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COMPARISON OF NINHYDRIN AND *o*-PHTHALALDEHYDE POST-COLUMN DETECTION TECHNIQUES FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FREE AMINO ACIDS

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SUMMARY

A comparative study of two post-column derivatization techniques for amino acid determination has been completed. Using conventional high-performance liquid chromatography, amino acids were separated on either sodium- or lithium-form, polystyrene-based, strong cation exchangers, then derivatized with either *o*-phthalaldehyde or ninhydrin. Amino acid detection limits with *o*-phthalaldehyde are near 5 pmoles for primary amines and 100 pmoles for secondary amines such as proline. Detection limits with ninhydrin are near 100 pmoles. The *o*-phthalaldehyde system should be the system chosen whenever detection of secondary amino acids is unimportant. Retention time reproducibility averaged 1–4% relative standard deviation for both systems with a median peak area relative standard deviation of 3%. A substantial benefit of amino acid determination by high-performance liquid chromatography is the ease with which one can apply this type of chromatography to solve other separation problems.

INTRODUCTION

Automated amino acid analysis was pioneered by Moore and Stein[1] in the early 1950s. Together with Spackman *et al.*[2], they developed an amino acid analyzer that automatically coupled the amino acid separation with quantitation based on the ninhydrin reaction. Roth reported derivatization of amino acids with *o*-phthalaldehyde (OPA) in 1971[3]. In the same paper, Roth noted that secondary amino acids such as proline could be detected after reaction with sodium hypochlorite (NaOCl).

In post-column derivatization amino acids are separated on an analytical column and subsequently allowed to react with the derivatizing reagent after they are eluted from the column. The post-column derivatization is performed "on line" by pumping the reagent solution(s) into a mixing manifold (Fig. 1). Such a procedure has been the technique of choice for commercial amino acid analyzers.

Three post-column derivatization reagents have found popular use in amino acid analyses: ninhydrin, fluorescamine, and OPA. Ninhydrin has been used the longest. The chemistry of the ninhydrin reaction with amino acids has been well de-

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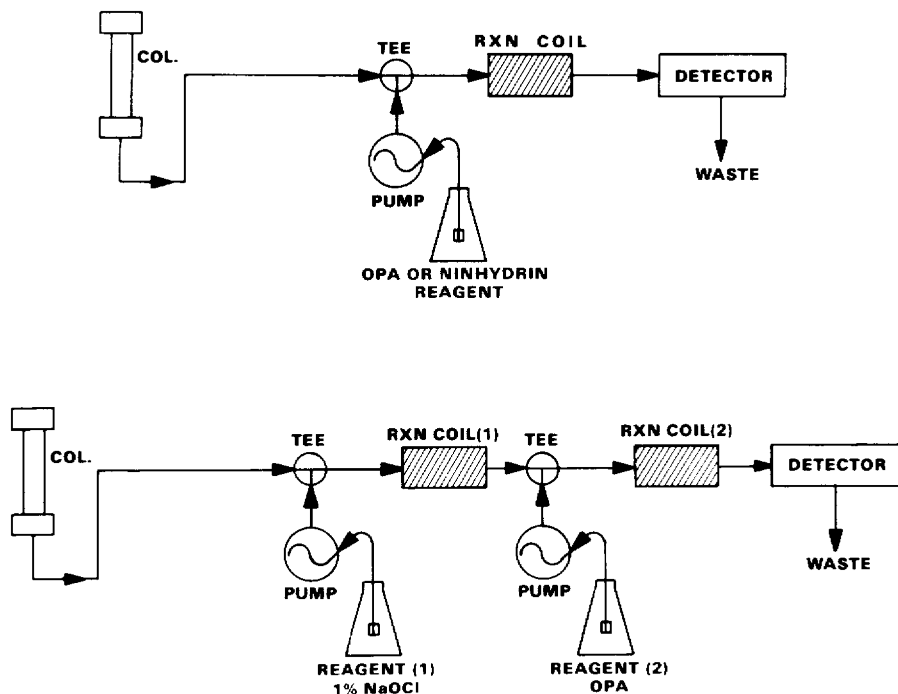

Fig. 1. Upper: post-column reaction for single pump OPA or ninhydrin; Reaction coil and tee are PTFE in the OPA system and stainless steel in the ninhydrin system. Lower: Dual-pump post-column reaction system for detection of secondary amines with OPA; delivery of NaOCl by Pump 2 (on or off) can be controlled by the HPLC apparatus.

scribed[4]. Particularly noteworthy is that ninhydrin reacts with both primary and secondary amino acids, yielding derivatives which adsorb strongly at 570 nm and 440 nm, respectively. For the complete amino acid assay, two detectors or one wavelength-programmable detector is necessary.

The fluorigenic reagent used in this study is OPA which has been shown to give better sensitivity than popular derivatization reagents such as fluorescamine or ninhydrin[6]. OPA meets two important criteria for post-column reagents. First, OPA is not fluorescent itself and thereby does not interfere with detection. Second, the reaction occurs quickly (half-life $\approx$ 1 sec) at room temperature, eliminating the need for long delay times between the mixing tee and detector which can seriously contribute to band broadening[3].

Another fluorescent reagent, fluorescamine (Fluram, tradename of Hoffmann–LaRoche product) was developed historically from the chemistry of the ninhydrin–amino acid reaction[5]. It provides a substantial increase (10–100-fold) in sensitivity over ninhydrin[6] but its drawbacks are that two post-column pumps are required and that it does not react with secondary amino acids. Since fluorescamine offers no particular advantage over OPA for post-column detection of amino acids, it was not included in this study.

EXPERIMENTAL

Instrumentation

High-performance liquid chromatography (HPLC). A Varian Model 5060 microprocessor-controlled single-pump liquid chromatograph capable of binary or ternary gradient formation was used. The external event option of these chromatographs, providing time-programmable a.c. power was used to control post-column pumps.

Detectors. A Fluorichrom (Varian) equipped with a tungsten lamp was the fluorescence detector. For OPA detection, the excitation wavelength was 340 nm (Corning 7-60 and 7-54 filters) and the emission wavelength was 450 nm (Corning 3-73 and 4-76 filters).

For visible detection with ninhydrin, a UV-100 (Varian) time-programmable detector was used. This detector was set to 570 nm for detection of primary amino acids and 440 nm for detection of secondary amino acids. The detector was programmed to reset automatically to zero after each wavelength change.

Post-column reaction systems

Fig. 1 depicts the post-column reaction (PCR) systems used in this study. Depending on the application, one or two PCR pumps (Varian Instruments, Walnut Creek, CA, U.S.A.) were used. The reaction coil (Varian) for the single-pump OPA system consists of 4.0 m $\times$ 0.3-mm I.D. PTFE tubing. For detection of secondary amino acids in the OPA system, an additional coil, 7.5 m $\times$ 0.3 mm I.D., was inserted into the system. A stainless-steel reaction tee and coil, 10.0 m $\times$ 0.25 mm I.D., was used in the ninhydrin system. The coil was heated in a water-bath or heater block (Pierce) to 100°C.

Columns. MicroPak (Varian) amino acid columns (15 cm $\times$ 4 mm) (9 μm polystyrene–divinylbenzene) in the sodium form were used for separation of amino acids commonly associated with acid hydrolyzates of peptides and proteins. The lithium form of this same column was used for separation of amino acids and amino acid metabolites commonly found in physiological fluids such as serum and urine. The sodium and lithium forms of the resin are not interchangeable *in situ*.

Chemicals and buffers. The OPA reagent was prepared by adding 0.5 mg/ml of OPA to potassium borate diluent (pH 10.4, 1.0 *M*), filtering through a 0.2-μm Nylon 66 filter (Ultipor) and then adding 0.1% (v/v) 2-mercaptoethanol and 0.10% (v/v) of Brij 35. All reagents were obtained through Varian. The NaOCl reagent was prepared by diluting a stock 5% solution (Fisher) with HPLC-grade water to give a 1% (w/v) solution.

For ninhydrin detection, a new commercially available reagent, Trione^{TM} (Pickering Labs., Mountain View, CA, U.S.A.) was used as supplied. Trione is a patented formulation which substitutes sulfolane for dimethyl sulfoxide (DMSO) as the primary solvent.

Hydrolyzate-derived amino acid separations were optimized using only two buffers in a continuous gradient. Two proprietary buffers comprised principally of sodium citrate were used in a gradient mode to elute the hydrolyzate-derived amino acids. Buffer 1 is a sodium citrate-based buffer (pH 3.25); Buffer 2 has pH 7.4. The sodium ion concentration is 0.2 *M* and 1.00 *M* in Buffers 1 and 2, respectively. Three

proprietary lithium citrate based buffers were used for elution of physiological amino acids. Lithium Buffer 1 contains 0.238 *M* Li^+ (pH 2.27); Buffer 2, 0.34 *M* Li^+ (pH 3.6); and Buffer 3, 0.643 M Li^+ (pH 5.3).

RESULTS AND DISCUSSION

Protein hydrolysate primary amino acids

Traditionally, protein hydrolysate amino acids have been separated on cation-exchange resin columns (Na^+ form) using a step gradient consisting of three sodium citrate buffers of increasing pH and ionic strength[6,7]. The buffer changes can be performed with a single pump using three on–off valves, each connected to a buffer at the low pressure side. Alternatively, three separate pumps could be controlled by

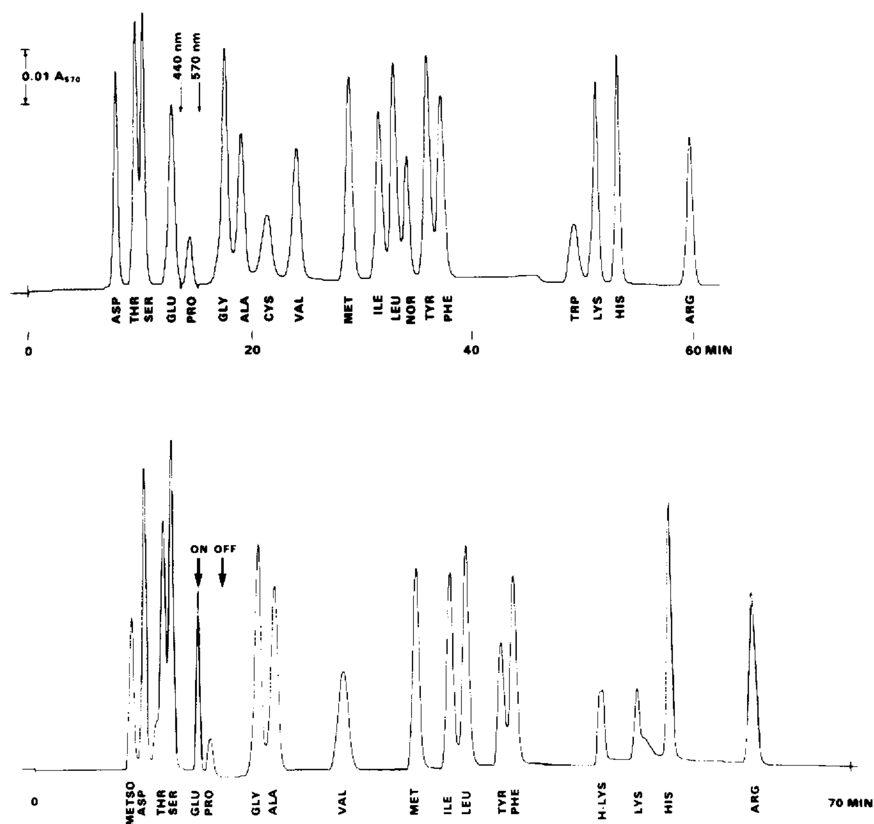

Fig. 2. Separation and detection of amino acid standards with ninhydrin (upper) and OPA (lower) post column detection. Chromatographic conditions (upper): solvent A = Na buffer 1; solvent B = Na buffer 2; column, MicoPak amino acid column, hydrolysate Na^+ form, 15 cm × 4 mm I.D.; 5 nmoles of each amino acid; temperature, 50°C (0–25 min), 90°C (25–60 min); flow-rate, 0.3 ml/min (0–46 min), 0.4 ml/min (46–60 min). Note wavelength change and autozero. Detector range 0.1 a.u.f.s.; T0 %B = 0, T5 %B = 0, T25 %B = 25, T25.1 %B = 30, T30 %B = 30, T45 %B = 65, T46 %B = 100. Chromatographic conditions (lower) same as chromatographic conditions (upper) except: 65°C (25–60 min), 0.3 ml/min (0–70 min).

timers. When stepwise buffer changes are made, baseline disturbances termed "buffer change peaks" are observed. These peaks undoubtedly result from chromatographic displacement of retained impurity peaks and also from a slight shift in background due to buffer refractive index effects[8]. The use of step gradients for amino acid analysis is also historical in that step changes were easier to implement in older liquid chromatographs and amino acid analyzers. The use of continuous gradients for the separation of amino acids is not new[9], but with modern high-performance liquid chromatographs, continuous gradients can now be formed reproducibly with minimal baseline perturbations.

Fig. 2 illustrates the separation of an amino acid hydrolyzate calibration standard achievable on a short (15 cm × 4 mm) MicroPak Protein Hydrolysate Amino

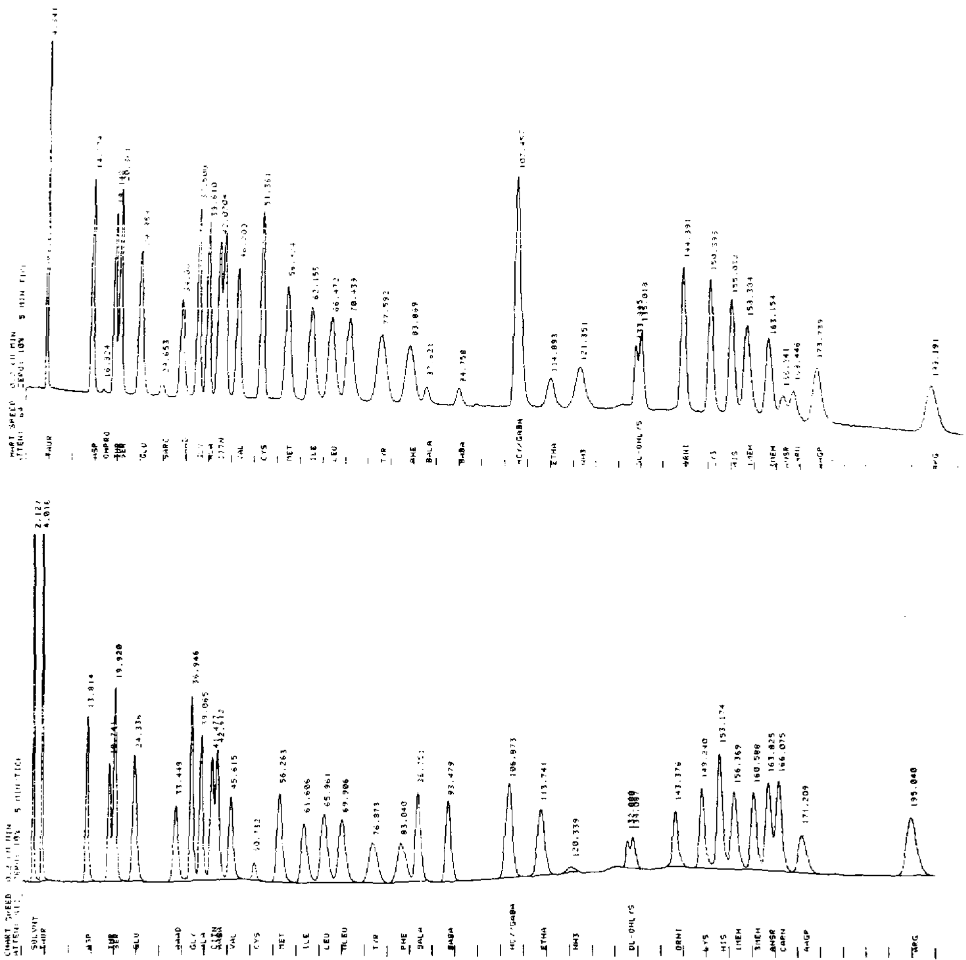

Fig. 3. Separation and detection of physiological amino acid standards with ninhydrin (upper) and OPA (lower) post-column detection. Upper chromatogram represents 5 nmoles of each amino acid, the lower 500 pmoles of each. Solvent A = Li^+ buffer 1, solvent B = Li^+ buffer 2, solvent C = Li^+ buffer 3. Column, Micropak Amino Acid column, biological fluid, Li^+ form (15 cm × 4 mm I.D.). Gradient program: *T0 %A = 100, T10 %A = 100, T80 %B = 100, T130 %C = 100*. Flow-rate, 0.3 ml/min. Temperature, 40°C (0–80 min), 60°C (80–200 min).

Acid column using a continuous binary gradient with flow and temperature programming. The use of a continuous gradient results in elimination of baseline disturbances that result when step-wise changes are made. The separation is complete in *ca.* 60 min. The single-pump OPA post-column reaction system (depicted in Fig. 1) was used for the detection of the primary amino acids. In a separate experiment, the minimum detectable quantity (MDQ) of aspartic acid was determined to be <3 picomoles (signal-to-noise ratio, $S/N = 3$).

In amino acid analyzers, the physiological fluid amino acids are usually separated in a step gradient consisting of live lithium citrate buffers which are directed onto the column at defined time intervals[10]. A continuous gradient was also applied to the separation of physiological fluid amino acids in a standard sample on a cation-exchange column with only three buffers (Fig. 3). Use of only three buffers simplifies the chromatographic instrumentation and separation conditions, is more convenient and is less expensive than the conventional five-buffer systems. The separation time was equivalent to the current five-step gradient physiological fluid amino acid analyses on particles of equivalent size. Selectivity effects in this system are principally due to a change in the ionization of the amino acids and the general order of elution is acidic, neutral and basic amino acids. Both Li^+ and Na^+ buffer systems were used to effect a separtion but Li^+ showed the best selectivity. Such selectivity differences can be observed, for example, by comparing the threonine–serine separation and the separation of the *allo* forms of hydroxylysine in Figs. 2 and 3.

OPA detection of secondary amino acids

The use of OPA derivatization for amino acids is particularly attractive since the sensitivity is enhanced compared to the classical ninhydrin[2] detection system based on visible absorbance. Unfortunately, most routine amino acid analyses of either protein hydrolysates or physiological fluids require the separation and quantitation of the secondary amino acids, proline and sometimes hydroxyproline.

Attempts have been made to convert these secondary amino acids first to primary amino acids by an alkaline hypochlorite oxidation but other OPA–amino acid adducts are degraded by hypochlorite oxidation[11]. By employing a valving scheme, Böhlen and Mellet[12] added the hypochlorite oxidant during the elution of the secondary amines only but baseline upsets produced when the valves were switched interfered with quantitation.

A two-stage post-column system (depicted in Fig. 1) was used to investigate the hypochlorite–OPA reaction. The first pump–coil arrangement is used to add the alkaline hypochlorite to the column effluent and the second pump–coil arrangement is used for the addition of OPA reagent.

Hypochlorite conversion of proline to a primary amine is temperature-dependent[12]. Heating the reaction coil markedly increases the amount of primary amine formed by the action of hypochlorite. The effect of temperature is clearly illustrated by Fig. 4a–c. Relative to reaction at room temperature (Fig. 4a), the proline response was enhanced about three-fold when the coil was heated to 40°C (Fig. 4b); above 50°C there was little increase, while above 60°C there was a marked drop in fluorescent response. It can be noted that there was a decreased response of the other primary amino acids with increased reaction coil temperature. In fact, in the experiment at 70°C, the primary amino acids were nearly eliminated, presumably by oxida-

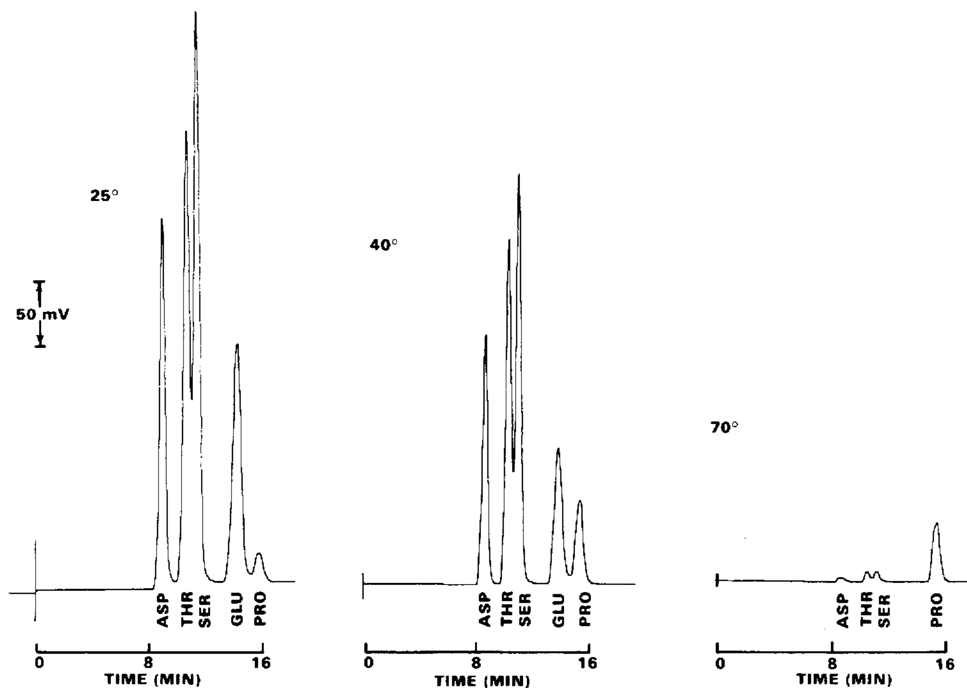

Fig. 4. Effect of hypochlorite reaction temperature on fluorescence of OPA primary amine and OPA proline adducts. NaOCl flow-rate, 0.3 ml/min. See Fig. 2 for chromatographic conditions. Sample, 0.5 μg of primary amino acids, 1.0 μg of proline.

tion of the primary amine group, leaving proline as the sole peak. Such an approach could prove useful for selectively detecting proline in the presence of a large amount of glutamic acid, which is eluted just prior to the proline peak. Under the conditions employed in the present study, a concentration of 1% NaOCl was found to give the best peak height response for proline conversion.

In agreement with previous results, the response of primary amino acids, with the exception of cystine, was found to decrease 15–30% at ambient temperature when a 1% (v/v) solution alkaline sodium hypochlorite is added to the column effluent. The relative cystine response actually increased about two-fold in the presence of hypochlorite. Cystine is probably converted into cysteic acid by hypochlorite oxidation. Cysteic acid, in turn, is more reactive toward the OPA reagent[12]. Not only did hypochlorite diminish the response of the other primary amino acids, it also contributed to a strong baseline elevation at the later part of the gradient. This baseline shift could have been caused by other strongly held secondary amine impurities on the column or in the strong buffer, since this baseline drift was not observed when the hypochlorite pump was turned off.

Because the strong oxidant, sodium hypochlorite, attacks the OPA and mercaptoethanol reagents, stabilizers and the primary amino acid–OPA adducts, there is some controversy as to how and when the hypochlorite should be added to the column effluent. One group[11] proposed adding the hypochlorite only during the

elution of proline (or hydroxyproline) while the other[13] claims that the hypochlorite degradation of the other primary amino acid–OPA adducts is reproducible and therefore the convenience of the switchless method is more advantageous.

Two procedures for adding hypochlorite only during the proline elution have been proposed. One procedure uses a switching valve on the low pressure end of the hypochlorite addition pump[13]. The purpose of this valve is to pump either make-up buffer into the post-column system during elution of all primary amino acids or hypochlorite during the elution of the proline or hydroxyproline. It is switched by timers. This set-up presumably avoids abrupt changes in flow-rate during the hypochlorite addition but is more complex. A second approach[11] is to turn the hypochlorite addition pump on only during the proline elution. This procedure is simpler, but can result in some baseline upset, depending on the fluorescent background and flow sensitivity of the fluorescence detector.

The simple approach of turning the hypochlorite reagent pump on and off at the proline elution time was found to yield satisfactory results as can be observed in Fig. 2. Reproducibility of peak height for proline was found to have a relative standard deviation (R.S.D.) of <5%. The response was linear from 25 to 1000 ng of injected proline with a correlation coefficient of 0.998. The MDQ of proline was found to be 10 ng (*ca.* 9 pmoles), but only when Brij 35 was removed from the OPA reagent. Brij was found to react with the hypochlorite and produce a baseline elevation that interfered with proline detection at low levels (less than 5 nmole). The absence of Brij 35 lowered the response of lysine and hydroxylysine.

Detection of amino acids with ninhydrin

Both primary and secondary amino acids can be detected by the classical ninhydrin reaction. With ninhydrin, the primary amino acids are monitored at 570 nm while the secondary amino acids are monitored at 440 nm. Thus, detection of all amino acids requires either two detectors or a time-programmable variable-wavelength detector with visible wavelength capability.

Table I compares results for both the OPA and ninhydrin system. Detection limits were determined at an S/N ratio of 2. It should be noted that the detection limit

TABLE I

COMPARISON OF OPA- AND NINHYDRIN-BASED SYSTEMS

Parameter	*Ninhydrin**	*OPA*
Detection limits, $S/N = 2$	< 100 pmoles for all amino acids except Pro (250 pmoles) and Trp (225 pmoles)	5 pmoles except Cys (30 pmoles) and Pro (100 pmoles) by NaOCl
Retention time reproducibility (R.S.D.), $n = 6$	1–4%	1–4%
Median peak height or area reproducibility (R.S.D.), $n = 6$	2%	3%

* Determined at 570 nm for all amino acids except proline (at 440 nm).

for the OPA system is limited, not by detector noise but by a rising baseline that results as Buffer 2 (in the Na^+ system) elutes amine contaminants contained in Buffer 1. Such baseline problems can be diminished by computer subtraction of a blank gradient. With the ninhydrin system, typical UV detector noise levels measured in the presence of reagent, *i.e.*, ninhydrin and buffer, are near 10^{-3} a.u.f.s. about 50 times higher than observed with water only in the flow cell. The larger noise level observed with the post-column system is due to reagent background absorbance and additional noise attributable to mixing effects. Detection limits for the HPLC systems are nevertheless comparable to those specified for dedicated amino acid analyzers.

Comparison with dedicated amino acid analyzers

A recent publication[14] reports that with a commercial amino acid analyzer the median intralaboratory R.S.D. is 9% (range 6–54%) for samples near the 100 nmole/ml level. Another publication[15] reports a median intralaboratory R.S.D. of 4% (range 2–40%). A similar test on Varian Equipment shows a median R.S.D. of 6% (range 3–24%). Such results indicate that when HPLC is used, the precision obtainable is somewhat greater than that with at least one commercially available amino acid analyzer. Proper calibration and sample preparation should result in comparable accuracy values.

Another study conducted in the laboratory compared results determined using the ninhydrin-based system for an acid hydrolysis of human growth hormone with results from a dedicated amino acid analyzer. Since the sequence of this hormone is known, it is possible to calculate expected values for each amino acid. Both systems yielded values within 10% of theoretical for all amino acids.

CONCLUSIONS

Post-column detection of amino acids can be accomplished easily, with either OPA or ninhydrin systems. The single-pump OPA system is more sensitive and presents fewer technical problems than the ninhydrin system but does not allow for detection of secondary amines. The single-pump OPA should be the system chosen whenever detection of secondary amino acids is not important. The ninhydrin system is the system of choice for detection of primary and secondary amines. It requires only one post-column pump and gives sensitivities for secondary amines that are comparable to dual-pump OPA–NaOCl systems. HPLC instrumentation can easily be adapted to provide separation and detection of amino acids comparable to dedicated amino acid analyzers.

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