Journal of Chromatography, 303 (1984) 238–243
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,994

Note

High-performance liquid chromatography with automated pre-column derivatization for amino acids

R. H. BUCK* and K. KRUMMEN
Analytical Research and Development, Pharmaceutical Department, Sandoz Ltd., CH-4002 Basle (Switzerland)
(Received May 17th, 1984)

A high-performance liquid chromatographic (HPLC) system with automated pre-column derivatization was established for the determination of amino acids in enzymatic digests of peptidic drugs. The amino acids are derivatized with *o*-phthalaldehyde–mercaptoethanol[1] and separated by reversed-phase HPLC. The derivatization reaction is rapid, but the fluorescent products are unstable and the reproducibility of the results is determined by the precision of the sample and reagent volumes and the reaction time. Internal standards have been used to eliminate these problems[2–6].

In this paper an automated derivatization system is described in which the sample and reagent volumes are controlled very precisely by two loop injection valves. The reaction occurs on the high pressure side of the HPLC system in a packed bed reactor. The reaction time is determined by the flow-rate. This method combines the techniques of flow injection and chromatography.

EXPERIMENTAL

Apparatus

The instrument comprised two Altex pumps Model 100 controlled by the Altex microprocessor system 420, a Kontron automatic sampling system ASI 45 connected to two Rheodyne pneumatic loop injection valves and a peristaltic pump. A Kontron SFM 23 LC spectrofluorimeter was used for detection and a Hewlett-Packard data system HP 3357 for data processing. The packed bed reactors were laboratory-made by packing glass beads of mean diameter 0.28 mm into stainless-steel tubes (4 cm × 4.6 mm I.D. or 25 cm × 4.6 mm I.D.).

Chemicals

Methanol (HPLC grade) and tetrahydrofuran (analytical grade) were from Rathburn (Walkerburn, U.K.) and the amino acids from Sigma (London, U.K.). All other chemicals were purchased from Merck (Darmstadt, F.R.G.). Water was twice distilled before use.

0021-9673/84/$03.00

Derivatization reagent

o-Phthalaldehyde (100 mg) was dissolved in 3 ml ethanol. To this solution 220 ml of 0.4 *M* sodium borate buffer pH 10 and 0.1 ml 2-mercaptoethanol were added. This reagent was freshly prepared every 2 days.

Samples

As standard reference, 30 μmol each of aspartic acid, asparagine, serine, glutamine, glycine, threonine, alanine, arginine, tyrosine, valine, phenylalanine, isoleucine, leucine and lysine were dissolved in 1 l water.

Chromatography

The chromatographic conditions were similar to those given in ref. 7. Mobile phase A was 0.05 *M* sodium phosphate buffer pH 7.0–tetrahydrofuran (99:1, v/v); mobile phase B was methanol. A reversed-phase HPLC cartridge column spheri 5, RP 8, 5 μm (22 cm × 4.6 mm I.D.), from Brownlee Labs. (Santa Clara, CA, U.S.A.) was used. The flow-rate was 1.4 ml/min and the column pressure *ca.* 150 bar. The derivatization process proceeded in 100% mobile phase A. The separation of the derivatives was done by gradient elution from 100% solvent A to 75% solvent B in 26 min (see Fig. 3 for gradient curve). Derivatization and chromatography were carried out at room temperature.

Derivatization and chromatographic system

Fig. 1 shows the arrangement of the components for the automated pre-column derivatization and reversed-phase HPLC. The mobile phase stream coming from the

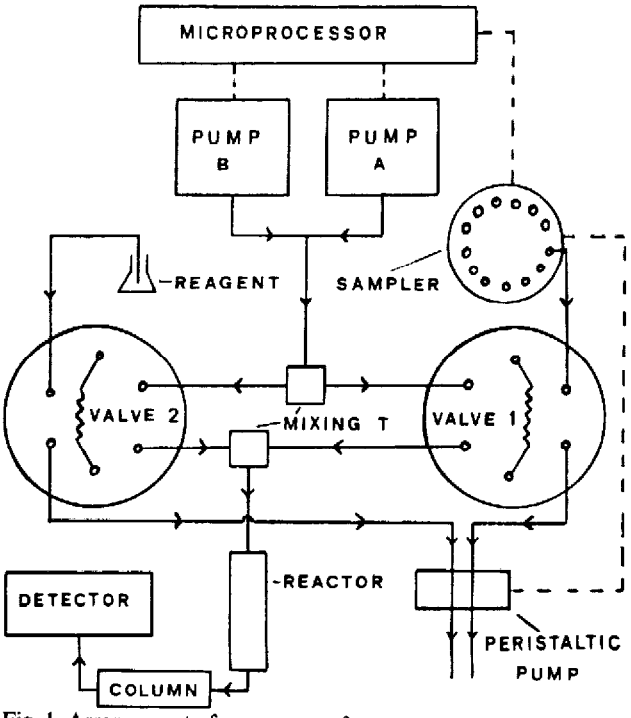

Fig. 1. Arrangement of components for automated pre-column derivatization and reversed-phase HPLC.

gradient system is split by a mixing T and led to two simultaneously acting pneumatic loop injection valves. One valve is equipped with a 10-μl loop and connected to an automatic sampling system, the other valve has a 50-μl loop and is connected to the reagent solution. After the valves, the eluent streams rejoin at the second mixing T and flow through the packed bed reactor and the chromatographic column.

At the beginning of each analysis, the sample and reagent loops are filled with the corresponding solutions. After switching the valves the sample segment is introduced into the reagent segment (see Fig. 2). The loop volumes and lengths of the capillaries are chosen such that the sample is completely enclosed by the reagent segment. The combined segments flow through the packed bed reactor where complete mixing and reaction occur. The reaction time is controlled by the flow-rate and the size of the packed bed reactor. When the chromatographic column is reached the reaction is stopped by the separation of sample and reagent. The derivatives are then separated by gradient elution and monitored by fluorescence detection.

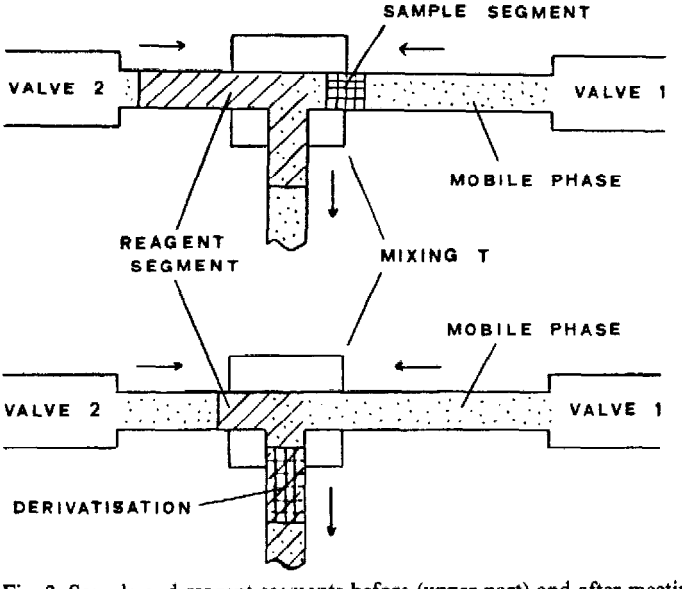

Fig. 2. Sample and reagent segments before (upper part) and after meeting in the mixing T (lower part).

RESULTS AND DISCUSSION

Derivatization of the amino acids and separation of the derivatives can be accomplished efficiently by the described system. An example of the separation of fourteen amino acids is given in Fig. 3.

The influence of the reaction time on the sensitivity and reproducibility was studied for the fourteen amino acids with packed bed reactors of two different lengths. The reaction times were calculated from the differences in retention times for the different packed bed reactors and are *ca.* 75 sec for the 25-cm reactor and *ca.* 13 sec for the 4-cm reactor at a flow-rate of 1.4 ml/min. The retention times, peak areas and standard deviations for both reactors are shown in Table I. All amino acids

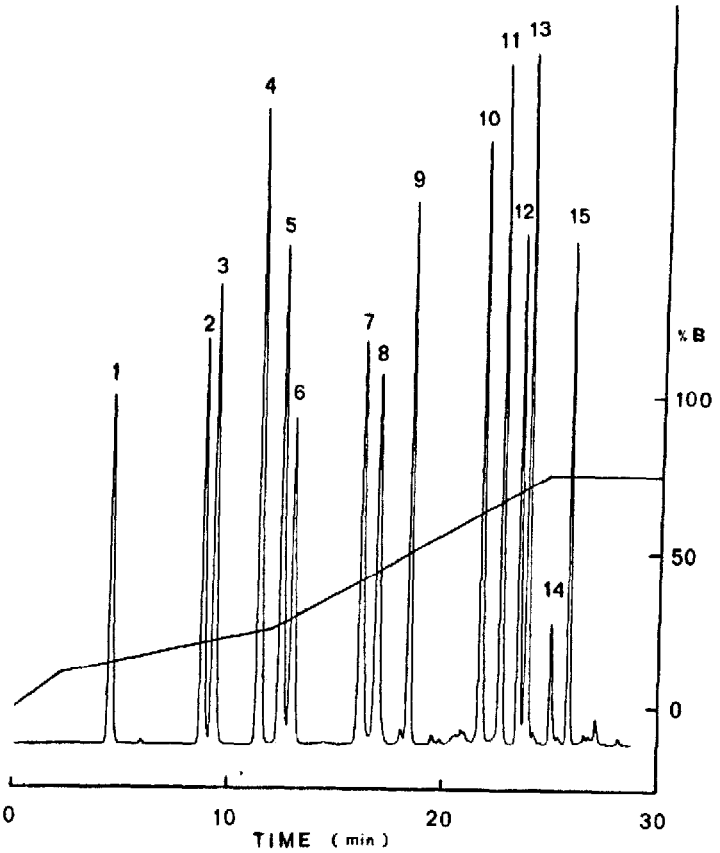

Fig. 3. Standard separation of amino acids after automated pre-column derivatization with *o*-phthalaldehyde–mercaptoethanol. Injected amount: each *ca.* 300 pmol. Packed bed reactor: column 25 cm × 4.6 mm I.D. filled with glass beads. For chromatographic conditions see text. Peaks: 1 = Asp; 2 = Asn; 3 = Ser; 4 = Gln; 5 = Gly; 6 = Thr; 7 = Ala; 8 = Arg; 9 = Tyr; 10 = Val; 11 = Phe; 12 = Ile; 13 = Leu; 14 = impurity; 15 = Lys.

investigated except Arg and Lys give a higher response after a reaction time of 75 sec than after 13 sec. The basic amino acids Arg and Lys react completely within 13 sec.

The reproducibility of the assay was tested with six consecutive injections (Table I). For ten amino acids the standard deviations were less than 3%, with a trend to improved reproducibilities with longer reaction times, whereas for Asp, Arg, Phe and Lys the standard deviations were above 3% with a trend to higher standard deviations with longer reaction times.

The linearity of the detector response was tested for glycine between 3 and 90 pmol per injection. An excellent correlation was found (r = 0.9999). The detection limit is below 0.3 pmol per injection and is determined by the purity of the reagents rather than by the signal to noise ratio.

Similar results were found by using 3-mercaptopropionic acid or ethanethiol instead of 2-mercaptoethanol in the derivatization reagent. With 3-mercaptopro-

TABLE I

REPRODUCIBILITY OF AUTOMATED PRE-COLUMN DERIVATIZATION ANALYSIS

Data from six consecutive analyses: I, reaction time *ca.* 13 sec; II, reaction time *ca.* 75 sec.

Amino acid	*I*				*II*			
	Retention time		*Peak area*		*Retention time*		*Peak area*	
	min	*Abs. S.D.*	*× 10⁴*	*Rel. S.D. (%)*	*min*	*Abs. S.D.*	*× 10⁴*	*Rel. S.D. (%)*
Asp	3.67	0.03	26	3.2	4.65	0.07	50	4.8
Asn	7.94	0.02	30	2.4	8.98	0.05	65	2.5
Ser	8.47	0.02	48	2.5	9.52	0.05	77	2.4
Gln	10.60	0.02	64	2.4	11.72	0.04	122	1.1
Gly	11.64	0.02	87	2.3	12.77	0.04	104	0.7
Thr	12.18	0.02	30	2.8	13.29	0.04	66	1.4
Ala	15.39	0.02	51	3.0	16.54	0.04	82	1.2
Arg	16.05	0.02	86	5.8	17.25	0.04	90	7.3
Tyr	17.62	0.02	42	2.3	18.72	0.03	76	1.5
Val	20.87	0.02	43	2-2	21.95	0.04	79	1.8
Phe	21.75	0.02	65	2.7	22.84	0.04	92	5.9
Ile	22.60	0.02	34	2.9	23.64	0.04	62	2.7
Leu	22.93	0.01	56	2.6	24.03	0.04	88	1.4
Lys	24.83	0.02	66	5.5	25.94	0.04	64	9.0

pionic acid the separation of the derivatives of Asp and Ser as well as of Gly and Thr is more difficult. With ethanethiol all fourteen amino acids could be derivatized and separated, but because of the unpleasant smell of ethanethiol no further investigations were carried out with this reagent.

The described system allows a fast, accurate and very sensitive determination of amino acids. It was successfully used for the assay of enzymatic digests of pharmaceutical peptides. A separation of fourteen amino acids takes only 30 min including reaction time and reequilibration of the column. The method is sensitive to picomol quantities of amino acids injected. Proline does not react with *o*-phthalaldehyde; cysteine should be oxidized[1] or treated with iodoacetic acid[8] prior to derivatization. Because of the dosage of sample and reagent by loop injection systems, pipetting errors are eliminated, and due to the flow-controlled reaction time, the derivatization is very reproducible. Consequently the method is very precise and internal standards are not necessary.

The flow-injection principle can also be used for other derivatization reactions, *e.g.*, the determination of enantiomers by reaction with a chiral reagent prior to chromatography. If necessary, the packed bed reactor can be heated in a column oven or a water-bath. As the derivatization occurs at high pressure, the operating temperature is not limited by the boiling points of the mobile phase components.

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