced from Krishnamurti et al. [116].

Amino acid	Fluorescent response		Standard error of the estimate (%)	Precision, C.V. (%)	
	Regression equation*	Coefficient of determination (r^2)		Within-run**	Between-run***
Asp	$y = 106.7x - 132.6$	0.967	0.155	3.0	6.1
Glu	$y = 184.8x + 101.4$	0.992	0.076	4.5	6.6
His	$y = 664.9x + 895.8$	0.977	0.068	6.3	5.6
Ser	$y = 569.8x + 274.4$	0.983	0.114	2.4	4.4
Thr	$y = 383.5x + 550.2$	0.959	0.164	6.3	5.3
Gly	$y = 3332.0x + 47.0$	0.999	0.049	3.3	7.0
Tyr	$y = 734.1x - 193.2$	0.995	0.083	8.4	5.7
Ala	$y = 601.8x + 792.6$	0.976	0.060	2.2	3.6
Arg	$y = 533.3x - 86.4$	0.999	0.030	4.0	3.9
Lys	$y = 1545.2x - 784.8$	0.993	0.117	1.8	6.8
Val	$y = 499.2x + 11.4$	0.999	0.060	3.6	5.4
Met	$y = 946.8x - 334.8$	0.999	0.032	1.5	6.1
Ile	$y = 478.2x + 678.4$	0.921	0.202	7.7	5.0
Leu	$y = 608.2x + 530.6$	0.987	0.061	2.3	3.6
Phe	$y = 638.1x - 514.0$	0.987	0.084	3.2	4.3

*Regression equation in the form $y = mx + b$, where y is the peak area, m is the slope, x is the amino acid concentration and b is the intercept on the y-axis.

**Within-run coefficient of variation is based on three injections of a standard mixture of amino acids on the same day.

***Between-run coefficient of variation is based on the mean coefficients of variation of six days over a 2-month period with $n = 14$.

a high response for the lysine derivative whereas the opposite was observed by Krishnamurti et al.

If peak areas are used as the basis for quantitation, the regression equations developed from the fluorescence response—concentration dependence for each amino acid summarized in Table 5 can be obtained.

Instead of the acetonitrile gradients, very good amino acid profiles can be obtained with gradients based on tetrahydrofuran—methanol. Using an Ultrasphere ODS or Resolve C_{18} column (250 × 4.6 mm I.D.), Qureshi et al. [121] were able to separate up to 23 free amino acids present in physiological fluids. The columns were conditioned in methanol—water (70:30) before use. With the Ultrasphere ODS system the gradient was created from solution A consisting of 20 m*M* phosphate buffer (pH 7.4)—tetrahydrofuran—methanol (90:1:9) and solution B consisting of methanol—20 m*M* phosphate buffer (pH 7.4) (75:25). The separation was carried out at ambient temperature with a flow-rate of 1 ml/min. The results are shown in Fig. 2. With the Resolve C_{18} column the recommended gradient consisted of solution A, which was 50 m*M* phosphate buffer (pH 7.4)—tetrahydrofuran—methanol (85:1:14), and solution B, consisting of 50 m*M* phosphate buffer—methanol (28:72). The time course of the gradient and the resulting amino acid profile are shown in Fig. 3.

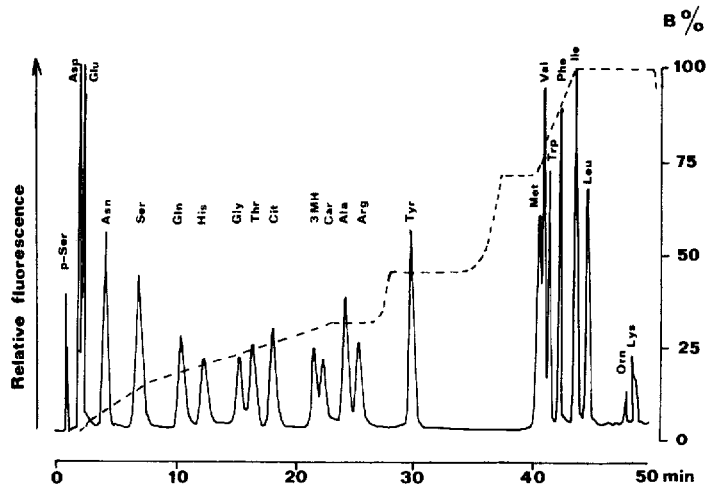


Fig. 2. Chromatographic separation of a series of *o*-phthalaldehyde amino acid derivatives. Details of separation conditions are given in the text. p-Ser = phosphoserine. Amount loaded, 10 μmol/ml. (From ref. 121 with permission.)

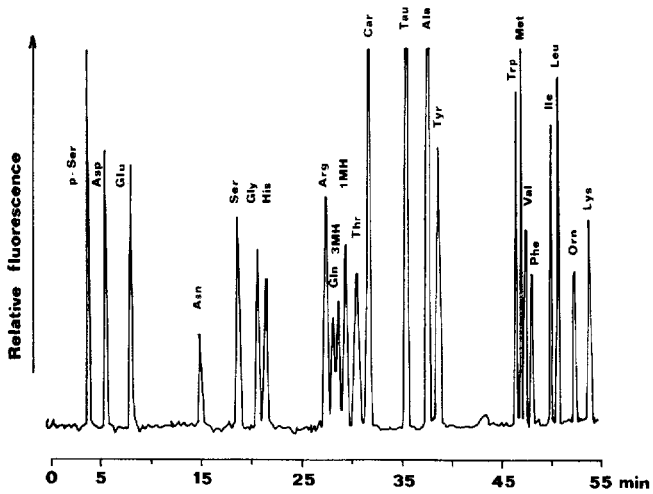


Fig. 3. Chromatographic profile of plasma of a uraemic patient spiked with 44 amino acids (as *o*-phthalaldehyde derivatives). Amount loaded, 5 μmol/ml. p-Ser = phosphoserine. (From ref. 121 with permission.)

Alternatively with the tetrahydrofuran gradient good resolutions can be obtained using the following system according to Hogan et al. [120]. In this instance the gradient programme consisted of two separate solvent systems, solvent A being tetrahydrofuran–methanol—0.05 *M* sodium acetate solution (pH 5.9) (8:192:800) and solvent B methanol—0.05 *M* sodium acetate solution (pH 5.9) (8:2). Solvent A was introduced for 1 min from the beginning of the programme, followed by a linear step to 14% solvent B within 5 min, then by an isocratic step at 14% solvent B for 7 min, a linear step to 60% solvent B in 9 min, followed by a linear step to 75% solvent B within 6 min and finally a linear step to 100% solvent B within 5 min (at a flow-rate of 1.5 ml/min). The procedure was used for amino acid profiles in human

blood. Jones and Gilligan [122] obtained profiles of *o*-phthalaldehyde-derivatized amino acids from human serum, adult urine and cerebrospinal fluid. Chromatographic separation was carried out in the reversed-phase mode using a 3 μm Ultrasphere ODS column (75 × 4.6 mm I.D.) at a flow-rate of 1.5 ml/min. The mobile phase used was based on the tetrahydrofuran—methanol system: solvent A was tetrahydrofuran—methanol—0.1 *M* potassium acetate solution (pH 7.2) (5:95:900) and solvent B was methanol. The proportions of the two solvents are evident from Fig. 4. The quantitative data presented in Table 6 provide some idea of the occurrence of different amino compounds in biological fluids.

The advantage of replacing methanol with acetonitrile in the gradients is a decrease in the back-pressure; however, this is achieved at the expense of lower selectivity. For instance, the separation of glycine and threonine may cause some problems.

In another variation of the separation of *o*-phthalaldehyde derivatives, elution is carried out with a complex gradient prepared from water–sodium propionate buffer (250 mmol of propionic acid and 350 mmol of anhydrous disodium hydrogen phosphate per litre)—acetonitrile (70:20:8) and from water—acetonitrile—methanol—dimethyl sulphoxide (42:30:25:3). The gradient programme is shown in Fig. 5. The reported between-run coefficients

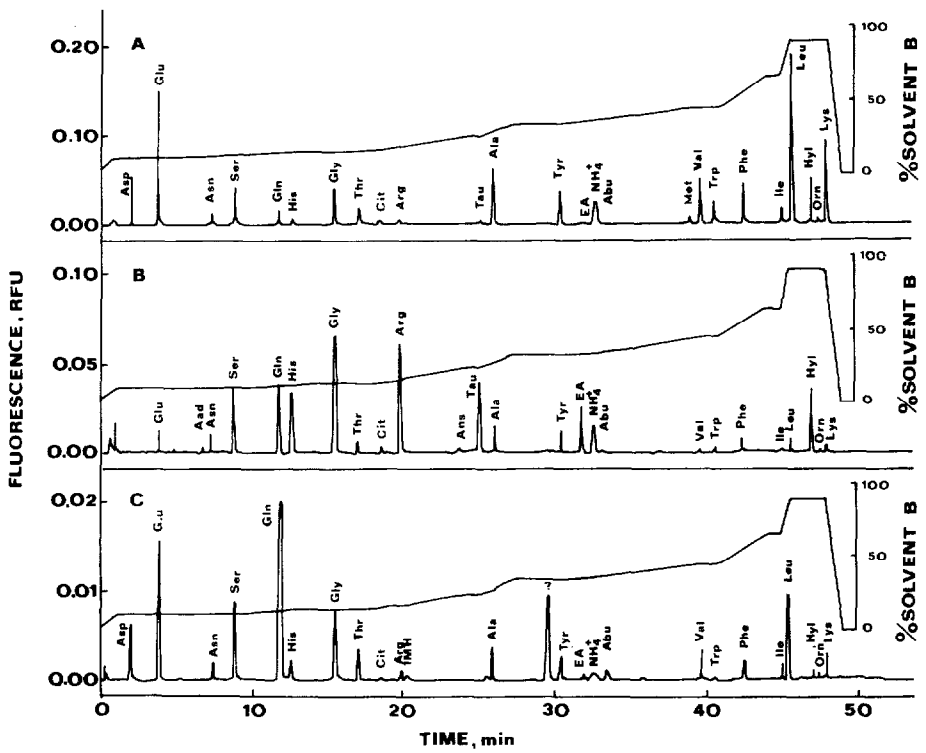


Fig. 4. Elution profiles of OPA-derivatized amino acids and biological amines from (A) adult serum, (B) adult urine and (C) rat CSF. The chromatography was performed on a 3-μm particle size Ultrasphere ODS column (75 × 4.6 mm I.D.) at a flow-rate of 1.5 ml/min. Hyl = hydroxylysine. (From ref. 122 with permission.)

TABLE 6

QUANTITATION OF AMINO ACIDS IN SERUM, URINE AND CEREBROSPINAL FLUID (CSF)

Reproduced from Jones and Gilligan [122] with permission.

Amine	Serum (nmol/ml)	Urine (nmol/ml)	CSF (nmol/ml)
Aspartic acid	38.85	—*	2.78
Glutamic acid	155.87	4.43	21.79
α-Aminoadipic acid	—*	3.95	—*
Asparagine	15.47	17.16	4.28
Serine	63.65	73.49	27.46
Glutamine	—*	104.32	398.94
Histidine	29.23	248.30	12.41
Glycine	94.51	253.39	34.75
Threonine	54.01	23.59	24.53
Citrulline	14.73	35.17	1.67
Arginine	22.36	356.20	5.83
1-Methylhistidine	—*	—*	0.60
3-Methylhistidine	—*	—*	—*
β-Alanine	—*	—*	—*
Anserine	—*	30.49	—*
Taurine	3.33	322.82	—*
Alanine	176.72	49.89	20.44
γ-Amino-*n*-butyric acid	—*	—*	—*
γ-Aminoisobutyric acid	—*	—*	—*
Tyrosine	40.51	19.96	6.61
Ethanolamine	4.66	41.23	1.13
α-Amino-*n*-butyric acid	0.71	6.14	2.12
Methionine	9.03	—*	—*
Valine	107.59	10.25	8.80
Tryptophan	28.15	11.71	1.13
Phenylalanine	66.72	9.56	5.71
Cystathionine	—*	—*	—*
Isoleucine	15.73	5.67	3.12
Leucine	176.72	11.70	11.22
Hydroxylysine	62.81	56.90	46.80
Ornithine	41.20	20.58	5.74
Lysine	206.82	37.44	29.79

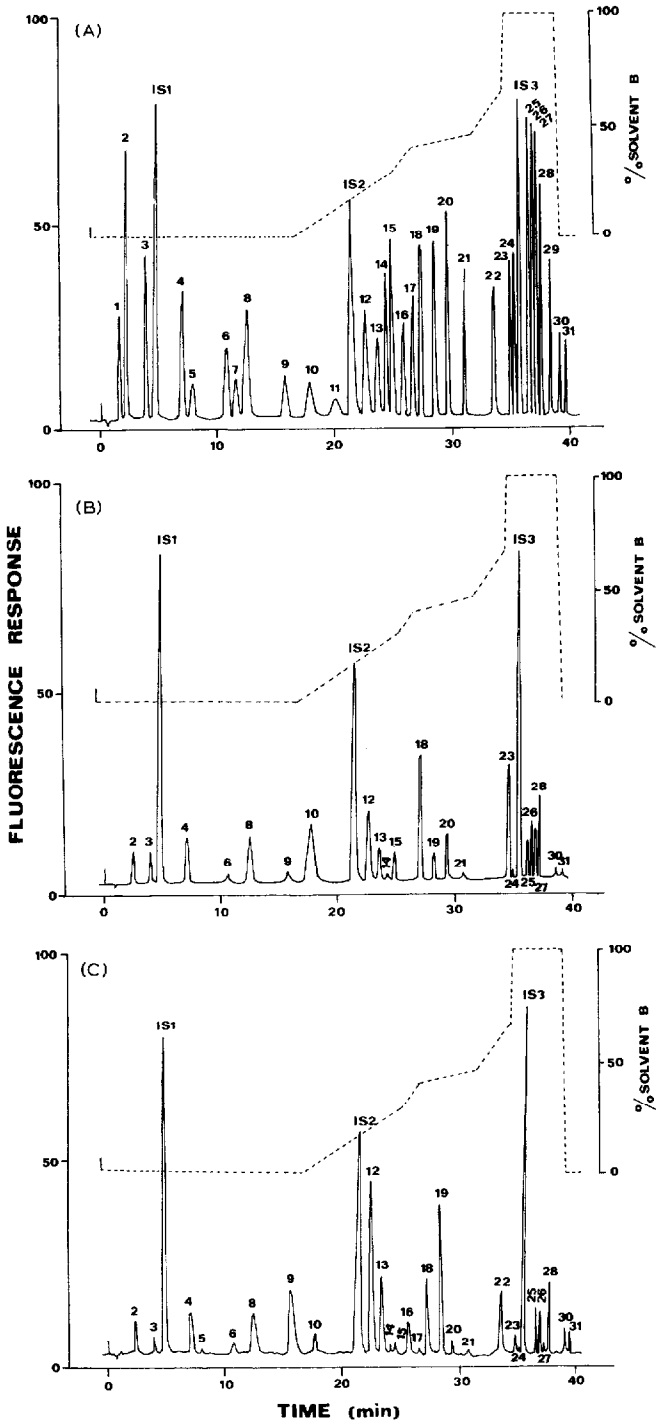
*Measurable peak not detected.

of variation ranged from 2.0 to 13.5% and the mean analytical recovery was 101%. The method is applicable to both urine and blood profiles with a detection limit of 38 fmol.

As mentioned already, *o*-phthalaldehyde derivatives can also be used in shortened versions of profiling when only a few amino acids are to be followed. For instance, as an aid in branched-chain ketoaciduria interest may be limited to the separation of valine, phenylalanine, isoleucine and leucine. The advantage of applying the *o*-phthalaldehyde procedure here is mainly the speed of analysis, which allows one to monitor the normalization of the plasma amino acid profiles during medical treatment (peritoneal dialysis).

o-Phthalaldehyde derivatives of amino acids can be used for the determination of free amino acids in tissues. The sensitivity of the method allows small

biopsy samples (10 mg) to be used. A typical procedure of this type was described by Godel et al. [123]. It differs from the above-described routines in two respects: the derivatization procedure is more thorough to ensure maxi-



mum reaction yields and the applied gradient is more complex. When using a Spherisorb II ODS (250 X 4.6 mm I.D.) column the profiles of free amino acids in plasma, striated muscle and liver can be obtained.

In order to maximize the precision and convenience of *o*-phthalaldehyde derivative profiling, Smith and Panico [124] developed an automated procedure that also eliminated the problems of degradation of the products formed. The instrumentation for such an amino acid analysis is shown in Fig. 6. A vial containing the derivatizing reagent is placed in position 1 of the autosampler. Typically, unknown samples and standards are placed in the rack in such a way that a standard will be analysed before and after every four samples. The derivatization is carried out according to the Waters Assoc. Auto TAG-OPA procedure. After equilibration of the whole system with the starting solvent, the flow is stopped for a few minutes to allow the pressure in the injection system to decrease, then an aliquot (5 μl) of the OPA solution is injected followed immediately by the same volume of the sample (or standard). The flow-rate is then increased to 0.1 ml/min for 2 min, during which the reaction mixture passes through a glass bead column where the derivatization reaction and mixing occur. The flow-rate is then increased to 1 ml/min and gradient elution of the *o*-phthalaldehyde derivatives formed is carried out. In the system described, the digitized output from the fluorescence detector is stored on a

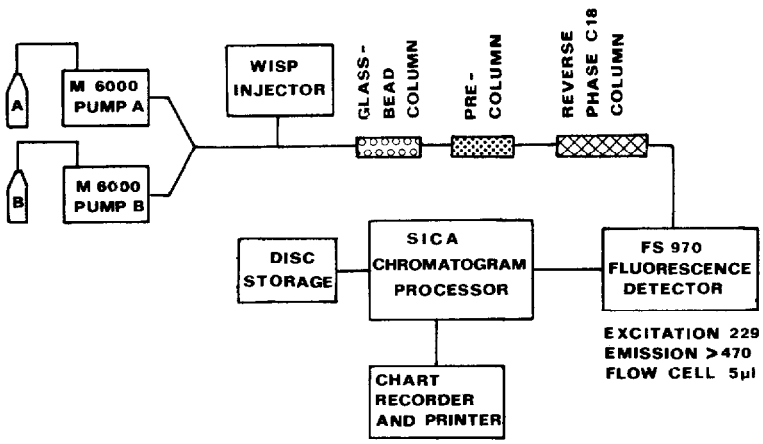


Fig. 6. Schematic outline of the automated system for the pre-column derivatization and separation of *o*-phthalaldehyde amino acid derivatives. (From ref. 124 with permission.)

Fig. 5. Amino acid profile obtained with pre-column derivatization with *o*-phthalaldehyde reagent. Separation in reversed-phase mode. (A) Standard solution containing 500 μmol/l of each amino acid; (B) adult serum; (C) adult urine. The gradient is indicated by the dashed line; for buffer composition, see text. IS refers to internal standards: IS1 = homocysteic acid; IS2 = homoserine; IS3 = norvaline. Peaks: 1 = phosphoserine; 2 = aspartic acid; 3 = glutamic acid; 4 = cystine; 5 = α-aminoadipic acid; 6 = asparagine; 7 = homocystine; 8 = serine; 9 = histidine; 10 = glutamine; 11 = ethanolaminephosphoric acid; 12 = glycine; 13 = threonine; 14 = citrulline; 15 = arginine; 16 = 3-methylhistidine; 17 = β-alanine; 18 = alanine; 19 = taurine; 20 = tyrosine; 21 = α-amino-*n*-butyric acid; 22 = ethanolamine; 23 = valine; 24 = methionine; 25 = tryptophan; 26 = phenylalanine; 27 = isoleucine; 28 = leucine; 29 = hydroxylysine; 30 = ornithine; 31 = lysine. (From ref. 115 with permission.)

disc and processed during the equilibration phase between individual runs. Individual amino acid peaks are identified by a two-step analysis of the chromatogram, the first covering the first 15 min of the run and the other the remainder. Reference peaks are identified in each of the two parts (glycine and valine) and the standard retention times are adjusted in accordance with the retention times found (correction for retention shift). The peaks are integrated and the peak areas are stored in the computer memory until a set of four analyses and two standards have been collected, then a correction for the changes in the internal standard peak areas is introduced. The corrected concentration factor for each peak is calculated and unknown sample data are finally corrected for dilution during preparation for chromatography. The coefficients of variation reportedly vary from 2.53 to 5.20%, being highest for aspartic acid and lowest for leucine.

Another detailed study of on-line pre-column derivatization with *o*-phthalaldehyde was reported by Fleury and Ashley [125]. In addition to the excellent resolution achieved by a complex gradient prepared from a sodium phosphate buffer and tetrahydrofuran, this work is particularly important as it compares the fluorescence decay of the *o*-phthalaldehyde derivatives in successive runs after a simple or automated derivatization.

4.1.1.2. The dansyl chloride procedure. The large amount of information that is available in the literature on the dansyl chloride amino acid reaction is clearly beyond the scope of this review, and we shall refer only to possible applications of this method to the separation and quantitation of amino acids in biological samples.

Briefly, several problems are encountered in the preparation of dansyl derivatives. The formation of dansyl derivatives of amino acids involves a series of reactions and the product yields are clearly dependent on the relative amounts of dansyl chloride and amino acids and on the incubation conditions. If this ratio is in the 1000 range then the formation of the derivatives is favoured over their decomposition. Also, the pH and temperature have to be controlled to optimize the product yield. An objection to the use of dansyl chloride for amino acid derivatization is that multiple derivatives of several amino acids may be formed. However, the conditions of the reaction may be selected in such a way that the formation of multiple derivatives does not pose a problem.

For the analysis of biological samples 1 g of tissue (brain, liver) or 1 ml of serum is required. After homogenization and deproteinization, aliquots (0.1 ml) of the extracts are mixed with 0.5 *M* sodium hydrogen carbonate solution (pH 8.5). Then 25 μl of the dansyl chloride reagent (6 mg/ml in acetone) are added (the pH should be 9.2—9.5 after the addition of the reagent) and the derivatization is carried out within 3–4 h in the dark. Then 0.8 ml of water is added, the precipitate is spun off and 50 μl of the supernatant are loaded on to the column.

Alternatively, the preparation of dansyl derivatives from body fluids can be carried out by the method described by Tapuhi et al. [126]. To 50 μl of cerebrospinal fluid (or other body fluid), 25 μl of saturated lithium carbonate solution and 50 μl of dansyl chloride solution in acetone (250 μg/ml) are added stepwise. The reaction vial is heated at 60°C for 15 min and, after cooling, an aliquot is injected into the chromatograph without further treatment.

Separation is typically carried out in the reversed-phase mode (Ultrasphere ODS, 5 μm, 250 X 4.6 mm I.D. column) using two mobile phases for gradient formation: buffer A consisting of 10 m*M* sodium acetate (pH 4.18)—tetrahydrofuran (95:5), and buffer B, consisting of acetonitrile—tetrahydrofuran (90:10). The mobile phase starts at 10% B and is increased to 40% B within 30 min (flow-rate 1.0 ml/min). Thereafter the separation is carried out isocratically. A typical example of the profiles that may be expected is shown in Fig. 7. Another variant suitable for plasma and saliva amino acid profiling is that published by Bongiovanni et al. [127] and Miller et al. [128].

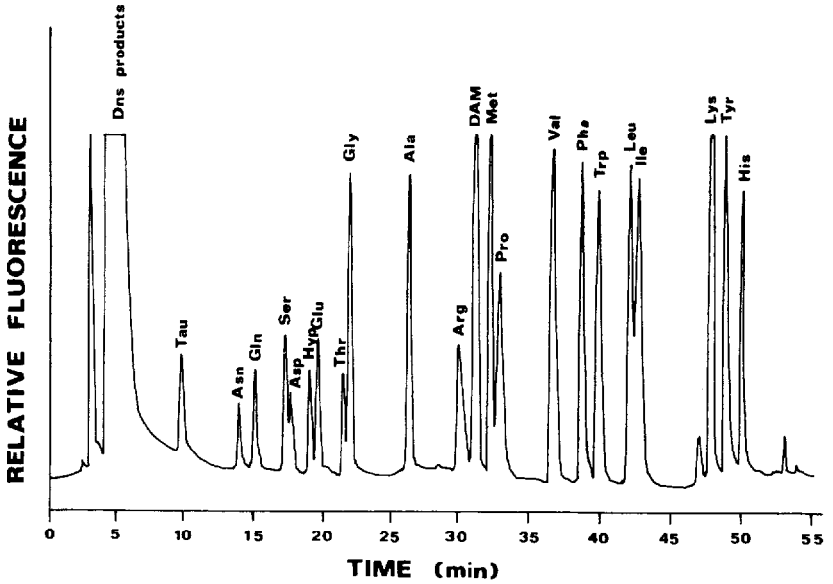
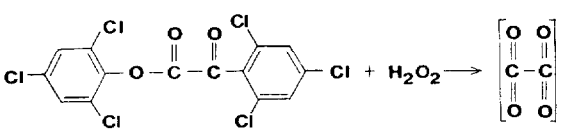


Fig. 7. Chromatographic profile of dansylated amino acids. Amount loaded, 0.5 nmole of each amino acid. Gradient from 10% B to 40% B over 30 min, then isocratic elution at 40% B. (A) 10 m*M* sodium acetate buffer (pH 4.18)—tetrahydrofuran (95:5); (B) acetonitrile—tetrahydrofuran (90:10). DAM = dansyl-amide. (From ref. 111 with permission.)

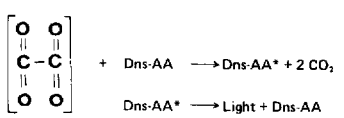
As indicated in many places in this review, conventional photoinduced fluorescence has found wide application in amino acid analysis. Generally, however, it suffers from fluctuations and stray radiation from the light source, which affects the fluorescence intensity and hence the sensitivity of detection. Recently a chemiluminescence system combined with HPLC of dansyl amino acids appeared to overcome this problem [129].

The chemiluminescence reaction scheme is as follows, an asterisk indicating the excited state:



2,4,6-trichlorophenyl oxalate

1,2-dioxethanedione



Imidazole buffer (0.1 *M*, pH 7.0, NO_3^-)—acetonitrile (90:10) and (55:45) were used for gradient formation as solvents A and B, respectively, and TSK gel type ODS 120 A was used as the sorbent in this particular instance. The solvent system is a modification of that described by Grego and Hearn [130] but has the disadvantage of not separating the derivatives of aspartic and glutamic acid. This, however, was done in order to avoid problems that may arise with baseline drift of the chemiluminescence system with an increasing proportion of the organic solvent in the mobile phase. It turned out that with reasonable column preconditioning the baseline drift during a 3-h separation procedure is still acceptable. It is unlikely that this system would find applicability in clinical practice as it is too slow and, except for an increased sensitivity (about 100 fmol), it has no other advantages over other available systems. However, if combined with a faster separation system it may be promising (Fig. 8).

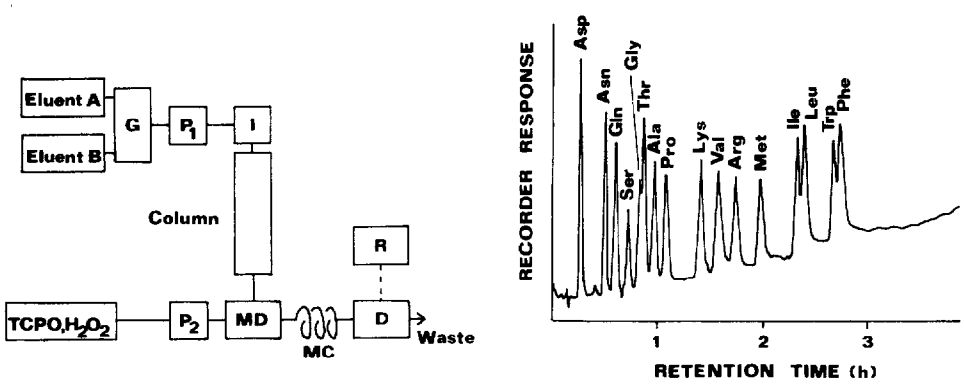


Fig. 8. Flow diagram of the apparatus and HPLC separation of dansyl amino acids with chemiluminescence detection. Eluent A, 0.1 *M* imidazole buffer (pH 7.0) (NO_3^-)—acetonitrile (90:10); eluent B, imidazole buffer (pH 7.0) (NO_3^-). P_1 = eluent pump; P_2 = reagent pump; TCPO = reagent reservoir; I = injector; column = TSK gel type ODS 120 A; MD = mixing device; MC = mixing coil; D = chemiluminescence detector; R = reactor. Linear gradient from 30% B to 99% B in 256 min. Each peak corresponds to 100 fmol of the particular amino acid derivative.

All the previously described separations of dansyl amino acids were carried out with a C_{18} reversed-phase column. Alternatively, it is also possible to use a C_8 reversed-phase column with 0.01 *M* Tris (pH 7.55, adjusted with HCl)—methanol (1:3) (A) and pure methanol (B) as solvents [131]. A linear gradient is applied by adding solvent B to solvent A at a rate of 0.5%/min until 25% of solvent B is reached and then the rate is increased to 5%/min. The column is recycled with solvent A within 15 min.

The application of dansyl derivatives for amino acid profiling appears less popular today than the separation of *o*-phthalaldehyde derivatives. If, however, there is a need for the resolution of individual enantiomers, dansyl derivatives may be the derivatives of choice. Lam and co-workers [132,133] described a method for the analysis of amino acid enantiomers that employs dansyl derivatives and mixed chelate complexation of the derivatives with Cu(II). Using a reversed-phase column and a mobile phase gradient of acetonitrile in a buffer

containing Cu(II) histidine methyl ester as the chiral component, sixteen amino acid pairs can be resolved within 2 h.

Before chromatography, the reversed-phase column (Nucleosil C_{18}, 15 × 0.43 cm I.D.) is conditioned by overnight pumping of a buffer containing 295 mg/l of aspartame and 100 mg/l of copper sulphate. The mobile phase used in the separation consists of $5 \cdot 10^{-3}$ mol of L-histidine methyl ester, $2.5 \cdot 10^{-3}$ mol of copper sulphate and 2 g of ammonium acetate per litre (pH 5.5). A stepwise gradient formed by blending the buffer with 45% acetonitrile solution (in the starting buffer) is applied. A typical separation of a standard mixture showing the resolution of individual enantiomers and a similar resolution of an amino acid profile of cerebrospinal fluid is shown in Fig. 9.

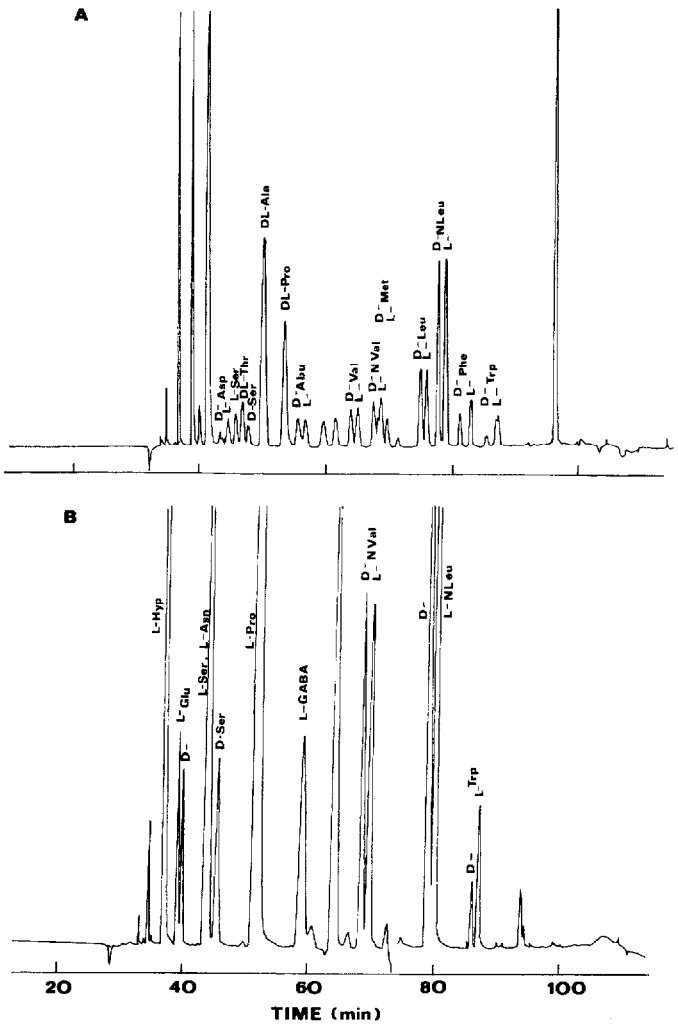


Fig. 9. Separation of D- and L-amino acids. (A) Standards; (B) cerebrospinal fluid of a meningitis patient. Separation as dansyl amino acid derivatives with $5 \cdot 10^{-3}$ *M* L-histidine methyl ester, 2.5×10^{-3} *M* copper sulphate and 2.0 g of ammonium acetate (pH 5.5) in the mobile phase as stepwise acetonitrile gradient. NVal = norvaline; NLeu = norleucine. (From ref. 132 with permission.)

A similar procedure was used by Lam et al. [134] for the separation of D- and L-pipecolic acid in urine and cerebrospinal fluid. Methods for the separation of enantiomeric amino acids without derivatization are also available [135,136].

4.1.1.3. The dabsyl chloride procedure. The dabsyl chloride (4-dimethylaminoazobenzene-4′-sulphonyl chloride) procedure is in its principle very similar to the reaction of amino acids with dansyl chloride. Although its sensitivity is about five times less than that of the dansyl chloride procedure, it is still occasionally used for amino acid profiling. The derivatization reaction is carried out in alkaline media (sodium hydrogen carbonate) in a manner almost identical with that of the dansyl chloride procedure.

Separation of the derivatives formed is typically carried out on a C_{18} reversed-phase column with ethanol—sodium acetate (20 mmol/l) (4:6) as the mobile phase. Dabsylated amino acids are conveniently detected by their absorbance at 425 nm. The sensitivity of analysis approached 5 μg per sample. However, the method was worked out in detail for the assay of branched amino acids and tryptophan only. This type of profiling reflects the observation that in some cancer patients high concentrations of urinary tryptophan and branched amino acids may be observed. Under the conditions described, highly polar amino acids such as glutamic acid, aspartic acid, threonine and serine are eluted immediately after the void volume; basic amino acids such as lysine and arginine remain on the column, from which they can be subsequently eluted with an increased concentration of ethanol.

Alternatively, a complete separation of all naturally occurring amino acids could be obtained in the reversed-phase mode with a concave acetate buffer—acetonitrile gradient [137].

4.1.1.4. Derivatization with 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole. Recently it has been found that 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole (NBD-F) is a useful derivatization reagent for amino and imino acids applicable to both pre-column and post-column reactions. (The chloro derivative can also be used, with minor modifications [141].) The main advantages of this reagent over the widely used *o*-phthalaldehyde are its ability to react with imino acids such as proline and its sensitivity. The sensitivity is one order of magnitude greater than that of the *o*-phthalaldehyde procedure. Aliquots of 5 μl of serum are sufficient for sample preparation. The derivatization reaction is rapid and simple: after deproteinization with ethanol, the supernatant is clarified by centrifugation and mixed with an equal volume (20 μl) of 0.1 *M* borate buffer (pH 8.0, Na^+) and 12 μl of 83 m*M* NBD-F in ethanol. The mixture is heated at 60°C for 1 min. After cooling, 150 μl of 0.05 *M* hydrochloric acid are added and 10 μl of the solution obtained are used for chromatography [139,140].

It is worth mentioning that the sensitivity of this method is high enough to allow amino acid analysis of, e.g., dried blood discs of 3 mm diameter for inborn errors of metabolism. Another advantage is the possibility of detecting imino acids.

Typically the separation is carried out in the reversed-phase mode using a μBondapak column (30 cm × 3.9 mm I.D.) attached to a fluorescence monitor set at 470/530 nm [141]. Three solvents are needed for establishing the gradient: solvent A is methanol—tetrahydrofuran—0.1 *M* phosphate buffer

(pH 6.0, Na^+) (3.75:16:94.65); solvent B is methanol—tetrahydrofuran—0.1 *M* phosphate buffer (pH 6.0, Na^+) (25:15:60); and solvent C is methanol—water (60:40). The elution programme is as follows: (1) isocratic elution with solvent A for 24 min; (2) a linear gradient from solvent A to 100% B over 30 min; (3) isocratic elution with solvent B for 6 min; and (4) isocratic elution with solvent C for 12 min. Elution profiles obtained with normal blood, a patient with phenylketonuria, maple syrup urine disease and tyrosinosis are presented in Fig. 10.

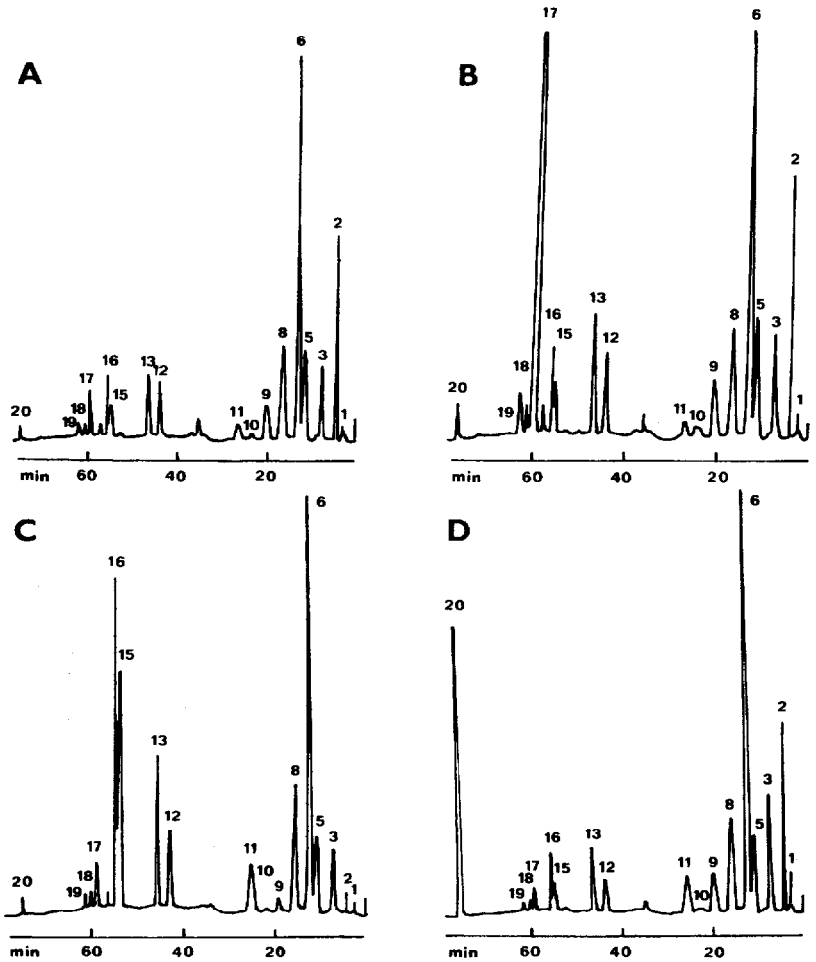


Fig. 10. Amino acid profile obtained with 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole amino acid derivatives. For separation conditions, see text. (A) Amino acids in a normal blood disc; (B) a blood disc obtained from a newborn with phenylketonuria; (C) a blood disc obtained from an individual with maple syrup urine disease; (D) a blood disc of a tyrosinosis patient. Peaks: 1 = NBD-Asp; 2 = NBD-Glu; 3 = NDB-Ser + NBD-Asn; 4 = NBD-His; 5 = NBD-Gly + NBD-Gln; 6 = NBD-OH; 7 = NBD-Arg; 8 = NBD—Thr + NBD-Tau; 9 = NBD-Ala; 10 = NBD-Pro; 11 = NBD-NH_2; 12 = NBD-ϵ-aminocaproic acid; 13 = NBD-Val; 14 = NBD-Met; 15 = NBD-Ile; 16 = NBD-Leu; 17 = NBD-Phe; 18 = NBD-Orn; 19 = NBD-Lys; 20 = NBD-Tyr. (From ref. 141 with permission.)

4.1.1.5. The phenylthiohydantoin procedure. The conversion of amino acids into phenylthiohydantoins is much less used than any of the previously described derivatization techniques in profiling studies. As follows from the nature of the reaction, this procedure has received much more attention in protein sequence studies. With regard to profiling, the thiohydantoin procedure requires much larger samples to obtain good results. Typically, 1 ml of serum or 5 ml of urine are mixed with phenyl isothiocyanate (PTH) and heated at 60°C for 40 min. The excess of PTH is removed by diethyl ether extraction (4 ml) and to the aqueous layer 250 μl of 12 *M* hydrochloric acid are added. Then the reaction mixture is incubated at 100°C for 17.5 min, the PTH derivatives are extracted with 4 ml of diethyl ether and the extracts are pooled and evaporated to dryness. The dry residue is dissolved in methanol—acetonitrile (4:1) and injected into the column.

The chromatography can be carried out in the reversed-phase mode (e.g., on Ultrasphere ODS) using a 250 × 4.6 mm I.D. column at 45°C. The gradient described by the manufacturer should also be suitable for purposes other than sequence analysis. Good amino acid profiles were obtained from blood, urine and hair, with high recoveries, low standard deviations and coefficients of variation and good intra- and interassay precision.

Alternatively, it is also possible to use a mixed nitrile—allylsilane mobile phase with a ternary gradient [142]. A number of other solvent systems can be found in refs. 143—155.

4.1.1.6. The phenylthiocarbamyl procedure. This procedure also belongs between those in which amino acids are converted into suitable derivatives before chromatography [156]. To our knowledge, this method has not yet been used for amino acid profiling, but it has some potential in this direction and is therefore mentioned here for completeness.

Derivatization is carried out with samples that are vacuum dried after being dissolved in ethanol—water—triethanolamine (2:2:1). The derivatization is then effected with ethanol—triethylamine—phenyl isothiocyanate (7:1:1:1). Phenylthiocarbamyl amino acids are formed after adding the derivatizing reagent to the dried samples and sealing them in vacuum vials for 20 min at room temperature. Excess of reagents is removed under vacuum.

The chromatographic separation of the derivatives formed can be achieved on Pico-Tag columns (15 cm × 3.9 mm I.D.) using 0.14 *M* sodium acetate containing 0.5 ml/l of triethanolamine (pH 6.35, titrated with acetic acid) and acetonitrile. A gradient is needed, traversing from 10% to 51% of acetonitrile using a convex curve.

The method has several disadvantages that prevent its wider adoption: first, trace amounts of oxygen interfere with the derivatization step; second, there is a slight loss of reliability in determining threonine, proline, phenylalanine and leucine at extremely low levels; third, the derivatization procedure, although it can be accomplished within 20 min, is complex in comparison with other derivatization procedures and therefore this technique is likely to find more applications in the area of sequence studies.

4.1.2. Chromatographic separation and profiling of amino acids without pre-column derivatization

Ion-exchange chromatography of underivatized amino acids with classical

TABLE 7

COMPOSITION OF LITHIUM CITRATE BUFFERS FOR ION-EXCHANGE CHROMATOGRAPHIC SEPARATION USING AMINCO JE5-7409 COLUMN PACKING

Buffer changes are indicated in Fig. 11. Reproduced from Drescher et al. [162] with permission.

Component	Buffer 1 (0.2 *M* lithium; 0.067 *M* citrate)	Buffer 2 (0.2 *M* lithium; 0.067 *M* citrate)	Buffer 3 (1.0 *M* lithium; 0.067 *M* citrate)	LiOH wash (0.2 *M* LiOH)
Citric acid, anhydrous (g)	12.87	12.87	12.87	—
Lithium hydroxide monohydrate (g)	8.39	8.39	12.59	8.39
30% Brij-35 (ml)	2.0	2.0	2.0	2.0
25% Thiodiglycol (ml)	5.0	5.0	5.0	—
Liquid phenol (ml)	0.1	0.1	0.1	—
Lithium chloride (g)	—	—	29.68	—
Boric acid (g)	—	—	0.62	—
EDTA, free acid (g)	—	—	—	1.0
Concentrated HCl (ml)	Approx. 12	Approx. 8	Approx. 8	—
pH at 25°C	3.20	4.26	7.70	13
Total volume (l)	1.0	1.0	1.0	1.0

ninhydrin detection can be useful even today [157,158]. Some procedures using this separation principle in combination with *o*-phthalaldehyde post-column derivatization may serve as an example. With, e.g., a Hitachi 835 high-speed amino acid analyser, a graphic printer and a recorder, excellent profiles can be obtained. The experimental conditions and buffer timings of such a system are summarized in Table 7.

With respect to the number of compounds separated within the profile, this classical approach is still highly valuable. As shown in Figs. 11—14, 52 ninhydrin-positive compounds can be separated. Excellent resolution of these compounds is obtained except for the pairs cystathionine—alloisoleucine and 5-hydroxylysine—allohydroxylysine. δ-Amino-*n*-valeric acid and D-glucosamine are used instead of norleucine as internal standards. The use of norleucine, the most widely used internal standard, is unsuitable here because in the system used it overlaps with the tyrosine peak.

The need to keep the analysis time reasonably short and to prevent secondary band spreading in the reaction coil introduces some problems, especially the shortening of the ninhydrin reaction, which results in low colour yields not only with proline and hydroxyproline, but also with carnosine, asparagine and anserine. However, the applicability of this approach was demonstrated recently in a case of keratopathy and in a case of Reye's syndrome patient [157].

One of the hidden problems in the amino acid profiling of tissues, particularly brain extracts, is the occurrence of one to three fast peaks at the beginning of the ion-exchange chromatograms. This fraction, called the "taurine fraction", represents a complex mixture of more than ten components, of which taurine, phosphoserine, phosphoethanolamine, glycerylphosphoethanolamine, hypotaurine, cysteic acid and cysteine sulphinic acid have been char-

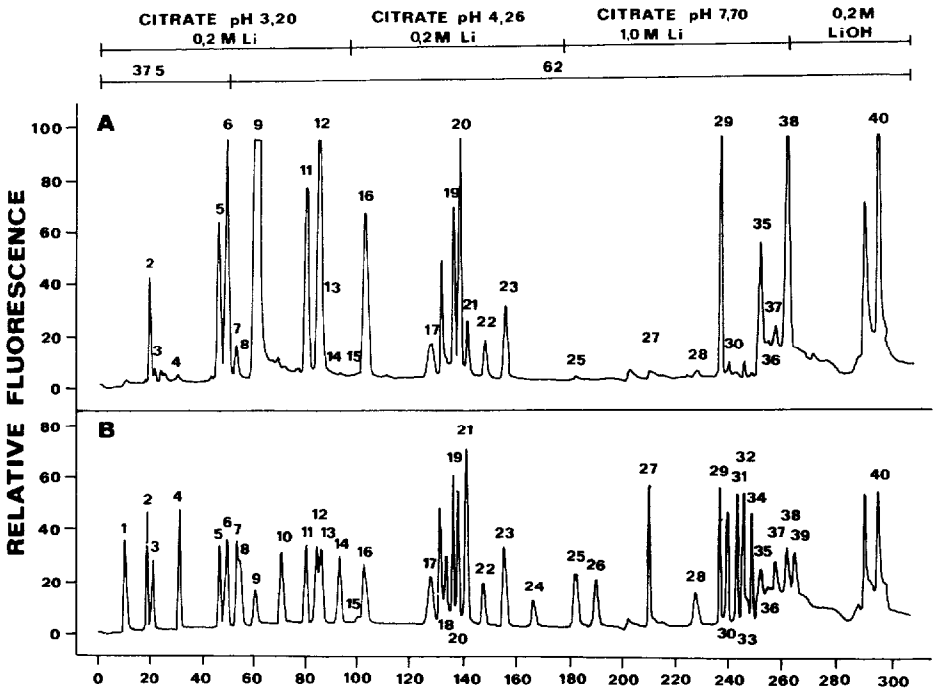


Fig. 11. Typical example of a chromatographic profile of amino acids present in guinea pig perilymph (A) compared with a standard mixture of amino acids (B). Ion-exchange separation using lithium citrate buffers (see Table 7). Detection with the *o*-phthaldialdehyde—mercaptoethanol reagent. Each component in the standard run was present at 100 μmol, except for norleucine, which was present at 150 pmol. For perilymph analyses, the numbers on the individual peaks represent the following: (1) phosphoserine; (2) taurine; (3) phosphoethanolamine; (4) aspartic acid; (5) threonine; (6) serine; (7) glutamic acid; (8) asparagine; (9) glutamine; (10) α-aminoadipic acid; (11) glycine; (12) alanine; (13) citrulline; (14) α-amino-*n*-butyric acid; (15) cystine; (16) valine; (17) methionine; (18) unknown; (19) isoleucine; (20) leucine; (21) norleucine; (22) tyrosine; (23) phenylalanine; (24) homocystine; (25) β-alanine; (26) β-aminoisobutyric acid; (27) γ-aminobutyric acid; (28) tryptophan; (29) histidine; (30) 3-methylhistidine; (31) 1-methylhistidine; (32) carnosine; (33) hydroxylysine; (34) anserine; (35) ethanolamine; (36) ammonia; (37) ornithine; (38) lysine; (39) α-amino-β-guanidinopropionic acid; (40) arginine. (From ref. 162 with permission.)

acterized. The individual highly acidic or highly ionized compounds have very close retention times. As ion-exchange chromatography fails to separate these compounds adequately, combination with another procedure, e.g., TLC on silica gel [two-dimensional: (1) 7% ethanol; (2) 75% phenol in water] has been applied for this purpose. To our knowledge, no problems of this type have been solved with any of the pre-column derivatization procedures.

4.1.2.1. o-Phthalaldehyde post-column derivatization. Except for increased sensitivity, post-column derivatization with *o*-phthalaldehyde offers little new in comparison with the classical ion-exchange ninhydrin procedure. Amino acid detection limits with the *o*-phthalaldehyde procedure are near 5 pmol for primary amines and near 100 pmol for secondary amines; detection limits with the ninhydrin procedure are close to 100 pmol. Another fluorescent reagent applied for the post-column derivatization, fluorescamine, provides a 10—100-

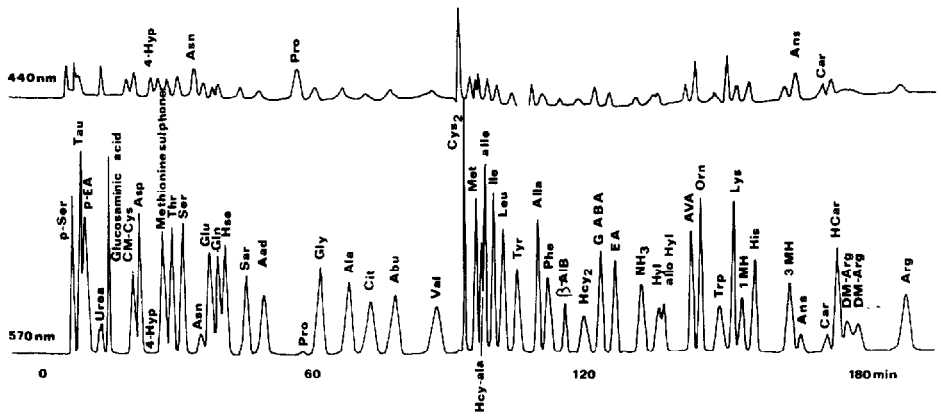


Fig. 12. Typical profiles of 52 ninhydrin-positive compounds obtained with a high-performance amino acid analyser. Amount loaded, 2.5 nmol of each amino acid. p-Ser = phosphoserine; p-EA = phosphoethanolamine; CM-Cys = carboxymethylcysteine; AVA = δ-aminovaleric acid; HCar = homocarnosine; DM-Arg = N^G,N^G-dimethylarginine. Recording was effected at two different wavelengths (440 nm and 570 nm). (From ref. 157 with permission.)

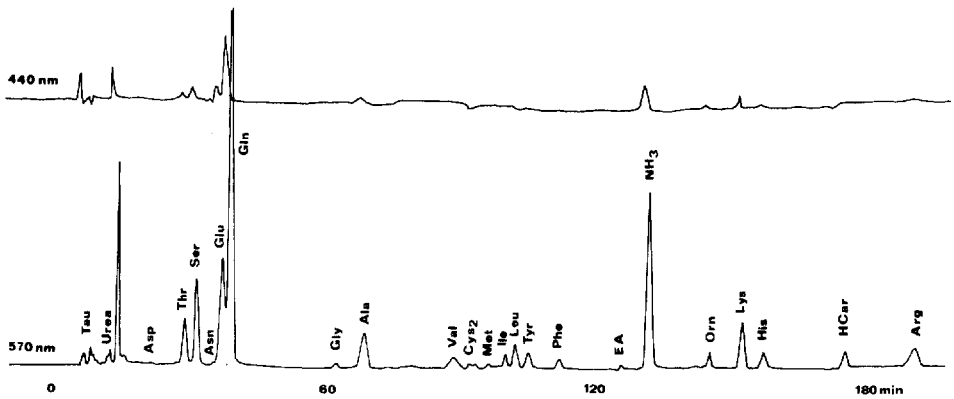


Fig. 13. Chromatographic profile of amino acids present in 33.3 μl of cerebrospinal fluid from a Reye's syndrome patient. Separation performed on a high-performance amino acid analyser (ion-exchange) with ninhydrin detection. HCar = homocarnosine. (From ref. 157 with permission.)

fold increase in sensitivity over ninhydrin but its drawbacks are that, similarly to *o*-phthalaldehyde, two post-column pumps are needed and that it does not react with secondary amines. In brief, fluorescamine does not offer particular advantages over the post-column *o*-phthalaldehyde derivatization and is used only rarely today.

The set-up of the detection system used with *o*-phthalaldehyde post-column derivatization is illustrated in Fig. 15. As mentioned, secondary amines do not form fluorescent derivatives with *o*-phthalaldehyde unless oxidative reagents are present in the reaction mixture. Using ion-exchange chromatography with a series of lithium citrate buffers, post-column *o*-phthalaldehyde detection and hypochlorite as the oxidizing reagent, Böhlen and Mellet [159] were able to overcome this disadvantage. The hypochlorite reagent was prepared by adding

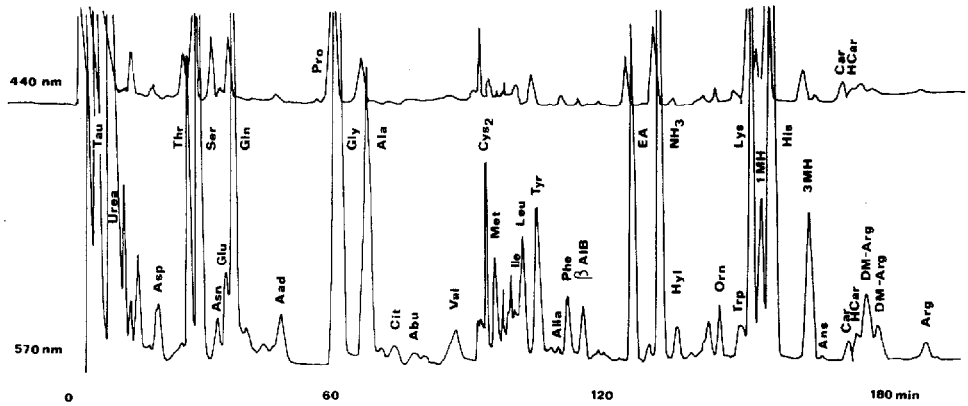


Fig. 14. Amino acid profile obtained with a high-performance analyser and ninhydrin detection from 50 μl of urine collected from a keratopathy patient. HCar = homocarnosine; DM-Arg = N^G, N^G-dimethylarginine. (From ref. 157 with permission.)

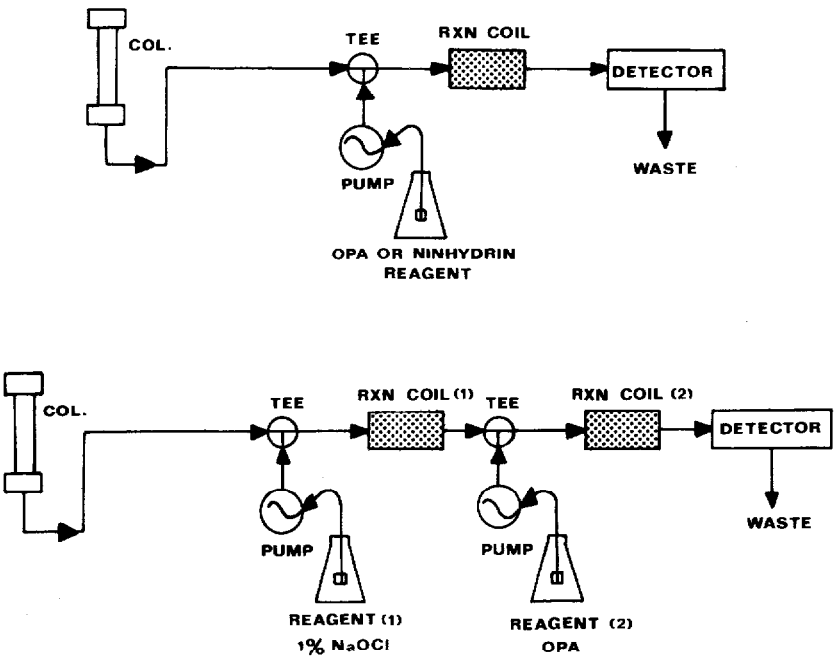


Fig. 15. Schematic diagram of a system applicable for post-column reaction with either *o*-phthalaldehyde or ninhydrin (above). In the lower part dual-pump post-column reaction system for the detection of secondary amines with *o*-phthalaldehyde is presented. The delivery of the hypochlorite solution can be controlled by HPLC apparatus. (From ref. 137 with permission.)

1.0 ml of commercial hypochlorite solution to 1 l of potassium borate buffer (pH 10.5). The flow-rate of the system was set at 8 ml/h and the hypochlorite solution was added at half this flow-rate (see also ref. 160).

As emphasized already, the separation of amino acids can be carried out in

any conventional way using lithium, citrate or borate buffers (e.g., see ref. 161). For example, Micro-Pak columns (15 × 0.4 cm I.D., polystyrene—divinylbenzene-based ion exchanger) in the lithium form can be used. It is noteworthy that the ion-exchange resins commercially available are frequently applied in their sodium form, which is not interchangeable with the lithium form in situ. Three proprietary lithium buffers were recommended for these separations for obtaining profiles of physiologically occurring amino acids: lithium buffer No. 1 contains 0.238 *M* Li^+ (pH 2.27), buffer No. 2 contains 0.34 *M* Li^+ (pH 3.6) and buffer No. 3 contains 0.643 *M* Li^+ (pH 5.3). Typically the following gradient can be applied: T_0 = 100% buffer 1, T_{80} = 100% buffer 2, T_{130} = 100% buffer 3 (the subscripts represent the running time in minutes, stepwise gradient). The analysis is lengthy, being completed in just over 3 h (see also ref. 161).

Fully automated ion-exchange procedures are available nowadays, not only for the separation of common amino acids but also for the separation of, e.g., basic amino acids, mono-, di- and polyamines, phenolic amines and indolamines in crude biological samples [163].

4.1.2.2. Post-column derivatization with 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole. The method of post-column derivatization of amino acids with 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole was devised by Watanabe and Imai

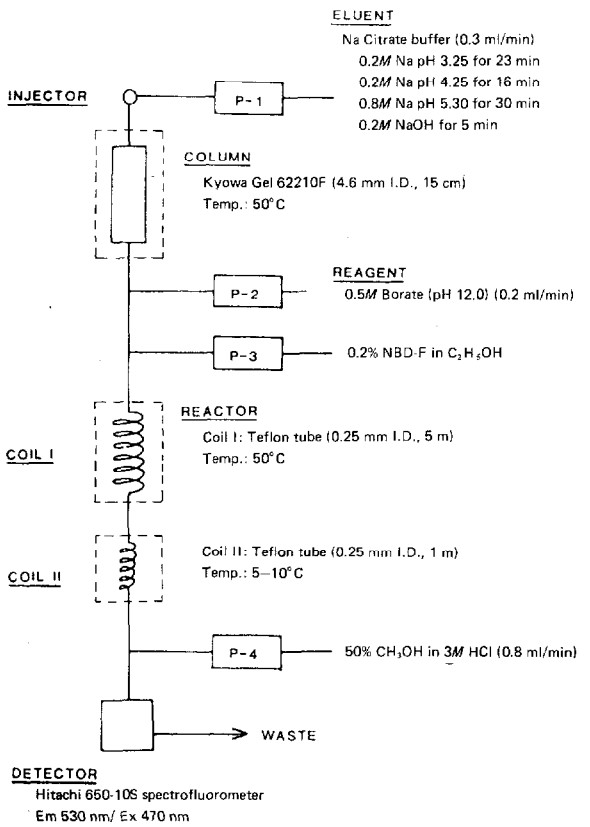


Fig. 16. Arrangement of the apparatus for post-column derivatization with 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole. (From ref. 138 with permission.)

[138]. The reagent is delivered in the form of a 0.2% solution in ethanol. Ethanol is used because it was shown to maximize the fluorescence yield. In the final set-up of the apparatus (Fig. 16), the column effluent is buffered with the addition of 0.5 *M* borate buffer (pH 13). The solutions of the reagent have to be prepared daily as they slowly react with ethanol and reduce its activity.

The separation applied in this instance was high-performance ion-exchange chromatography using a series of sodium citrate buffers and a stepwise gradient system. The timings and composition are evident from Fig. 16. After having passed through the column, amino acids are derivatized in the buffered eluate and the reaction is carried out in reaction coil No. I, where the reaction mixture is heated to 50°C, the reaction is completed and the derivatized amino acids pass through a cooling coil (No. II) before entering the spectrofluorimeter [141]. Fluorescence detection was carried out at 470/530 nm for amino and imino acids. The method is also applicable to the detection of thiols, where the fluorescence maxima in the detection step are shifted to 450/520 nm. The sensitivity of this method is generally in the picomole range (e.g., 10 pmol for tyrosine, proline and cysteine). In contrast to many of the advanced procedures, this one was tested for clinical applicability and proved suitable for the analysis of, e.g., free amino acids in blood discs.

4.1.2.3. Ion-pairing procedures for amino acid profiling. A recent strategy in separating underivatized amino acids is that of Walker and Pietrzyk [63]. In this procedure alkylsulphonate salts are used as additives to the mobile phase while the whole separation is carried out in the reversed-phase mode (Hamilton Prp-1). The reason for using the above additives is to enhance the retention of underivatized amino acids during the reversed-phase separation, where generally the retention of these compounds is low. The increased amino acid retention is the result of two major interactions, namely that caused by the formation of the arylsulphonate salts of amino acids and that resulting from the ion-exchange selectivity between the cationic form of the amino acid and the RSO_3^- counter cation (or other counter cations present in the mobile phase).

A good example of such an approach is that in which the ion-pair separation mechanism is combined with *o*-phthalaldehyde post-column derivatization [63]. This can be achieved by using LiChrosorb C_8 (5 μm) as the sorbent. Separation is carried out by means of a binary convex gradient. The starting mobile phase (buffer A) is 0.05 *M* sodium lauryl sulphate acidified with monochloroacetic acid to pH 3.0; the second solvent (buffer B) is 0.05 *M* sodium lauryl sulphate—acetonitrile (55:45). The effluent is mixed with the *o*-phthalaldehyde reagent (mobile phase flow-rate 0.7 ml/min; reagent flow-rate, 1.2 ml/min). The procedure is applicable to the analysis of blood stains on filter-paper discs and typical examples refer to maple syrup disease and phenylketonuria.

Radjai and Hatch [165] applied a similar technique using two kinds of counter-ion solutions to achieve the separation of amino acids. Their technique in comparison is much more complicated than that described above and is not easily applicable to routine analysis.

In the reversed-phase ion-pair separation of amino acids, several points are of importance. First, the column temperature influences the quality of the separation considerably; in general, the lower the column temperature, the better the separation that is obtained, particularly with isoleucine,

phenylalanine and leucine. The second problem to be overcome is the baseline drift caused by the reagent blank from the *o*-phthalaldehyde—mercaptoethanol reagent. This can be overcome on the basis of Roth's experience simply by adjusting the pH of the reagent solution to 9.0 [166]. The ratio of the mobile phase and reagent is also critical in this respect. The third problem (common to ion-exchange separations) is the need to eliminate amine contaminants present in the buffers used for elution. The procedure described below takes advantage of the fact that the product of the reaction of fluorescamine with an amine is fluorescent at basic pH, but rearranges to a non-fluorescent tetramic acid at acidic pH. According to Nadi and Margolis [167], this is effected by adding fluorescamine in a calculated amount (not in excess) to the lithium citrate buffers. As the impurities present in the chemicals used for buffer preparation vary from one batch to another, the amount needed for purification has to be calculated for every batch used. Typically, to 300 ml of Pierce lithium citrate buffer 100 ml of acetone containing 1 mg/ml of fluorescamine are added, the reaction mixture is stirred for 5 min, the yellow acetone phase is removed and the residual acetone is removed by keeping the mobile phase under reduced pressure overnight. Fluorescent products in the buffer are converted into the non-fluorescent derivatives by acidifying the solution to pH 1.5 with redistilled hydrochloric acid and the solution is diluted to 1 l with ultrapure water. If stored in hydrochloric acid-washed bottles, the mobile phase remains stable for over 6 months, encompassing 200 runs. The preparation of elution buffers from this stock solution is carried out in the routine manner.

A thorough investigation of problems arising from commercial buffers was published by Barbarash and Quarles [168].

4.1.2.4. Problems encountered with post-column derivatization. A serious disadvantage in using post-column derivatization of amino acids with, e.g., *o*-phthalaldehyde is the additional peak broadening occurring in the reaction coil (or even two coils in the hypochlorite system, see p. 216). Hence the efficiency of profiling by the HPLC procedure may be partly lost. Therefore, packed-bed reactors are preferable to open-tubular reactors. In practice, packed-bed reactors containing immobilized enzymes offer good prospects. A good example of such an approach was published by Kiba and Kaneko [169]. In their procedure, the properties of L-amino acid oxidase type III (E.C. 1.4.3.2), which catalyses the reaction

$$\text{L-amino acid} + O_2 + H_2O \rightarrow \text{2-keto acid} + NH_3 + H_2O_2$$

are made use of. Of the products formed, hydrogen peroxide is detected using another fixed-bed reactor containing the peroxidase—homovanillic system (E.C. 1.11.1.7). The fluorescence arising in the second reaction is recorded. In practice, both reactions occur in a single combined device called an AAO-PO reactor, in which the respective enzymes are coupled with a controlled-pore glass packing.

As the respective keto acids arising after deamination are physiologically present in plasma samples, they have to be eliminated first. This is achieved by passing the sample through a short (5 × 1 cm I.D.) ion-exchange column from which the originally present keto acids are eluted by four 1-ml aliquots of 0.01 *M* hydrochloric acid (and may be used for separate profiling). The amino

acids are eluted from the washed column with 4 ml of 4 *M* ammonia solution and the eluate is frozen and taken to dryness by lyophilization. The samples are then mixed with 1 ml of a solution containing amino acid oxidase and catalase (0.1 ml of amino acid oxidase and 0.05 mg catalase in 1 ml of 0.5 *M* Tris buffer, pH 7.6). The sample, after being flushed with oxygen, is incubated at 37°C for 1.5 h and 1 *M* hydrochloric acid is added to lower the pH below 1.0. HPLC is accomplished on a 5 μm C_{18} column and detection is carried out at 214 nm. Isocratic elution is performed with 0.05 *M* sodium phosphate (pH 7.0)—acetonitrile (90:10). A typical profile of plasma branched amino acids as the respective keto acids is shown in Fig. 17.

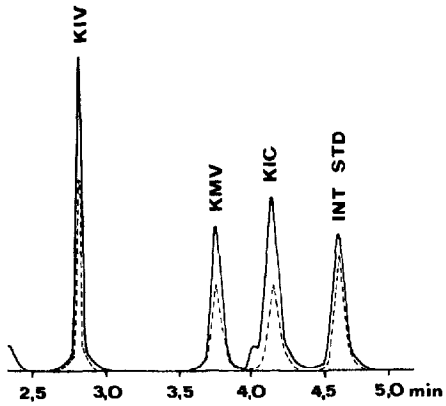


Fig. 17. Chromatographic profile of branched-chain amino acids after their conversion into the corresponding α-keto acids. Broken line, standards; solid line, plasma. KIV = α-keto-isovalerate; KMV = α-ketomethylvalerate; KIC = α-ketoisocaproate; INT STD = α-keto-caproate (internal standard). (From ref. 163 with permission.)

4.1.3. Separation of amino acid enantiomers

As the separation of enantiomers plays an important role in pharmaceutical chemistry and as in the future it is expected to invade the area of biochemical research more intensely than up to now, let us at least outline the possibilities in this area.

Many attempts have been made to separate amino acid enantiomers by liquid chromatographic techniques using chiral derivatization reagents [170—173], chiral eluents [174—176] or chiral stationary phases [177—179]. However, no practical method capable of resolving all the enantiomers related at least to common protein amino acids has been reported. Up to 1984 only two systems have hitherto been reported involving common liquid chromatographic separation of amino acids followed by optical resolution of amino acid enantiomers. The two-step separation needed to resolve these compounds made these procedures tedious and time consuming [180].

Nimura et al. [181] described a chiral derivatization method using O-acetylated sugar isothiocyanates, such as 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate. The other approach reported by the same group [181] was to use a chiral mobile phase with the N-tosyl amino acid—copper complexes such as N-(*p*-toluenesulphonyl)-D-phenylglycine—copper(II). By

using these two approaches most of the common amino acids could be resolved.

In the chiral derivatization method, derivatization with isothiocyanate reagent gave diastereomeric thiourea derivatives during the derivatization of enantiomeric amino acids. These derivatives were efficiently separated in the reversed-phase mode using an octadecyl-silica column (Develosil ODS, 200 × 6 mm I.D., particle size 5 μm). A typical example of a separation is shown in Fig. 18. The high-performance apparatus used for this analysis was equipped

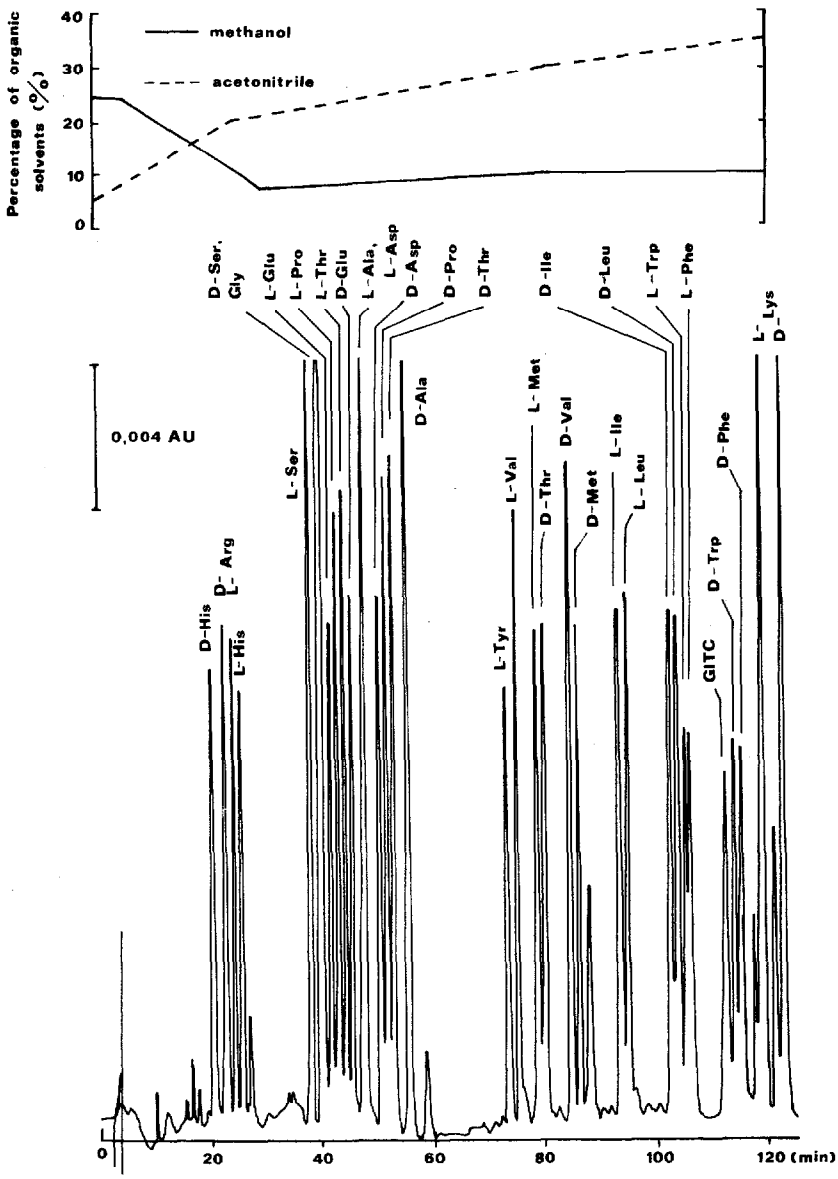


Fig. 18. Chromatographic separation of enantiomeric amino acids as diastereomeric thiourea derivatives. The gradient composition is obvious from the upper part of the figure. (From ref. 181 with permission.)

with the possibility to use a ternary solvent gradient; in this particular instance 0.1% aqueous phosphoric acid, methanol and acetonitrile were the three eluents used. The gradient programme is evident from Fig. 18. UV absorbance detection at 250 nm was used. The resolution of individual pairs can be described as good, although some peaks overlap. The separation of cysteine by this method is not possible.

In the chiral mobile phase method described by the same group, the optically active binary copper complex N-(*p*-toluenesulphonyl)-D-phenylglycine—copper(II) was used as a chiral additive to the mobile phase for the ligand-exchange separation of underivatized amino acids. In this instance, however, a column switching technique has to be applied, in combination with a chiral solvent—acetonitrile gradient. The assembly is complex and the reader is directed to the original paper [181] for more information. Detection in this instance was achieved by the post column *o*-phthalaldehyde method.

Another possibility for separating amino acid enantiomers was described by Buck and Krummen [182]. In their method, fluorescent diastereomers are formed by the reaction with *o*-phthalaldehyde and Boc-L-cysteine prior to chromatography. The reaction occurs at ambient temperature and can be performed in an automated pre-column derivatization device. Reversed-phase chromatography was carried out on Spheri 5 RP-8 in a 220 × 4.6 mm I.D. column. Mobile phase A was sodium phosphate buffer (pH 7.5) containing 1% of tetrahydrofuran and mobile phase B was methanol. The gradient separation was accomplished by running from 0 to 64% over 40 min. Derivatization was carried out in 100% A. The derivatives were monitored by fluorescence detection at 344/443 nm.

For completeness, it should be remembered that dansyl derivatives can also be exploited for the separation of amino acid enantiomers. This is discussed in more detail on p. 208.

4.2. Planar techniques

The possibility of running large numbers of samples in planar chromatography makes this technique a useful tool in screening programmes. In the planar arrangement both underivatized and derivatized amino acids have been separated both in their free form and as derivatives. In addition to paper, thin layers of starch, cellulose, acetylated cellulose, silica gel, aluminium oxide, calcium oxide, iron oxide hydrate, charcoal, calcium hydroxide, calcium phosphate, calcium sulphate, magnesium oxide, magnesium trisilicate, alginic acid, polyacrylonitrile, polyamide, Sephadex, Kieselguhr, anion and cation exchangers and more recently reversed-phase layers have been used. To survey the vast number of papers published in this area is beyond the scope of this review. The aspects that can well be made use of even today with underivatized amino acids can be found in the excellent review by Ersser and Smith [55].

The number of derivatives that have been used for planar amino acid separations is large (see the reviews by Rosmus and Deyl [164] and Horáková and Deyl [183]). Of these, let us mention at least 2,4-dinitrophenyl derivatives, phenylthiohydantoins, 1-dimethylamino-5-naphthalenesulphonyl (dansyl) derivatives and carbobenzoxy, *tert.*-butyloxycarbonyl, dinitropyridyl and

nitropyrimidyl derivatives. Most of these techniques are a matter of history today, although they might find application in special situations, and readers are directed to the relevant compilations (see also Niederwieser and Pataki [184]). Fairly recent information in this respect can be obtained from a book by Kirchner and Perry [185,186].

As a general introduction to these types of separations, Tables 8 and 9 on

TABLE 8

R_F X 100 VALUES OF AMINO ACIDS IN VARIOUS SOLVENTS ON SILICA GEL G

Reproduced from ref. 185 with permission.

Amino acid	Solvent* [23]					Solvent* [34]		
	A	B	C	D	E	F	G	C
α-Alanine	47	37	27	39	40	49	25	32
β-Alanine	33	26	27	30	29	49	20	33
α-Aminobutyric acid						54	25	32
β-Aminobutyric acid						48	32	36
γ-Aminobutyric acid						38	30	39
α-Aminoisobutyric acid						57	26	35
β-Aminoisobutyric acid						46	29	38
α-Aminocaprylic acid	66	65	60	58	60			
Arginine	4	2	8	10	6	6	14	13
Asparagine						46	19	22
Aspartic acid	55	33	21	9	7	56	5	26
Citrulline						46	29	26
Cysteic acid	69	50	14	17	21	61	5	20
Cystine	39	32	16	27	22	8	9	7
Dihydroxyphenylalanine			45					
Glutamic acid	63	35	27	14	15	55	7	32
Glutamine						55	28	24
Glycine	43	32	22	29	34	50	18	28
Histidine	33	20	6	38	42	33	24	10
Hydroxyproline	44	34	20	28	31	63	33	26
Isoleucine	60	53	46	52	58	60	36	47
Leucine	61	55	47	53	58	63	37	53
Lysine	3	2	5	18	11	5	8	10
Methionine	59	51	40	51	60	62	36	43
Norleucine	61	57	49	53	59	66	54	55
Norvaline	56	50	38	49	57	67	56	54
Ornithine						6	5	8
Phenylalanine	63	58	49	54	60	63	41	54
Proline	35	26	19	37	30	48	45	24
Sarcosine	31	22	17	34	31			
Serine	48	35	22	27	31	52	19	29
Taurine						59	22	29
Threonine	50	37	25	37	40	66	18	28
Tryptophan	65	62	56	55	58	69	45	56
Tyrosine	65	62	56	55	58	65	36	50
Valine	55	45	35	48	56	56	29	38

*A = 96% ethanol—water (7:3); B = *n*-propanol—water (7:3); C = *n*-butanol—acetic acid—water (4:1:1); D = *n*-propanol—34% ammonia solution (7:3); E = 96% ethanol– 34% ammonia solution (7:3); F = *n*-propanol—water (1:1); G = phenol—water (3:1). Development distance, 10 cm in all solvents.

TABLE 9

$R_{DANSYL\text{-}NH_2}$ VALUES × 100 OF DANSYL AMINO ACIDS IN VARIOUS SOLVENTS ON SILICA GEL

Reproduced from ref. 185 with permission.

Dansyl amino acid	Solvent*					
	A	B	C	D	E	F
NH_2	100.0	100.0	100.0	100.0	100.0	100.0
Ileu	90.5	83.5	92	100.0	100.0	89
Leu	87.5	67.5	89	100.0	99	63
Val	82.5	75.0	85	100	97	75
Pro	78.5	51.5	79	93	96	50
Phe	73.0	71.0	69	94	99	42
Met	66.0	69.0	65	92	99	33
Ala	62	56	61	89	96	29
Lys	55.0	82.0	48	93	96	33
Orn	49.0	79.0				
Tyr	47.0	49.0				
Gly	44.0	46.0	38	65	93	12
Try	41.0	64.5	34	87	95	25
Thr	25.0	45.0	18	25	57	2
Hypro	30.0	36.0				
Glu	24.0	0.5	18	42	85	8
Ser	19.0	36.0	15	20	52	2
Met-SO	15.0	58.0				
$MetSO_2$	14.0	57.0				
Asp	8.0	0	6	2	60	0
Asn	8.0	20.0	2	2	10	3
Gln	8.0	29.0	3	7	14	0
Orn	0	14.0				
ϵ-Lys	0	20.0	2	8	5	4
$CySO_3H$	0	4.0				
OH	0	67.0				
Di-$(cys)_2$	5.0	9.0				
Di-His	4.0	42.0				
Arg	0	29.0	0	0	0	0

*Solvents: A = toluene—pyridine—acetic acid (150:50:3.5); B = toluene—2-chloroethanol—25% ammonia solution (100:80:6.7); C = benzene—pyridine—acetic acid (40:10:1); D = chloroform—*tert.*-amyl alcohol—acetic acid (70:30:3); E = chloroform-*tert.*-amyl alcohol—formic acid (70:30:1); F = chloroform—*tert.*-amyl alcohol—acetic acid (70:30:0.5).

the chromatography of underivatized amino acids and their derivatives on silica gel may be informative.

The application of either one- or two-dimensional separations depends on the purpose of the analysis. For targeted profiles directed to a search for the change in a particular amino acid, usually one-dimensional separations are sufficient, as in most instances the separation conditions can be selected in such a way as to offer a good resolution of the amino acid in question. For more general screening, naturally two-dimensional maps are essential.

Of the numerous solvent systems used for underivatized amino acids, only a few have established themselves and are still in use. It is probably worth mentioning here some general rules or hints that should be borne in mind.

Butanol—acetic acid—water (120:30:50) and butanol—acetic acid—acetone—water (70:70:20:40) systems. These systems have been universally applied for amino acid analysis because the individual members of the amino acid family are more or less uniformly distributed along the chromatogram. There is little destruction of compounds during chromatography and the solvents are easy to remove. Replacing of some of the butanol with acetone produced more satisfactory results, particularly with microcrystalline cellulose.

Ethanol—ammonia—water (180:10:10) system. This is almost as good as those with acetic acid, although the spots, particularly on paper, may be more diffuse. The vital condition to be met in this system is vapour saturation of the chromatographic chamber.

tert.-Amyl alcohol—methyl ethyl ketone—water (120:40:40) system. This is the best solvent system for separating leucine from isoleucine. Generally it can be recommended for the separation of amino acids that show high R_F values in butanol—acetic acid—water systems. Addition of methyl ethyl ketone alters the R_F values very slightly, but its main role is to give more acceptable running times, which without this modifier are exceedingly long.

Phenol—ammonia (200:1) system. This system, in addition to producing a strong smell in the laboratory, has several disadvantages, mainly that some compounds are destroyed in contact with phenol, which is also difficult to remove from the chromatograms. The high background is another drawback. Nevertheless, it has withstood the years, particularly because of its ability to separate proline and hydroxyproline. When used today it is in two-dimensional separations with butanol—acetic acid—water systems.

tert.-Butanol—methyl ethyl ketone—25% ammonia—water (50:30:10:10) system. The advantage of this system is that desalting of specimens is not needed and thus it can be recommended for amino acid analysis in urine, because salts move little in this solvent system. It should be noted that sometimes a dark background occurs after ninhydrin detection because of the presence of impurities in the *tert.*-butanol. Removal of these impurities can be achieved by shaking the solvent with a dry cation-exchange resin in the H^+ form.

Pyridine—acetone—ammonia (100:60:40) and isopropanol—formic acid—water (160:20:20) systems. These are again systems suitable for the second run in two-dimensional development. In the flourishing era of thin-layer chromatography they were extremely popular, although with pyridine—acetone—ammonia vigorous shaking during mixing of the components leads to an explosion.

It should be recalled here that it was particularly in amino acid analysis where repeated development and over-run development were applied, and may still be successfully applied even today.

Unbelievable as it may seem, this field remained almost unchanged for years in clinical practice and the advanced planar techniques known today under the name high-performance planar (thin-layer) chromatography remained nearly unnoticed. The new achievements in this direction were excellently reviewed in 1977 by Zlatkis and Kaiser and the reader searching for more information about the general methodology is directed to individual chapters of their book [268].

TABLE 10

R_X VALUES OF DANSYL AMINO ACIDS SEPARATED BY TWO-DIMENSIONAL CHROMATOGRAPHY ON MICROPOLYAMIDE PLATES

Reproduced from Biou et al. [187] with permission.

Dansyl amino acid	R_{X_1}*	Dansyl amino acid	R_{X_2}**
Tyrosine (bis-dansyl)	0	Arginosuccinic acid	0
Cysteine (bis-dansyl)	0.11	α- or β-dansylcysteine	0
Cystine (bis-dansyl)	0.11	Taurine	0
Homocysteine	0.11	Taurocholic acid	0
Homocystine	0.11	Cysteic acid	0
Tryptophan	0.13	α-Aminodansylhistidine	0.05
Lysine (bis-dansyl)	0.13	Cysteine (bis-dansyl)	0.17
Histidine (bis-dansyl)	0.19	Cystine	0.17
Leucine	0.25	Homocysteine	0.17
Isoleucine	0.29	Homocystine	0.17
Taurine	0.30	Arginine	0.24
Taurocholic acid	0.30	Tyrosine (O-dansyltyrosine)	0.27
Ornithine	0.32	1-Methylhistidine	0.30
Cysteic acid	0.38	Aspartic acid	0.32
Phenylalanine	0.38	Asparagine	0.42
Tyrosine (O-dansyltyrosine)	0.39	Serine	0.46
Norvaline	0.42	Glutamic acid	0.53
Methionine	0.49	Glutamine	0.58
Valine	0.56	Citrulline	0.59
γ-Aminoisobutyric acid	0.75	DL-Methionine sulphone	0.66
DL-β-Aminoisobutyric acid	0.80	Threonine	0.67
Proline	0.88	Tryptophan	0.68
Alanine	0.92	Hydroxyproline	0.73
Glutamic acid	0.92	DL-α-Aminoadipic acid	0.80
Aspartic acid	0.93	3-Methylhistidine	0.80
β-Alanine	1.00	Glycine	1.00
Glycine	1.00	L-Methionine sulphoxide	1.08
DL-α-Aminoadipic acid	1.00	Ornithine	1.12
Hydroxyproline	1.19	Lysine (bis-dansyl)	1.32
DL-Methionine sulphone	1.33	Methionine	1.54
Threonine	1.35	Alanine	1.55
Serine	1.38	Phenylalanine	1.64
Citrulline	1.45	β-Alanine	1.70
L-Methionine sulphoxide	1.50	Tyrosine (bis-dansyl)	1.75
Glutamine	1.57	γ-Aminoisobutyric acid	1.82
Asparagine	1.72	Histidine (bis-dansyl)	1.86
Arginosuccinic acid	1.80	Norvaline	1.91
α- or β-dansylcysteine	2.00	Leucine	1.95
1-Methylhistidine	2.02	DL-β-Aminoisobutyric acid	2.00
α-Aminodansylhistidine	2.02	Valine	2.00
3-Methylhistidine	2.02	Isoleucine	2.09
Arginine	2.08	Proline	2.09

*R_{X_1} = (migration distance of dansyl amino acid)/(migration distance of dansyl glycine) in solvent system I [formic acid—water (1.5:98.5)].

**R_{X_2} = (migration distance of dansyl amino acid)/(migration distance of dansyl glycine) in solvent system II [benzene—acetic acid (4.5:1)].

As an example of what kind of methods can be expected in this respect today, we can refer to the method of Biou et al. [187], which is a typical procedure that can be used for screening large numbers of samples. Its advantages are an excellent resolution and high sensitivity (amount deposited in the range of 50 pmol), and it can mainly be applied to non-deionized urine and non-deproteinized ultrafiltrated serum. Separation is achieved on micro-polyacrylamide sheets using two-dimensional development with (I) formic acid—water (1.5:98.5) and (II) benzene—acetic acid (4.5:1). The running distance is small (3.5 cm). The spots are detected under UV light. Samples of 1 μl are spotted on the origin. A survey of R_X values obtained is presented in Table 10.

5. TARGETED PROFILING OF AMINO ACIDS IN BODY FLUIDS AND TISSUES

All biologically important amino acids, no matter whether proteinaceous in nature or not, are of clinical interest today. Of the about 170 naturally occurring compounds that can be described as amino acids, about 80 play a role in clinical practice [21,81]. Naturally, some of these are more diagnostically important than others [23,24,26]. With many of these there are also biologically important derivatives or metabolites that can be exploited for diagnostic purposes. Here we try to summarize the changes in body fluid amino acids that are bound to different physiological or pathological situations [20,22,24, 26, 188, 322, 347, 360, 361, 365].

α-Alanine (Ala). There is no typical disease accompanied by changes in levels of this amino acid; its decrease is combined with deficits of pyruvate dehydrogenase and pyruvate carboxylase complexes [189,190], with lactic acidosis and in glycogenosis type I. In contrast, it is increased in some shock situations and in some types of acyl-CoA dehydrogenase deficiency [191,192].

β-Alanine (Alla). This occurs physiologically in biological fluids in trace amounts only [76,113]. Its level is increased in β-alaninaemia, which is accompanied by β-aminoisobutyricaciduria and elevated levels of taurine (after kidney transplantation, after 6-azauridine administration) [193,194].

α-Aminoadipic acid (Aad). An increased content of Aad in blood and urine is observed in α-aminoadipicaciduria [195,196]. In urine there may be a concomitantly increased level of α-oxoadipic acid. Slight elevations are seen in saccharopinuria [197] and glutaric aciduria [198].

α-Amino-n-butyric acid (Abu). This is physiologically present in body fluids in trace amounts only [101]; pathological changes are unknown.

γ-Aminobutyric acid (GABA). This amino acid is physiologically high in the central nervous system [199], kidney and intestine tissues [37]. In body fluids particularly high levels are seen in cerebrospinal fluid in psychiatric disorders [200,201] and in hyper-β-alaninaemia [202]. Increased levels are also seen after 6-azauridine treatment [194].

β-Aminoisobutyric acid (β-AIB). This is present physiologically in large amounts in the so-called "excretors" [81,203]. Its occurrence probably results from β-AIB pyruvate—amino transferase deficiency [204]. The incidence of this phenomenon is particularly high in Japanese and Chinese

populations (up to 25%) [205]. Pathologically increased values are related to excessive tissue breakdown and to different neoplasias [206]. In these situations its increase may be accompanied with increased levels of uric acid.

Anserine (Ans). Ans occurs physiologically in biological fluids in trace amounts only [91,101]. Increased excretion is seen after ingestion of rabbit and poultry meat [207]. Pathologically increased values are seen in carnosinaemia resulting from carnosinase (E.C. 3.4.13.3) deficiency [208].

L-*Arginine (Arg).* This occurs physiologically in body fluids in trace amounts only [101]; pathologically increased values are seen in argininaemia [209] resulting from arginase (E.C. 3.5.3) deficiency, in lysinuric intolerance [210] and in dibasic hyperaminoaciduria [211]. In urine increased levels are seen in cystinuria together with increased levels of lysine, ornithine, cystine and cysteine—homocysteine disulphide [24,26,212,213].

L-*Argininosuccinic acid (ASA).* ASA occurs physiologically in biological fluids in trace amounts only [26]. Pathologically high excretion is observed in argininosuccinic aciduria resulting from a deficiency of argininosuccinase (E.C. 4.3.2.1) [214]. From the analytical point of view the existence of its two anhydrides is important [215].

L-*Asparagine (Asn).* This amino acid is present in all body fluids and organs; its content is particularly high in erythrocytes [216]. Decreased plasma levels are seen in leukaemias [217]; specific inborn error is not known.

L-*Aspartic acid (Asp).* This is a ubiquitous amino acid; its concentration in erythrocytes and leucocytes is about 100 times higher than in plasma [50, 218]. False increases are seen after mild haemolysis [69]. Pathologically increased values are seen in urine in dicarboxylic aciduria, a defect of renal glutamate and aspartate transport [219].

β-Aspartylglucosamine. This occurs physiologically in urine in trace amounts only [220]. Pathologically increased excretion is seen in aspartylglucosaminuria, a defect resulting from a deficiency of aspartylglycosaminidase (E.C. 3.5.1.26) [221,222].

γ-Carboxyglutamic acid. This occurs physiologically in trace amounts in urine. It is functionally active in blood coagulation [223]. It may serve as a marker of bone intermediate metabolism [224].

L-*Carnosine (Car).* Car occurs physiologically in human muscle as the only histidine dipeptide [225]. In plasma or urine it is present in trace amounts in the newborn period [75]. Pathologically increased values are seen in carnosinaemia resulting from a deficiency of carnosinase (E.C. 3.4.3.3) [207, 226]. Moderate excretion values are observed after ingestion of meat.

L-*Citrulline (Cit).* This occurs physiologically in low concentrations in plasma or urine. Premature individuals and newborns excrete constant amounts in the first month [74]. Pathological increases can be observed in citrullinaemia resulting from a deficiency of argininosuccinate synthase (E.C. 6.3.4.5) [227], argininosuccinic aciduria [228], sacchropinuria [229], hyperargininaemia [230] and cystinuria [231].

L-*Cystathionine (Hcy-ala).* This occurs physiologically in body fluids of newborns that are fed with protein-rich cow milk [232] and in prematures [233]; cow milk contains large amount of this amino acid [234]. Pathologically increased values are seen in primary or secondary cystathioninuria resulting

from a deficiency of β-cystationinase (E.C. 4.4.1.1) [235,—237]. The urine of these patients also contains cystathionine sulphoxide as the product of autoxidation [238]. N-Acetyl-L-cystathionine is excreted in massive amounts in both cystathioninurias [239].

L-Cysteic acid ($CySO_3H$). This amino acid represents the main artificial oxidation product of cysteine, cystine, cysteine—homocysteine disulphide and other disulphides [21,81].

L-Cysteine (Cys). Cys is present in plasma and urine but is rapidly oxidized to cystine [81].

The following cysteine derivatives occur in small amounts in the urine of healthy individuals: S-methyl-L-cysteine and its oxidation products, S-methyl-L-cysteine sulphoxide. S-Carboxymethylcysteine and S-(2-carboxy-*n*-propyl)-cysteine occur in the urine of patients with cystathionuria. S-(2-Carboxyisopropyl)cysteine, S-(3-glutaryl)cysteine, 5-S-cysteinyl-dopa, L-cysteinylglycine, L-cystinylglycine and S-sulpho-L-cysteine are markedly increased in sulphite oxidase (E.C. 1.8.3.1) deficiency [26,232,238—241].

L-Cystine (Cys—Cys). This always occurs physiologically in plasma or urine [24,26]. In newborns it is frequently accompanied by lysine [242]. Plasma levels are pathologically decreased in cystinuria [23] and cystathionurias [243] and in cystinosis. It also occurs in cells as such and tissues where it is accumulated [244]. High urine levels are seen in cystinuria (particularly types I and II) together with elevated levels of lysine, arginine and ornithine [20,26, 235,243]. N-Acetyl-L-cystine occurs in the urine of both healthy individuals and cystinurics [236,245].

3,4-Dihydroxyphenylalanine (Dopa). Under physiological conditions this amino acid is not present in urine. Pathologically increased levels are observed in patients with neuroblastoma [217,239], malanoma [237,246], in malnutrition [238,247] and in hyperpigmentation [240,248].

Disulphides. Cysteine—homocysteine disulphide is the main disulphide increased in homocystinurias [241,249], cystinurias [20,26] and argininaemia [209]. These disulphides also occur in biological fluids physiologically after L-methionine load [242]. β-Mercaptolactate—cysteine disulphide is present in trace amounts in healthy individuals. Large amounts of this disulphide are excreted in β-mercaptolactate—cysteine disulphiduria resulting from a deficiency of β-mercaptopyruvate—sulphur transferase [250]. Equally, high levels are seen in homocystinuria [251]. S-(Carboxymethylthio)cysteine is present in normal urine [252]. In contrast, S-(3-hydroxy-3-carboxy-*n*-propylthio)cysteine occurs mainly in the urine of homocystinuria subjects [238]. Cysteine—penicillamine disulphides are present in the urine of cystinurics during their treatment with D-penicillamine. Glutathione—cysteine mixed disulphide can be observed in glutathione synthase deficiency [217,253]. S-(3-Hydroxy-3-carboxy-*n*-propylthio)homocysteine and S-(2-hydroxy-2-carboxyethylthio)homocysteine are present in the urine of homocystinuria patients [238].

Ethanolamine (EA). This occurs only in trace amounts under physiological conditions. Pathologically its increase may be as an artefact formed by bacteria from phosphoethanolamine in hypophosphatasia [254]. Elevated levels have been seen in hyperlysinaemia [255], sarcosinaemia [256] and liver tumours [257].

Formimino-L-glutamic acid (FIGLU). Trace amounts of this amino acid are present in normal urine [258]. Typically, high increases are seen in formiminoglutamic aciduria resulting from formiminotransferase (E.C. 2.1.2.5) deficiency. Increased values are also seen in patients with folate and vitamin B_{12} deficiency [259,260], in pregnant women and in cases of liver cirrhosis [20,26]. This amino acid does not react with ninhydrin and must be assayed with nitroprusside—ferricyanide reagent (orange-red colour) [258].

L-*Glutamic acid (Glu).* This is a ubiquitous amino acid present in all human tissues and cells at high concentrations [37]. High levels are also seen in body fluids [27,53,81]. Increased concentrations may be artificial, as a consequence of decomposition of glutamine and its conjugates, in particular in urine not stored at −80°C [26,69]. Pathologically elevated levels are observed in dicarboxylic aciduria resulting from impaired renal tabular transport [261, 262]. The following glutamyl peptides occur in normal urine: γ-glutamylornithine, γ-glutamylleucine, γ-glutamylisoleucine and α-glutamylproline. γ-Glutamylcysteine is a precursor of glutathione and is present in all human tissues and fluids [26]. Pathologically increased values are observed in γ-glutamylcysteine synthase deficiency [263].

L-*Glutamine (Gln).* This amino acid is present in all body fluids and tissues [81]. It represents the most prominent amino acid in the cerebrospinal fluid [264,265]. Pathologically increased values in urine reflect urea cycle disorders frequently accompanied by hyperammonaemia [20,23]. L-Glutamine also represents the main amino acid occurring in generalized hyperaminoacidurias [262] (Lowe syndrome [266], Hartnup disease [267]).

Glutathione (GSH—GSSG). This compound occurs in all tissue cells, but is absent from urine [268]. Pathologically increased values in urine are observed in haemolytic anaemias, in 5-oxoprolinuria due to glutathione synthase deficiency [269] and γ-glutamylcysteine synthase deficiency [263]. High levels also occur in glutathionuria resulting from glutamyltranspeptidase deficiency 270].

Glycine (Gly). This is one of the main amino acids in all human tissues and body fluids [50,81]. Armstrong and Stave [27] reported healthy women with increased plasma glycine levels. Plasma levels of glycine increase during starvation (2—3-fold increase) [26,271]. Pathologically increased values are observed in nonketotic hyperglycinaemia [272]. Permanent or intermittent increases are seen in ketotic hyperglycinaemias [273], i.e., organic acidurias [272,274], propionic acidaemia, methylmalonic acidaemia, isovaleric aciduria, glutaric aciduria [275,276] and generalized hyperaminoacidurias [262]. In glycylprolinuria increased levels of glycylproline are observed [24]. In iminoglycinuria large amounts of glycine together with proline and hydroxyproline are excreted [277]. Many generalized hyperaminoacidurias except Hartnup disease have an obligatory high level of glycine in body fluids.

Hawkinsine. This amino acid was isolated from the urine of a patient with tyrosinaemia [241].

L-*Histidine (His).* His occurs in all body fluids and tissues and represents the main basic amino acid in urine [24,26,27,50]. Physiologically its level is increased in pregnancy [278]. A moderate increase is observed in the plasma and urine of subjects with benign metabolic error, histidinaemia, due to

histidase (E.C. 4.3.1.3) deficiency [279,280]. Histidinaemia also occurs in all types of generalized hyperaminoacidurias [262]. Histidylleucine and histidylproline are present in trace amounts in urine under normal circumstances [26,273,281].

N^1-Methylhistidine (1MH). This amino acid is a typical constituent of muscle actin and myosin [37]. Its occurrence in urine can be used as a marker of muscle breakdown [282]. Decreased excretion is observed in malnourished patients and during acute starvation [283]. In contrast, increased levels are found in multiple trauma, severe burns and renal insufficiency [282,284].

N^3-Methylhistidine (3MH). This occurs normally in the dipeptide anserine [81]. Urinary excretion originates from anserine present in food [27]. Pathologically increased levels are seen in patients with renal insufficiency [284–286].

L-*Homoarginine (Har).* This occurs in trace amounts in normal urine. Increased levels are found in hyperlysinaemia [287] and in cystinuria [288].

L-*Homocarnosine (Hca).* Hca occurs only in cerebrospinal fluid due to its high content in the brain [289]. Its levels are increased in homocarnosinosis [281] and classical phenylketonuria [20].

L-*Homocitrulline (Hci).* This is present in trace amounts in urine. Pathologically increased values are detected in hyperammonaemia [290], hyperornithinaemia [283] and argininosuccinic aciduria [290].

L-*Homocysteine (Hcy).* This compound is present pathologically only in biological fluids of homocystinurics [241,291].

L-*Homocysteic acid ($HcySO_3H$).* This represents an artificial oxidation product of homocysteinesulphinic acid. Small amounts are present in the urine of homocystinurics [291].

L-*Homocysteinesulphinic acid ($HcySO_2H$).* This represents an unstable oxidation product present in homocystinuria urine [241,291].

S-Adenosylhomocysteine (Ad-Hcy). Ad-Hcy is present only under pathological circumstances during homocystinuria [292]. S-(Carboxymethyl)homocysteine and S-(1,2-dicarboxyethyl)homocysteine accompany this amino acid in homocystinuria urine [291]. In cystathioninuria S-(2-hydroxy-2-carboxyethyl)homocysteine is a typical urine constituent [293].

L-*Homocystine (Hcy_2).* This is absent from healthy urine or plasma. Pathologically increased values are found in homocystinuria due to the deficiency of cystathionine-β-synthase (E.C. 4.2.1.22) [241,294] and in homocystinuria due to methylenetetrahydrofolate reductase deficiency (E.C. 1.1.1.68) [295]. It is also present in the urine of homocystinurics with methylmalonic aciduria due to N^5-methyltetrahydrofolate—homocysteine methyltransferase (E.C. 2.1.1.10) deficiency [296]. Secondary occurrence results in neuroblastoma [297], after 6-azauridine treatment [194] and in vitamin B_6 deficiency [298].

Homolanthionine. This occurs only in the urine of homocystinurics [291, 294].

L-*Homoserine (Hse).* Hse occurs in the urine of neuroblastoma patients [299].

L-*3-Hydroxykynurenine.* This amino acid is excreted pathologically in urine during vitamin B_6 deficiency [299], xanthurenic aciduria [300] and in hydroxykynureninuria resulting from the kynureninase deficiency (E.C. 3.7.1.3) [301].

*5-Hydroxy-*L*-lysine (Hyl).* Trace amounts of this amino acid are found in plasma. In urine, total hydroxylysine consists of free and peptide-bound forms [302], free hydroxylysine representing about 10% of the total. Free hydroxylysine is known to decrease gradually with age. Peptidically bound hydroxylysine reaches extremely high values in sucklings and gradually decreases towards adulthood. Enormous excretion values are seen in hydroxylysinuria [303], Paget's disease and fibrous dysplasia [304]. In contrast, reduced hydroxylysine excretion is found in hydrolysine deficient collagen diseases [305,306].

L-*3-(4-Hydroxy-3-methoxyphenyl)alanine.* This occurs in physiological urine in trace amounts and an increase is observed in patients with neuroblastoma and melanoma [307,308].

*3-Hydroxy-*L*-proline (3-Hyp).* This amino acid occurs in collagen in addition to 4-hydroxyproline. Trace amounts of its bound form are found in urine [309].

*4-Hydroxy-*L*-proline (Hyp).* This is detectable under physiological conditions in high concentrations in the plasma of newborns and its concentration increases up to 18 years of age [74]. In urine it occurs in both free and peptide-bound forms. Elevated excretion is observed in newborns and prematures [310]. Elevated concentrations in blood and urine are found in hydroxyprolinuria, probably resulting from a deficiency of hydroxyproline oxidase. In iminoglycinuria elevated levels of this amino acid are found together with elevated levels of proline and glycine [20,24,277].

*5-Hydroxy-*L*-tryptophan (Hyt).* This amino acid is not present in plasma under physiological conditions. However, it occurs as an important supplementary metabolite during the treatment of tetrahydrobiopterine deficiencies [311].

L-*Isoleucine (Ile).* This ubiquitous amino acid is present in all body tissues and fluids [27,81]. Pathological increases are seen in all variants of maple syrup urine disease, where its presence is caused by a deficiency of branched-chain oxoacid dehydrogenase and impaired decarboxylation of the corresponding 2-oxo acids [26,312]. In Hartnup disease increased levels of this amino acid are found together with increases in all natural amino acids [267]. Its levels are also increased in malnutrition and terminal phases of metabolic disbalances such as shock, sepsis and coma [262,313].

Allo-isoleucine (alle). This occurs only in the blood and urine of patients with maple syrup urine disease.

Isovalthine. This occurs only in the urine of patients with hypercholesterolaemia and hypothyreoidism [314]. Administration of the drug bromisoval also causes isovalthinuria [315].

L-*Kynurenine (Kyn).* Kyn is physiologically present in urine [50] and pathologically increased in vitamin B_6 deficiency [299] and in kynunerinase deficiency (E.C. 3.7.1.3) [301].

L-*Leucine (Leu).* Leu is found in all tissues and body fluids [50,81]. Increased levels occur in the plasma and urine of all types of maple syrup urine disease [20,312], Hartnup disease [24,267,316] and other generalized hyperaminoacidurias [262].

L-*Lysine (Lys).* The urinary excretion of this ubiquitous amino acid depends

on age [50,73]; high excretion is found in sucklings where it is combined with cystine (physiological cystine—lysinuria) [26,30,50]. Pathologically elevated excretion in hyperlysinaemic patients results from lysine—oxoglutarate reductase (E.C. 1.5.1.8) deficiency [317]. High levels are also found in dibasic hyperaminoaciduria [211], cystinuria [24,26,213], generalized hyperaminoacidurias [315] and familial pancreatitis [313,318].

*N-Acetyl-*L*-lysine.* This is physiologically present in urine and pathologically increased values are found in hyperlysinaemia [255,317].

*Methyl-*L*-lysine, dimethyl-*L*-lysine and trimethyl-*L*-lysine.* These are regularly present in blood and urine in small amounts and are related to muscle metabolism [319].

L-*Methionine (Met).* Met is present in almost all human tissues and fluids [27,30,75,81]. Pathological elevations result from adenosyltransferase deficiency (E.C. 2.5.1.6) [320] and occur in transitory hypertyrosinaemias and hypermethioninaemias [284,321], in tyrosinoses [20,23,26] and in all types of homocystinuria [20,22,23].

Methionine sulphoxide (MetO). This is physiologically present in body fluids only in trace amounts. Larger amounts result from methionine oxidation during storage [26,69]. S-Adenosyl-L-methionine (AdMet) is present in urine and blood under normal conditions and elevated levels are found in neuroblastmastoma [307,308] and homocystinurias [26,238].

L-*Ornithine (Orn).* This occurs in plasma, urine, sweat and cerebrospinal fluid [50,81]. Increased values can be found in hyperornithinaemia, resulting from a deficit of L-ornithine: oxo acid aminotransferase (E.C. 2.6.1.13) [323]. Particularly high levels are found in cerebrospinal fluid. Increased urinary levels are also found in cystinurias [213,215] and dibasic hyperaminoaciduria [211].

L-*Phenylalanine (Phe).* The metabolism of this essential amino acid has been studied in great detail [20,22—24,26]. It is present in all body fluids and tissues [27,50]. Blood levels over 242 μmol/l are indicative of hyperphenylalaninaemia and such a situation should be further differentiated [20,26]. With regard to this amino acid many syndromes have been studied: transient, permanent, classical, material [318,324], malignant [311], benign, etc. Phenylalanine, together with other amino acids, is increased in Hartnup disease and other generalized hyperaminoacidurias [262].

Phosphoethanolamine (PEA). Trace amounts are present in blood and urine under physiological circumstances; increased values are indicative of hypophosphatasia [21,26,254].

L-*Pipecolic acid (Pip).* This is normally present in trace amounts and is elevated in pipecolicacidaemia [317,325].

L-*Proline (Pro).* In contrast to other species, this is not an essential amino acid in humans [81]. In childhood elevated levels in urine are physiological [27,74]. Pathologically increased levels in blood are found in hyperprolinaemia type I resulting from proline oxidase (E.C. 1.5.1.2) deficiency [24] and in hyperprolinaemia type II resulting from a deficiency of pyrroline-5-carboxylate oxidoreductase (E.C. 1.5.1.12) [319,326]. Increased values in urine are also found in hyperprolinuria [26], iminoglycinuria [264,277] and both types of hyperprolinaemia [26]. Several proline peptides are also biologically important. This holds particularly for glycylproline present in ricketts, iminodipep-

tiduria [327], Paget's disease, chronic osteopathy [328], hereditary hyperphosphatasia [329] and other bone diseases. 1-Pyrroline-5-carboxylic acid is present in the urine of patients with hyperprolinaemia type II together with Δ^1-pyrroline-3-hydroxy-5-carboxylic acid [326].

L-*Saccharopine (Sac).* This occurs in urine together with citrulline, homocitrulline, homoarginine and α-aminoadipic acid in saccharopinuria [20,23,26].

Sarcosine (Sar). Sar is normally present in trace amounts only; elevation results in hypersarcosinaemia from a deficit of sarcosine dehydrogenase (E.C. 1.5.3.1) [330].

L-*Serine (Ser).* This occurs in all body tissues and body fluids but specific metabolic disorders are unknown [20,28].

Taurine (Tau). Physiologically high levels of this amino acid are found in plasma briefly after birth and decrease rapidly in a few days [27,74]. Thrombocytes and leucocytes contain considerably higher concentrations than plasma [40,45]. Pathological increases are seen only in sulphite oxidase deficiency (E.C. 1.5.3.1) [331]. It is perhaps worth mentioning that in contrast to human milk, cow milk is extremely rich in taurine [321].

L-*Threonine (Thr).* This represents an essential amino acid for human beings [81]. It is ubiquitous, but pathological alterations in its level are unknown.

L-*Tryptophan (Trp).* This occurs in plasma in free and albumin-bound forms [27,30,50]. Pathologically increased levels are found in Hartnup disease [267] and tryptophanuria [332]. In contrast, in pellagra and malnutrition tryptophan levels decrease [333].

L-*Tyrosine (Tyr).* This is present in all human tissues and fluids [50,81]. In newborns the plasma level depends on the maturity of the liver [74]; further, it depends on the level of protein intake and ascorbate deficiency [24,26]. Neonatal transient hypertyrosinaemia is the most common finding of systematic newborn screening [328,334]. Pathologically increased values are observed in tyrosinosis type I, resulting from a deficiency of fumarylacetoacetate hydrolase (E.C. 3.7.1.2) [335] together with *p*-hydroxyphenylpyruvate dioxygenase (E.C. 1.13.11.27), and in tyrosinosis type II from a deficiency of tyrosine aminotransferase (E.C. 2.6.1.5) [336].

L-*Valine (Val).* Val is an essential amino acid present in all body fluids and tissues [81]. Increased levels are found in hypervalinuria, resulting from decreased valine transferase activity (E.C. 2.6.1.32) [337]. Elevated levels are also found in maple syrup urine disease [20]. It can be constantly found in generalized hyperaminoacidurias.

6. APPLICATION TO METABOLIC DISORDERS

The whole of the above armoury of individual sampling techniques, separation, screening and identification techniques, detection methods and frequently the sophisticated equipment used is valuable in the early and rapid diagnosis of the numerous hereditary disorders of amino acid metabolism. This is one of the limited number of applications where chromatographic techniques clearly dominate the clinico-chemical routine without any competition.

Clinically, hereditary disorders of amino acid metabolism manifest themselves by acute, chronic and intermittent symptoms and many of these diseases,

if not recognized early, are fatal, and it is here where chromatographic techniques are invaluable. Returning to the symptomatology, the following classification can be outlined:

(a) Acute symptoms of metabolic disruption: metabolic acidosis, coma, convulsions, dyspnoe and cyanosis. If the disease is not diagnosed in time and therapy is not started, these disorders are fatal to the patient already in the neonatal period.

(b) Chronic symptoms that manifest themselves stepwise after the first 6 months of life: psychomotoric retardation, hepatosplenomegaly, cataract and diverse damage to organs.

(c) Intermittent symptoms of a metabolic disease that are provoked by stress situations (increased intake of proteins, acute infections, vitamins carency, drug intake, febrility, disturbances to school attendance, pubertal alterations and alterations during pregnancy, etc.): timely diagnosis prevents a repeat of metabolic crises that make the clinical state of the patients worse step-by-step.

Table 11 summarizes the known disorders and types of metabolites observed, and provides literature references in which readers may find more details about the methods of choice for the analysis of deviations in amino acid metabolism. It should be noted here that alterations in amino acid metabolism may lead to metabolites of the fatty acid type (particularly with Ile, Leu, Val, Ala and Gly), resulting in organic aciduria. These problems, however, exceed the scope of this review and are considered (at least in part) elsewhere in this volume.

7. SUMMARY

The needs of urgent diagnoses and the needs emerging from acute forms of diseases have directed progress in amino acid profiling to modern, rapid, automated analyses that can be done at reasonable cost. The first step in this direction was the short programmes of classical ion-exchange chromatography. At the beginning of this review we attempted to survey methods of sample preparation and sample treatment, as these are frequently neglected stages where artefacts or erroneous results may arise.

There are basically the following approaches in amino acid profiling by liquid chromatographic techniques. For preliminary screening of a large number of samples in clinical routine planar procedures are the methods of choice, as they allow large numbers of samples to be handled with minimum effort and at very reasonable cost.

For more precise profiling, particularly where quantitative data are essential, one can choose between some of the modern procedures for separating underivatized amino acids using modern equipment for cation-exchange chromatography, by making use of a stepped series of lithium citrate buffers with ninhydrin, *o*-phthalaldehyde or 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole detection. Ninhydrin detection is preferred in those situations where the demands on sensitivity are not high. Where, however, only small amounts of samples are available or high sensitivity is required, one of the latter two methods is preferred. The *o*-phthalaldehyde procedure is not suitable for the detection of

TABLE 11

SURVEY OF THE MOST COMMON HEREDITARY DISORDERS OF AMINO ACID METABOLISM

In situations where gas chromatography or gas chromatography—mass spectrometry were used for following the metabolite(s), they are also listed under method of determination for completeness.

Disease	Enzymic defect	Diagnostically important metabolite	Method of determination	Reference
Alkaptonuria	Homogentisic acid 1,2-dioxygenase (E.C. 1.13.11.5)	Homogentisic acid	GC, PC, TLC	13, 30, 92, 338, 339
α-Aminoadipic aciduria	Unknown	α-Aminoadipic acid	LC, TLC	95, 97, 193
α-Ketoadipic aciduria	Unknown	α-Ketoadipic acid	LC, GC	
β-Aminoisobutyric aciduria	Unknown	β-Aminoisobutyric acid	LC, GC, PC, TLC	24, 30, 201, 339
Argininosuccinic aciduria*	Arginine succinate lyase (E.C. 4.3.2.1)	Argininesuccinate, ammonia	LC, GC—MS	215, 332, 339
Argininuria (hyperargininaemia)*	Arginase (E.C. 3.5.3.1)	Arginine	LC, TLC	95, 339
Aspartylglycosaminuria	N-Aspartylglycosaminidase (E.C. 3.5.1.6)	Aspartylglycosamine	LC, GC, GC—MS	62, 221, 222, 338, 340
Carnosinaemia	Carnosinase (E.C. 3.4.3.3)	Carnosine	LC, GC, TLC	84, 95, 97, 341
Chinese restaurant syndrome	Unknown	Glutamic acid	LC, TLC	342—344
Citrullinaemia	Arginine succinate synthetase (E.C. 6.3.4.5)	Citrulline, homocitrulline, N-acetylcitrulline, homoarginine, glutamine, orotic acid, uracil	LC, GC, TLC	95, 97
Cystathionuria	L-Cystathionine—cysteine lyase (E.C. 4.4.1.1)	Cystathionine, homocysteine and additional sulphur-containing amino acids	LC, LC—MS, GC, GC—MS, TLC	95, 238, 339, 343—345
Cystinosis	Cystine reductase?	Cystine in tissues; generalized hyperamino-aciduria	LC, GC—MS	310, 338

Cystinuria	Deficiency of amino acid absorption in the proximal tubule and intestine	Cystine, lysine, arginine, ornithine, homoarginine, homocysteine—cysteine disulphide, homoarginine, citrulline	LC, PC, TLC	40, 95, 97, 238, 343, 344, 346, 347
Cystine-lysinuria	Deficiency of amino acid absorption in the proximal tubule	Cystine, lysine	LC, PC, TLC	242
Cystinuria (isolated)	Deficiency of cystine absorption in the proximal tubule	Cystine	LC, PC, TLC	23, 243
Diaminoaciduria	Deficiency of intestinal and kidney absorption of lysine, arginine and ornithine	Lysine, arginine, ornithine	LC, TLC	30, 95, 339
Dicarboxylic aciduria	Transport deficiency of dicarboxylic acids	Glutamic acid, aspartic acid	LC, TLC	95, 339, 347
Disulphiduria of cysteine β-mercaptolactate*	β-Mercaptopyruvate sulphur transferase?	β-Mercaptolactate cysteine disulphide	LC, GC, GC—MS, TLC	95, 238, 250, 345
Formiminoglutamic aciduria	Glutamate formimino transferase (E.C. 2.1.2.5)	Formiminoglutamic acid, folic acid	GC, PC, TLC	258, 341, 349
Glutathionaemia (-uria)	γ-Glutamyltransferase (E.C. 2.3.2.2)	Glutathione	LC, TLC	30, 95, 62, 221, 222, 344
Hartnup disease	Deficiency of kidney and intestinal transport of neutral amino acids	Alanine, serine, taurine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, histidine, indole-3-acetic acid, indole-3-acetylglutamine, indole-3-lactate, indikan	LC, GC, TLC	30, 61, 97, 267, 316, 341, 344
Histidinaemia	L-Histidine ammonia lyase (E.C. 4.3.1.3)	Histidine, imidazolylpyruvic acid	LC, GC, TLC	30, 95, 341, 350
Histidinuria	Deficiency of histidine absorption	Histidine	LC, TLC	30, 95, 341, 350

(Continued on p. 238)

TABLE 11 (continued)

Disease	Enzymic defect	Diagnostically important metabolite	Method of determination	Reference
Homocarnosinosis	Unknown	Homocarnosine in spinal fluid	LC, TLC	95, 97, 341, 350
Homocystinuria	Cystathionine-β-synthase (E.C. 4.2.1.22)	Homocystine, S-adenosyl-homocysteine, methionine, S-adenosylmethionine, disulphides of homo-cysteine and other S-containing amino acids	LC, GC, TLC	95, 238, 291, 293, 341, 345
Homocystinuria	5,10-Methylenetetra-hydrofolate reductase (E.C. 1.1.1.68)	Homocystine	LC, GC, TLC	95, 238, 291, 293, 341, 345
Homocystinuria	Results from the defective resorption of vitamin B_{12}	Homocystine	LC, GC, TLC	95, 238, 291, 293, 341, 345
Hydroxykynureninuria	Kynureninase (E.C. 3.7.1.3)	Kynurenine, xanthurenine, 3-hydroxykynurenine	LC, TLC	98, 344, 351
Hydroxylysinaemia	Unknown	Hydroxylysine, acetyl-lysine	LC, PC, TLC	95, 97, 98, 346, 347
Hydroxyprolinaemia	Hydroxyproline oxidase (E.C. 1.5.1.2)	Hydroxyproline	LC, PC, TLC	95, 97, 346
Hyperamonaemia* type I, congenital	Ornithine carbamyl transferase (E.C. 2.1.3.3)	Ammonia, glutamine, orotic acid	LC, GC, GC—MS	62, 338, 352—354
Hyperamonaemia* type II, congenital	Carbamyl phosphate synthetase (E.C. 2.7.2.5)	Ammonia, glutamine	LC	95, 355
Hyperalaninaemia*	Pyruvate dehydrogenase (E.C. 1.2.4.1), pyruvate decarboxylase (E.C. 4.1.1.1), pyruvate carboxylase	Pyruvic acid, alanine	LC, LC—MS, GC, GC—MS, TLC	30, 95, 330, 331, 347

Hyper-β-alaninaemia*	β-Alanine pyruvate amino-transferase (E.C. 2.6.1.18)	β-Alanine	LC, TLC	343, 344
Hyperglycinaemia	Serine hydroxymethyl-transferase (E.C. 2.1.2.1)	Glycine	LC, TLC	30, 61, 95, 97, 356, 357
Hyperphenylalaninaemia (phenylketonuria, classical)	Phenylalanine-4-mono-oxygenase (E.C. 1.14.16.2)	Phenylalanine, phenylpyruvate, *o*-hydroxyphenylacetate	LC, GC, TLC	30, 342, 346, 347, 355—359
Hyperphenylalaninaemia, malignant	Dihydrobiopterin reductase (E.C. 1.6.99.7)	Biopterine, phenylalanine, phenolic acid	LC, GC, GC—MS, TLC	30, 87, 97, 342, 346, 347, 355—359
Hyperphenylalaninaemia, malignant	Dihydrobiopterin "synthase"	Neopterin, phenylalanine, phenolic acid	LC, GC, GC—MS, TLC	30, 87, 97, 342, 346, 347, 355—359
Hyperlysinaemia	L-Lysine:2-oxoglutarate (NADPH) oxidoreductase (E.C. 1.5.1.8)	Lysine, N-acetyllysine, citrulline, homocitrulline	LC, TLC	95, 341, 343, 346, 350
Hyperlysinuria	Transport deficiency	Lysine, ammonia	LC, TLC	95, 341, 343, 346
Hypermethioninaemia	Methionine adenosyl-transferase (E.C. 2.5.1.6)	Methionine	LC, GC, GC—MS, TLC	24, 56, 95, 291, 293, 294, 343, 345, 352, 354
Hypermethioninaemia	Transport deficiency	Methionine	LC, GC, GC—MS, TLC	24, 56, 95, 291, 293, 294, 343, 345, 352
Hyperornithinaemia*, type I	Ornithine oxoacid transferase (E.C. 2.6.1.13)	Ornithine, ammonia, homocitrulline, lysine, arginine	LC, GC, GC—MS, TLC	24, 56, 95, 291, 293, 294, 343, 345, 352, 354
Hyperornithinaemia, type II	Ornithine carbamoyl transferase (E.C. 2.1.3.3)	Ornithine, ammonia, homocitrulline	LC, TLC	58, 95, 335, 346, 347
Hyperprolinaemia, type I	Pyrroline-5-carboxylic acid reductase (E.C. 1.5.1.2)	Proline, hydroxyproline, glycine	LC, GC, TLC	30, 95, 338, 339
Hyperprolinaemia, type II	L-Pyrroline dehydrogenase (E.C. 1.5.1.12)	Proline, hydroxyproline, glycine, Δ^1-pyrroline-5-carboxylic acid	LC, GC, TLC	30, 95, 338, 339
Hypersarcosinaemia	Sarcosine oxidase (E.C. 1.5.3.1)	Sarcosine	LC, TLC	30, 95, 98

(Continued on p. 240)

TABLE 11 (continued)

Disease	Enzymic defect	Diagnostically important metabolite	Method of determination	Reference
Hypervalinaemia*	Valine-3-methyl-2-oxovalerate amino-transferase (E.C. 2.6.1.32)	Valine	LC, TLC	30, 95
Hypertyrosinaemia (transitory)	4-Hydroxyphenylpyruvate dioxygenase (E.C. 1.13.11.27)	Tyrosine, *p*-hydroxyphenyl-pyruvic acid, *p*-hydroxy-phenylacetic acid, *p*-hydroxyphenyllactic acid	LC, GC, GC—MS, TLC	30, 334, 341, 343, 352, 356, 357, 363
Hypophosphatasaemia	O-Phosphorylethanolamine phosphorylase?	Phosphoethanolamine	LC, GC	30, 95, 98, 352
Indikanuria (blue diaper syndrome)	Tryptophan degradation	Indoles, indikan	GC, TLC	95, 339, 347
Iminoglycinuria	Transport of amino acids (glycine)	Proline, hydroxyproline, glycine	LC, TLC	30, 98, 336
Iminopeptiduria	Prolidase (E.C. 3.4.3.7)	Proline hydroxyproline, proline-containing peptides	LC, TLC	20, 98, 336
Imidazolepyruvic aciduria	Unknown	Imidazolylpyruvic acid, carnosine, anserine, 1-methylhistidine	LC, GC, TLC	338, 341, 354, 355
Indolylacroylglycinuria	Unknown	Indolylacroylglycine	GC, TLC	95, 339, 364
Lysine intolerance	Unknown	Lysine, arginine, ammonia	LC, TLC	95, 343, 346, 352
Lysine malabsorption	Isolated deficit of intestinal absorption	Lysine	LC, PC, TLC	95, 343, 346, 352
Maple syrup urine disease (leucinosis)	Leucine decarboxylation complex	Leucine, isoleucine, alloisoleucine, valine, 2-oxoisocaproic acid, 2-oxo-3-methylvaleric acid	LC, GC, GC—MS, TLC	30, 56, 95, 97, 338, 339, 343, 346, 347, 362
Methionine malabsorption, type I	Isolated deficit of intestinal absorption	Methionine, leucine, isoleucine, valine, phenylalanine, tyrosine, 2-hydroxybutyric acid	LC, LC—MS, GC, GC—MS, TLC	30, 95, 238, 240, 291, 294, 339, 352

Methionine malabsorption, type II (oast house urine disease)	Unknown	Methionine, phenylpyruvic acid, 2-hydroxybutyric acid, 2-oxoisovaleric acid	LC, LC—MS, GC, GC—MS, TLC	30, 95, 238, 240, 291, 294, 339, 352
Saccharopinuria	Saccharopine dehydrogenase (E.C. 1.5.1.9)	Saccharopine, lysine, citrulline, homocitrulline, homoarginine, 2-amino-adipic acid	LC, GC, GC—MS, TLC	95, 328, 366
Trimethylaminuria	Deficit of intestinal cell transport, deficit of liver oxidation	Trimethylamine	GC, TLC	328
Tryptophanuria	Tryptophan-2,3-dioxygenase (E.C. 1.13.11.11)	Tryptophan		332
Tyrosinosis, type I	Fumaryl acetoacetate hydrolase (E.C. 3.7.1.2), 4-hydroxypyruvate dioxygenase (E.C. 1.13.11.27)	Tyrosine, methionine, 4-hydroxyphenyllactic acid, 4-hydroxyphenylpyruvic acid, N-acetyltyrosine, succinylacetoacetic acid, succinylacetone	LC—MS, GC, GC—MS, PC, TLC	57, 58, 95, 340, 341, 343, 346, 352, 354, 358, 361
Tyrosinosis, type II	Tyrosine aminotransferase (cytosol fraction) (E.C. 2.6.1.5)	Tyrosine, 4-hydroxyphenyllactic acid, 4-hydroxyphenylpyruvic acid, 4-hydroxyphenylacetic acid		57, 58, 95, 341, 343, 346, 352, 354, 358, 361
Tyrosinosis (Maedes)	Unknown	*p*-Hydroxyphenylpyruvic acid		335, 336

*Acute metabolic diseases.

secondary amines and, if these are of interest, then diazole derivatization is to be preferred. At present, however, the ninhydrin and *o*-phthalaldehyde detection procedures are the most popular.

The other choice is to use one of the sophisticated HPLC systems equipped with fluorescence detection and to separate amino acids as derivatives. Here *o*-phthalaldehyde and 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole derivatives offer the most versatile possibilities.

Automation and computerization have penetrated both categories of liquid column separation and are applied to automated sample delivery, automated and computerized gradient formation and quantitation of the data obtained.

The tables of metabolic disorders of amino acids and the roles of different amino acids in these disorders should provide preliminary information for clinical chemists.

8. ACKNOWLEDGEMENTS

We acknowledge the cooperation of a number of colleagues during the different stages of the preparation of this manuscript. Thanks are due to Dr. Karel Macek of the Institute of Physiology, Czechoslovak Academy of Sciences, Prague, for stimulating discussions of the manuscript and to Mrs. J. Horáčková, J. Krausová and J. Koldová of the same institution for technical help, typing and graphics.

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