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Note

Automatic amino acid analysis utilizing 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole

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In previous studies [1–3], amino acids including proline were labelled with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F), separated by high-performance liquid chromatography (HPLC) and sensitively detected. However, the manual labelling procedure used in these studies was time-consuming, and involved a risk of inducing experimental errors. The recent instrumental progress in HPLC is remarkable. Highly automated labelling, if achieved, would lead to further progress in amino acid analysis by eliminating experimental errors and by affording reproducible results.

This paper describes a method for and results of automatic pre-column labelling of amino acids with NBD-F using a programmable auto-sampling injector.

EXPERIMENTAL

NBD-F (Dojindo Labs., Kumamoto, Japan) solution (5–50 m*M*) in acetonitrile and 30 μl of amino acid solution in 50 m*M* borate buffer (pH 8.0, with 0.05 m*M* EDTA in certain instances) were separately placed in different brown test vials (Chromacol 07-CPVA, 0.7 ml) and placed on the rack in a Model 231-401

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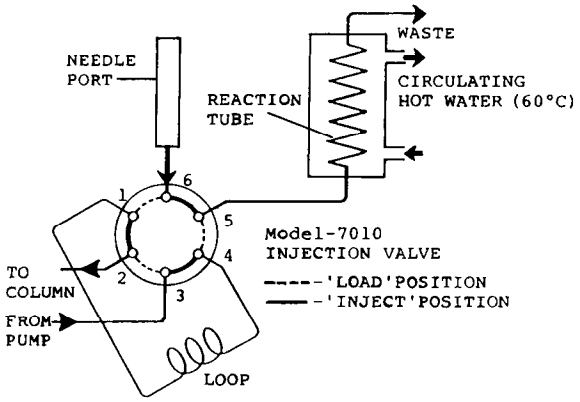

Fig. 1. Schematic diagram of the labelling process.

auto-sampling injector (ASI) (Gilson Medical Electronics). A 10-μl volume of NBD-F solution was aspirated from the vial, transported and ejected into a sample vial containing amino acids. The solution was mixed by bubbling with nitrogen gas. Then, an aliquot of more than 25 μl (programmable) was aspirated and transferred to the reaction tube (500×0.5 mm I.D. PTFE tubing), enclosed in a plastic pipe (100×30 mm I.D.), after passing through the injection valve at the "Inject" position and incubated for 1 min at 60°C. The reaction tube was connected to port No. 5 of a Model 7010 injection valve (Rheodyne) in the ASI with the shortest connection length using 0.25 mm I.D. PTFE tubing. The reaction mixture was carried to the 20-μl sample loop in the injection valve at the "Load" position, and then injected into the analytical column (Fig. 1). Sample solution was always held between air gaps during transportation to prevent diffusion or dilution. The needle, the needle port and the reaction tube were washed with ethanol used as a rinsing solution in the dilutor unit of the ASI, to prevent cross-contamination between samples and reagent. The next sample was treated in the same way as above. The reagent to be used for four to eight samples was placed in a reagent vial.

Amino acids automatically labelled with NBD-F as mentioned above were separated on an M&S Pack C_{18} reversed-phase column (M&S Instruments Trading) (150 mm×4.6 mm I.D., 5 μm, 100 Å, temperature 30°C) with a gradient HPLC system (Gilson Medical Electronics).

RESULTS AND DISCUSSION

Previously used eluents [1] contained phosphate buffer (pH 6.0), which afforded faster elution of acidic amino acids such as Asp and Glu. However, the peaks of interests were often preceded and affected by impurities present in the samples. Moreover, NBD-Asn, NBD-Gln, NBD-taurine, NBD-Ser, NBD-Gly and NBD-Thr were not well separated under these elution conditions [2]. Therefore, eluents containing acidic buffers were adopted in the present experiment. By using a starting buffer A, 0.15 *M* phosphoric acid–acetonitrile (84:16, v/v), and

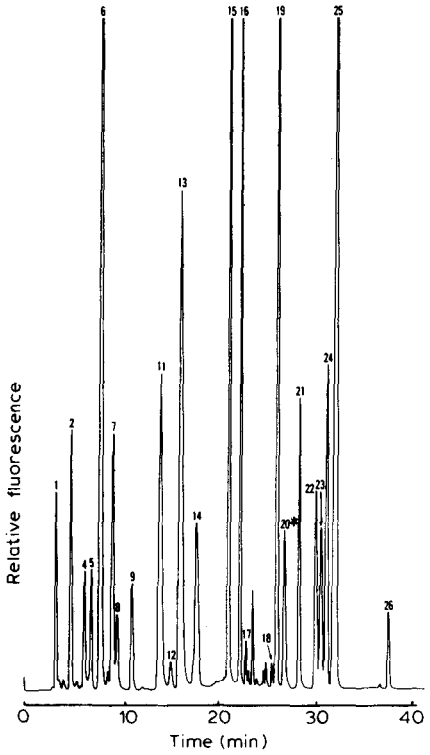

Fig. 2. Amino acid analysis of normal human serum. Free amino acids in normal human serum were analysed by the proposed method. Elution conditions: 0% B from 0 to 15 min, 0 to 80% B from 15 to 20 min, 80% B from 20 to 30 min, 80 to 100% B from 30 to 31 min, 100% B from 31 to 40 min. The flow-rate was 1.2 ml/min. The fluorimeter was operated at λ_{ex} 470 nm and λ_{em} 540 nm. Peaks: 1 = NBD-His; 2 = NBD-Arg; 3 = NBD-cysteic acid (the peak is not present); 4 = NBD-Tau; 5 = NBD-Asn; 6 = NBD-Gln; 7 = NBD-Ser; (8 = NBD-Ctr); 9 = NBD-NH_2; 10 = NBD-Asp; 11 = NBD-Gly; 12 = NBD-Glu; 13 = NBD-OH; 14 = NBD-Thr; 15 = NBD-Ala; 16 = NBD-Pro; 17 = NBD-Cys; 18 = NBD-Met; 19 = NBD-Val; 20 = NBD-$(Cys)_2$ [*No. 20 contains NBD-ornithine and NBD-$(Cys)_2$]; 21 = NBD-Lys; 22 = NBD-Phe; 23 = NBD-Ile; 24 = NBD-Leu; 25 = NBD-nor-Leu; 26 = NBD-Tyr.

a second buffer B, 0.1 *M* potassium phosphate–acetonitrile–methanol (40:21:39, v/v/v), and the elution conditions described in the legend of Fig. 2, the capacity factors of NBD-Asp and NBD-Glu were increased and the NBD-amino acids were well separated in 40 min. This separation was faster than the previous one [3], indicating that the presently used eluents would be suitable for the amino acid analysis of biological samples in general. According to a preliminary experiment, an increase in pH was necessary in the course of elution for adequate separation of NBD-Lys, NBD-Phe, NBD-Ile and NBD-Leu, and so potassium phosphate was used in buffer B to change the pH during the run.

Ten replicate samples containing 24 standard amino acids, each at 100 pmol, and another ten samples containing the same 24 amino acids, each at 20 pmol, were labelled and analysed in about 9 h. There were no amino acids for which the peak areas decreased from the first analysis to the last, suggesting that degrada-

TABLE I

FREE AMINO ACIDS IN NORMAL HUMAN SERUM (μM)

Amino acid	Sample No. 1	Sample No. 2	Sample No. 3
His	78	77	75
Arg	84	90	100
Taurine	44	53	84
Asn	46	51	53
Gln	883	881	890
Ser	126	120	142
Ctr	23	39	37
Asp	1	3	4
Gly	181	219	206
Glu	9	25	17
Thr	138	117	104
Ala	245	303	427
Pro	83	160	156
Met	26	22	33
Val	197	178	182
Lys	154	150	119
Phe	51	50	49
Ile	50	53	51
Leu	95	91	93
Tyr	55	59	63

tion of amino acids did not occur during test runs of about 9 h at room temperature. The average coefficient of variation (C.V.) of the peak areas relative to the internal standard (nor-Leu) of all but one amino acids (Cys) was 0.71% at 100 pmol. This average included 2.1% for Asp and 2.6% for Glu. At 20 pmol, the corresponding average was 1.18%, including 3.2% for Gly. Conversely, the C.V.s of Cys were 1.29% at 100 pmol and 4.47% at 20 pmol. It seems that a comparatively large amount of Gly in sample blanks (as mentioned below) and the instability of NBD-Cys affected the measurement at the 20-pmol level. The C.V. obtained previously with a manual method was 2.78% at 500 pmol of each amino acid [1]. The automatic pre-column labelling with *o*-phthalaldehyde (OPA) resulted in average C.V.s of 5.2–5.9%. This large variability was ascribed to instability of OPA adducts [4]. In a recent study using OPA [5], the C.V.s for Ser, Thr, Tyr and Met were 1.8, 1.9, 1.5 and 1.1%, respectively, when relatively large amounts of standard amino acids (500 μM each) were used. The excellent features of the presently used system (accurate volumetric transfer of samples and reagents, well controlled column and reaction temperatures and correct timing) might contribute to the high reproducibility of the method.

Various amounts of amino acids, each from 10 to 1000 pmol in 30 μl, were derivatized and analysed. Samples were analysed twice and the average peak areas were calculated. The amino acids gave a linear response in the range 10–500 pmol or more, except for Asp, Glu, Pro and Val, for which the responses were linear from 10 to 300 pmol. Cys gave a linear response between 50 and 200 pmol.

The detection limits for the amino acids using NBD-F were reported to be in

the range 10–100 fmol when a small aliquot of the reaction mixture containing relatively large amounts of amino acids was tested [1]. The detection limit in the present method depended greatly on the background derived from the reagent, buffer constituents and vial, etc., as a large portion of the reaction mixture was subjected to HPLC analysis (ca. 50% of 30 μl of the solution). In our laboratory, the background was less than 500 fmol for all the amino acids, except for Ser and Gly, for which it was 1 pmol. Accordingly, 50–100 pmol of each amino acid in a sample can be easily determined. Additional purification and clean-up of buffer constituents and reagents would be desirable for more sensitive detection of amino acids.

Free amino acids in adult human serum were extracted with methanol. The solvent was evaporated with a stream of nitrogen and the residue was dissolved in water as described previously [3] and analysed by the proposed method. The chromatogram and the results obtained are shown in Fig. 2 and Table I, respectively. Under the present conditions, the peaks of $(Cys)_2$ and ornithine were not separated. As the C.V. for Cys was higher than those of other amino acids, the Cys level in serum was not measured in this study. The C.V.s of each amino acid normalized by the internal standard were less than 4.4% ($n=4$). This value was about half of that obtained with manual labelling [3].

In conclusion, the automatic analyser using NBD-F will be useful for the routine analysis of amino acids in protein hydrolysates and body fluids. It might also be applicable to monitoring drugs with amino functional groups.

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