ON-LINE HPLC ANALYSIS OF PTH-AMINO ACIDS DERIVED FROM EDMAN DEGRADATION OF PROTEINS AND PEPTIDES: OPTICAL SENSOR CONTROLLED SAMPLE INJECTION

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A new principle in the system of identification and quantitation of amino acid phenylthiohydantoins derived from automated Edman degradation of proteins and peptides is described. The beginning of the on-line HPLC analysis of PTH's, the actuation of the valve and the sample injection are controlled by an optoelectronic sensor placed at the sample loop outlet. At the moment the loop has been loaded the sensor actuates the injection valve thus starting the chromatography run. This arrangement eliminates the possibility of incomplete (irreproducible) loading of the loop or of void injection. The chromatographic separation of the PTH's is carried out by isocratic elution.

The stepwise degradation of proteins and peptides by the automated Edman method[1,2] has received still increasing application in studies on protein sequences because of its reliability and high efficiency. The PTH's derived from the degradation are assayed almost exclusively by HPLC and principles have been reported of integration of the sequencer with the liquid chromatograph permitting direct on-line detection of PTH-amino acids[3-5].

The present study describes the coupling of the Applied Biosystems Model 470A Gas Phase Protein Sequencer to Beckman Series 340 High Performance Liquid Chromatograph.

EXPERIMENTAL

Material and Methods

Chemicals. Sperm-whale myoglobin was from Beckman, Austria, SDS from Serva, F.R.G., acetonitrile from Merck, F.R.G. The chemicals for sequencing were purchased from the sequencer manufacturer.

Apparatus and chromatographic conditions. The Edman degradation was carried out in Model 470A Gas Phase Protein Sequencer (Applied Biosystems, U.S.A.) according to a modified standard program 03CPTH supplied by the manufacturer. The content of PTH's in the individual cycles was assayed by HPLC using a system consisting of Beckman 114 Solvent Delivery Module, Beckman 421 Controller with 420 Pneumatic Interface Card, Beckman 340 Organizer and Altex

210 injection valve assembled with a 20 μl or 50 μl loop. The effluents were monitored at 269 nm in Shimadzu SPD-2A Spectrophotometric Detector and quantitated in Shimadzu Chromatopac C-R3A integrator. For the chromatographic separation a Beckman Ultrasphere ODS column (4·6 mm × 250 mm, particle size 5 μm) or a DuPont Zorbax ODS column of the same dimensions were used at 40°C and a flow rate of 1 ml/min. Isocratic elution[6] and recycling of the eluent were used in both cases. The eluent was a 40% acetonitrile solution in 0·032M sodium acetate, pH 4·5, containing 100 mg of sodium dodecyl sulfate (SDS) per one liter. The injection valve was controlled by Beckman 420 penumatic actuator. The optoelectronic sensor for the detection of liquid flow through the capillary was manufactured in the electronics shop of the Institute.

Principle and Function of Sensor.

The automatic feeding of samples to the column was effected by placing the sample loop of the injection valve between the conversion vessel and the fraction collector of the sequencer (Fig. 1). The inlet port of the valve was connected to the conversion vessel through a 40 cm teflon capillary of 0·5 mm i.d. The injection needle of the chromatograph was placed at one end of the capillary. The outlet port of the injection valve was connected to the fraction collector through a capillary 60 cm long, 0·3 mm i.d. and 1·5 mm e.d. The optoelectronic sensor was placed 40 mm apart

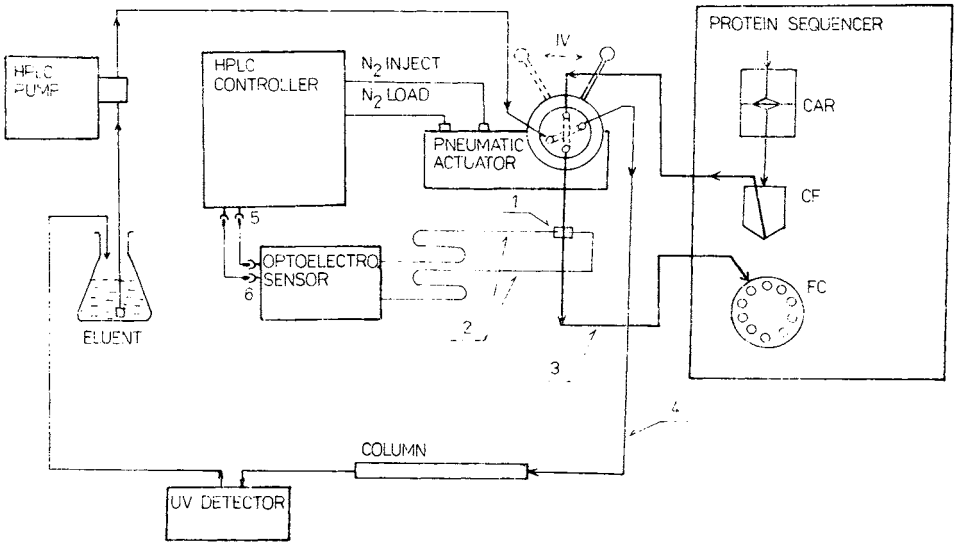

FIG. 1

Schematic representation of on-line HPLC of PTH's and of optoelectronic sensor-controlled sample injection. Individual modules: Applied Biosystems Model 470A Protein Sequencer; Beckman 114M Delivery Module; Beckman 421 Controller; Shimadzu SPD-2A Spectrophotometric Detector; Beckman Ultrasphere ODS 4·6 × 250 mm column (particle size 5 μm); Beckman 420 Pneumatic Actuator; Altex 210 injection valve. CAR cartridge, CF conversion flask, FC fraction collector, IV injection valve, 1 holder, 2 optical fiber, 3 teflon capillary, 4 HPLC stainless steel capillary, 5 remote-input response of HPLC controller, 6 optoelectronic sensor output

from the valve. PTH derivatives in acetonitrile–water solution are passed through the injection loop to the sensor which after the flow start and adjustment of the time lag (0·4 s to eliminate bubbles in the loop) pneumatically actuates external flags 1 and 2 of the HPLC controller programmed as shown schematically in Table I.

The sensor (Fig. 2) is a teflon capillary of 1·5 mm e.d. and 0·3 mm i.d. placed radially in a cylindrical holder and accommodating two optical fibers axially to both sides of the holder with ends in close contact with the capillary wall. The axis of both optical fibers (PCS type, core diameter 0·2 mm, e.d. 0·4 mm, numerical aperture 0·27, length 300 mm) must pass through the center of the capillary. One of the fibers transmits (with type WK 16 403 LED at its terminus) light into the capillary. The optical output coupled to the fiber is 50 μW and the light wavelength is 810 nm. The other fiber (with type KPX 81 phototransisotr at its terminus) acts as

TABLE I

Program of HPLC control

Time, min	Function	Value	Duration
0·00	FLOW	1·0	0·5
0·00	% B	0·0	0·5
4·90	ALARM		0·1
5·00	HOLD		
5·01	EXT. FLAG	1	0·02
5·01	START INT		
5·40	EXT. FLAG	2	0·02
30·0	STOP INT		
30·1	LOOP TO	0	

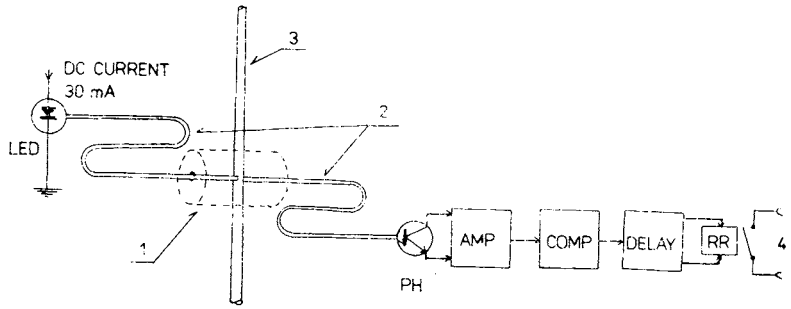

FIG. 2

Optoelectronic sensor of liquid flow through capillary. See text for explanation of sensor function. PH phototransistor, AMP amplifier, COMP comparator, DELAY delay 0···1 s, RR reed relay, 1 holder, 2 optical fiber, 3 teflon capillary, 4 output

light receiver. All these parts are products of TESLA-Blatná, Czechoslovakia. The light intensity varies with the liquid content of the capillary and these variations are converted into changes of electric current. The latter are amplified and then converted into voltage changes in MAA 741 amplifier and the signal treated in MA 1458 comparator. The polarity change of the voltage indicating that the capillary has been filled up is delayed by an adjustable (0—1 s) MAA 741, KF 506 time-lag element to compensate for the presence of air bubbles in the injection loop.

The degradation programs were made compatible with the on-line principle of the injection as shown in Table II. After the sample loop has been loaded and the loop content injected into the column the sample flow to the collector is discontinued and the loop is flushed. Approximately 40 s thereafter the valve is returned to its original position and the excess of the fraction solution delivered to the collector. Since the system does not respond to the flow of liquid through the loop during the sample analysis, the capillary and the loop can be flushed by another portion of the eluent and dried to prevent the sensor from switching-on too early because of traces of liquid in the capillary. Drying is effected by argon (additional command „collect", flask function 12 of sequencer program).

Another modification of the procedure involves the replacement of acetonitrile by its 40% aqueous solution used for PTH transfer. The volume of the loop can then be increased to 50 µl and 25 to 50% of the PTH solution in the conversion vessel can be injected. With undiluted acetonitrile blurred signals appear on the chromatogram. The replacement of acetonitrile by its aqueous solution has no effect on the degradation yields.

RESULTS AND DISCUSSION

On-line injection based on time synchronization only was found to be little reliable in our experiments. The time necessary for the loop to fill up varied with ambient temperature, with the presence of additional components in the sample and with other factors and the valve became actuated even when the loop was empty or half-empty. These difficulties are completely eliminated by the sensor control of liquid

TABLE II
Modification of standard program 03CPTH

Step	Cartridge function	Flask function	Time, s
1—4	cf. program 03CPTH		
5	Pause	Collect	120
6—27	cf. steps No 5—26 of program 03CPTH		
28	Deliver R3	Collect	180
29—48	cf. steps 28—47 of program 03CPTH		

flow. The capillary must be perfectly dry before the injection since traces of the liquid could actuate the sensor too early.

We have been using this system of sample injection into the HPLC column for several years without any major problems. The system has several advantages: *a*) larger numbers of samples can be analyzed, the capacity of HPLC is increased about two times, *b*) the system is fully automatic, no personal control is necessary, *c*) the storage of fractions, their concentration and dissolution are eliminated, *d*) the practically instantaneous analysis has a favorable effect on the yields of several, less stable PTH's, *e*) the original functions of both the sequencer and of the chromatograph are unaltered, the individual fractions are collected in the collector and can be used for manual injection whenever necessary, and *f*) the isocratic elution systems permits the eluent to be recycled several times and thus the quantity of acetonitrile to be decreased.

The autosampling system described here differs from the commercial systems, such as, e.g. Applied Biosystems Model 120A On-Line PTH Amino Acid Analyzer in various parameters because of differences in construction, principle of elution, column dimensions, etc. The cost of the PTH analyses is several times lower compared to the commercial instrument.

Our procedure is reproducible to $\pm 3{\cdot}0\%$ and as yet about 2 000 samples have been analyzed without any failure of the system which is thus comparable to the commercial instrument. Its another advantage is that any HPLC apparatus can be coupled to the sequencer; hence, there is no need for buying a specialized module.

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