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COLUMN CHROMATOGRAPHY OF AMINO ACIDS WITH FLUORESCENCE DETECTION

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SUMMARY

A highly sensitive fluorescence reaction for amino acids using *o*-phthalaldehyde and 2-mercaptoethanol permits the detection of amino acids separated on small ion-exchange columns. The advantages are: (1) good precision at the level of 0.5 nmole; (2) high speed due to the smaller height of the column; and (3) linear relationship between meter response and amino acid concentration.

The same reaction is also applicable to amino sugars.

INTRODUCTION

In the amino acid analyzers commonly used at present, absorptiometric detection is performed on the effluent with the classical ninhydrin colour reaction. More sensitive detection methods, however, are highly desirable in view of recent progress in ion-exchange chromatography showing that fast separation is possible in small columns at high pressure.

Following the discovery, a few years ago, of a very sensitive fluorescence reaction for α-amino acids[1], we developed a fluorimetric method for the detection of amino acids in column effluents following ion-exchange separation. The reaction utilizes a combination of *o*-phthalaldehyde and 2-mercaptoethanol, which, at pH 9, gives an intense blue fluorescence with all α-amino acids. The obvious advantage over the ninhydrin reaction is the sensitivity, which is increased by a factor of at least 50. Another advantage is that the fluorescent product forms at room temperature within 2 min, which makes the lengthy heating coil used in ninhydrin detectors unnecessary.

EXPERIMENTAL AND RESULTS

Fig. 1 shows the scheme of the analysis system. Separation is achieved on a 0.6 × 25 cm column filled with Aminex 6 ion-exchange resin. For elution, three citrate buffers of pH 3.20, 4.25 and 6.40 are pumped successively at the rate of 25 ml/h, the first switch being made after glutamic acid and the second after phenylalanine.

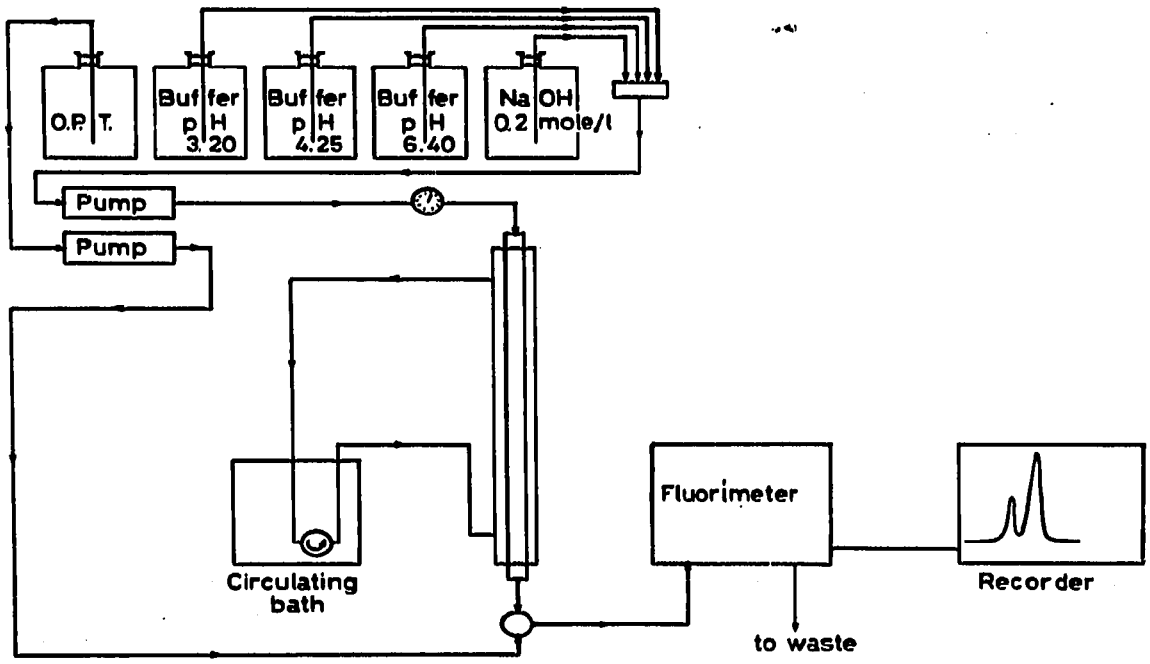

Fig. 1. Flow diagram of the analysis system.

The jacket temperature is set initially at 34° and is raised to 55° after the passage of phenylalanine. The reagent, which is pumped at the rate of 30 ml/h, consists of a solution of *o*-phthalaldehyde (0.8 g/l), ethanol (10 ml/l) and 2-mercaptoethanol (200 μl/l) in 0.1 *M* borate buffer of pH 10.

Mixture with the effluent occurs just past the outlet of the column. The reaction mixture is made to flow in a polyethylene tube for 5 min at room temperature, and then to enter the flow cell of an Aminco fluoromicrophotometer, which is a round cell of 2 mm diameter. The fluorescence intensity is recorded on a linear recorder with an integrator.

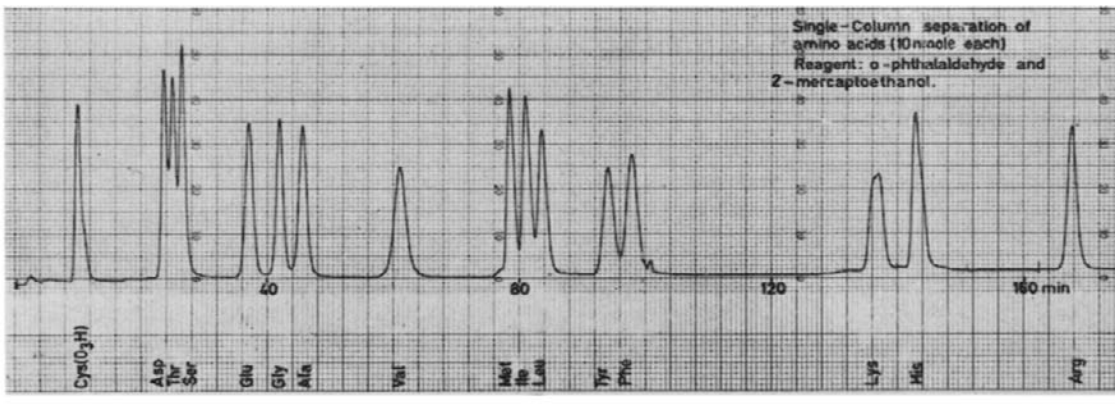

Fig. 2. Single-column separation of amino acids (10 nmole each). A linear recorder was used to monitor the fluorescence intensity.

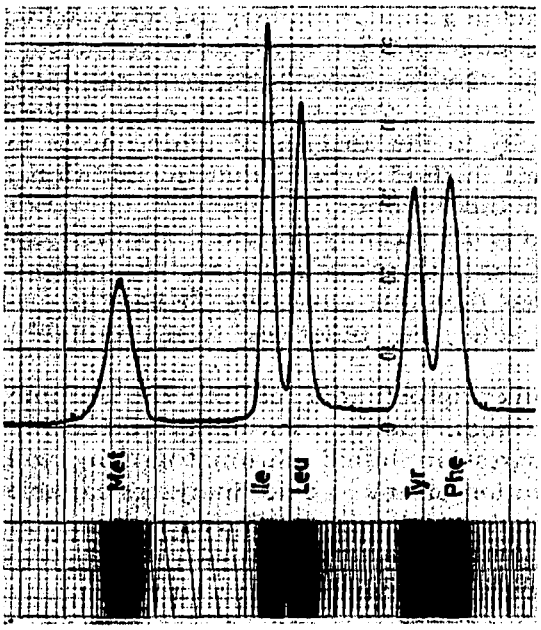

Fig. 3. Fragment of a chromatogram with integration of peak areas.

Fig. 2 shows a chromatogram in which 10 nmole each of different amino acids were separated. At this level, the amplifier capacity is far from being fully exploited. The base line is extremely stable. Much smaller amounts of amino acids, down to 0.5 nmole, can easily be detected with this system.

An advantage of fluorimetric detection is that the signal is a linear function of the concentration, which permits the easy evaluation of peaks after linear recording.

Fig. 3 shows the fragment of another chromatogram where fluorescence intensity is quantitated with an integrator.

We have experienced no difficulties with the reagent mixture stored in a stoppered erlenmeyer flask for 48 h at room temperature. Storage for longer periods is possible under nitrogen.

The small size of the column permits short elution times. The chromatogram in Fig. 2 took 170 min to complete, but this time could certainly be reduced further by the use of even smaller columns operated at high pressure.

The specificity of the reaction resembles that of ninhydrin, with the advantage that ammonia virtually does not interfere. Of the common natural amino acids, only proline and hydroxyproline do not react. Cysteine reacts poorly, but its oxidation product, cysteic acid, gives a bright fluorescence.

Proline and hydroxyproline can be oxidized with chloramine T, N-bromosuccinimide or hypochlorite to give compounds capable of giving the fluorescence reaction with *o*-phthalaldehyde. The excess of oxidizing agent must be destroyed before the fluorigenic reaction takes place, as otherwise *o*-phthalaldehyde would be oxidized to a fluorescent compound. Development of an auxiliary system permitting the determination of proline and hydroxyproline in column effluents is in progress.

Amino sugars, such as glucosamine, react in a similar manner to amino acids, and therefore the same principle can be used to monitor small amounts of amino sugars fractionated on chromatographic columns. On the other hand, peptides react much less than amino acids.

The system has been applied successfully to the analysis of amino acids in urine. Comparison with the classical ninhydrin method showed that no additional interfering peak is encountered in the fluorimetric method.

It should be mentioned that Udenfriend *et al.*[2] recently described another sensitive fluorigenic reagent for amino acids, which they call fluorescamine. This reagent is less specific than the present reagent, and has the same sensitivity. It has the disadvantage of being rapidly decomposed by water, but otherwise seems valuable, especially for the analysis of peptides.

In conclusion, the *o*-phthalaldehyde–2-mercaptoethanol reaction for α-amino acids appears to be a very promising analytical tool. The high sensitivity of fluorimetry combined with other technological progress in liquid chromatography will contribute to the development of a new generation of amino acid analyzers capable of handling large numbers of samples with excellent resolution and precision.

REFERENCES

1 M. Roth, *Anal. Chem.*, 43 (1971) 880.
2 S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leimgruber and M. Weigele, *Science*, 178 (1972) 871.