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Note

Separation of enkephalins from their metabolites and precursor by a single pump gradient high-performance liquid chromatographic method

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The rapid peptidase hydrolysis is the major cause of the brief physiological action of the enkephalins (Tyr-Gly-Gly-Phe-Met/Leu)[1]. Three amide bonds of the enkephalins are susceptible to be cleaved by peptidases[2,3]. Tyr is the metabolite of enkephalin hydrolyzed by the aminoenkephalinases, Tyr-Gly is the product by enkephalinase B and dipeptidylaminopeptidase, and Tyr-Gly-Gly is the product by enkephalinase, metallo-endopeptidase, and angiotensin-converting enzyme. Since Tyr-Gly and Tyr-Gly-Gly are resistant to further hydrolysis by brain membranes, their formation after the incubation of enkephalin with the brain membranes can be a direct measure for the activity of the different enzymes involved[4]. Tyr, Tyr-Gly, and Tyr-Gly-Gly can be determined by high-performance liquid chromatography (HPLC) with a C_{18} column eluted with acetonitrile–0.1 *M* phosphate buffer, pH 3 (4:96)[5]. Because the intact peptides (enkephalins) are retained on the column, their unexpected release after the column is fully loaded interferes with the determination of the enkephalin metabolites. The incubated enkephalin can be quantitated by the same type of column eluted with 27.5% of acetonitrile in the phosphate buffer[5]. But this mobile phase is too hydrophobic to withhold Tyr, Tyr-Gly, and Tyr-Gly-Gly on the column. In order to study the role that the different peptidases play in the enkephalin inactivation, it is desirable to separate and quantitate Tyr, Tyr-Gly, Tyr-Gly-Gly, enkephalins, and enkephalin precursor (Arg^0-Met-enkephalin)[5] in the same run. We report here a single pump gradient HPLC method to achieve this goal.

EXPERIMENTAL

The single pump gradient HPLC system consists of two parts, the gradient generating system and the conventional single pump HPLC.

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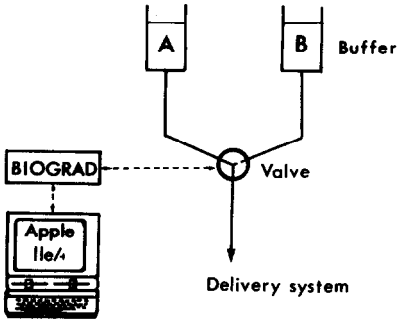

Fig. 1. Schematic diagram of the gradient system. Through the Apple IIe, BIOGRAD controls the high-speed three-way valve which selects the flow from buffer A or buffer B. The buffer then flows directly into the delivery system.

Gradient generating system

The gradient was generated by a Biograd gradient system controlled by an Apple IIe microcomputer (Cupertino, CA, U.S.A.) with a Duodrive and a monitor. The Biograd gradient system purchased from Bioputa Innovations (Palisades, NY, U.S.A.) contains an interface card, a control box, a high-speed three-way valve and an accompanied software BIOGRAD.

The principle mechanism of the gradient is as follows: the initial buffer (buffer A) is pumped when the valve is in the "on" position and the final buffer (buffer B) is pumped when in the "off" position (see Fig. 1). The software BIOGRAD contains four interactive programs. The program varies the on–off ratio of the valve in order to produce the desired compositions. The software is able to generate up to 100 000 on/off cycles for each run. The program SETUP is used to set up the gradient file by the user. Up to nineteen linear segments of gradient can be compiled in each run and the run time is user definable from minutes to ten days. The gradient file can be saved on a floppy disk which can be retrieved as needed and re-run as many times as desired. After the gradient file is established, the program GRADIENT is used to run and control the system. Current status is continuously displayed on the monitor during the run. The program DIRECTORY is used for file management and upkeep the created gradient files. Finally, the program RECORD is used for making hard copies of the gradient files.

High-performance liquid chromatography

The mixture of acetonitrile and 0.1 *M* phosphate buffer, pH 3.0 was used as elution buffer. Buffer A contains 3% of acetonitrile where buffer B contains 60% of acetonitrile. Tyr, Tyr-Gly, Tyr-Gly-Gly were obtained from Sigma (St. Louis, MO, U.S.A.); Met-enkephalin and Leu-enkephalin from Boehringer Mannheim (Indianapolis, IN, U.S.A.), and Arg^6-Met-enkephalin from Peninsula Laboratories (San Carols, CA, U.S.A.). HPLC-grade acetonitrile was purchased from Fisher (Fairlawn, NJ, U.S.A.). The pump employed was a Waters (Milford, MA, U.S.A.) 6000 pump or a Milton Roy (Chicago, IL, U.S.A.) minipump with a flow-rate of 0.7 ml/min. The sample was loaded onto a LiChrosorb RP-18 column (250 × 4.6 mm, particle size 7 μm, Unimetrics, Westwood, NJ, U.S.A.) through a Waters septumless U6K injector. The sample was eluted at ambient temperature resulted with a back pressure

of 500 p.s.i. The eluate was monitored for Tyr by UV absorbance at 280 nm with a LKB Uvicord S (Bromma, Sweden) equipped with a HPLC flow cell (volume 8 μl).

Accuracy and reproducibility test of the gradient

Water was used as the starting solvent (buffer A) and 0.1 m*M* Tyr solution was used as the final solvent (buffer B). The solvent mixture, with a flow-rate of 2.5 ml/min by gravity force, was monitored by UV at 280 nm (specific for Tyr) with a LKB Uvicord S equipped with a quartz flow cell (volume 70 μl). Under these conditions, the relative composition of the two solvents could be continuously determined. The electronic signal sent from the computer to the valve was compared with the actual UV tracing of Tyr in the mixture.

RESULTS AND DISCUSSION

Using the present gradient system for separation of the opioid peptides from their metabolites and precursors with a reversed-phase HPLC C_{18} column, Tyr, Tyr-Gly, Tyr-Gly-Gly, Met-enkephalin, Leu-enkephalin, Arg^0-Met-enkephalin were well resolved from each other with the retention times of 5.2, 7.7, 6.6, 26.2, 28.6, and

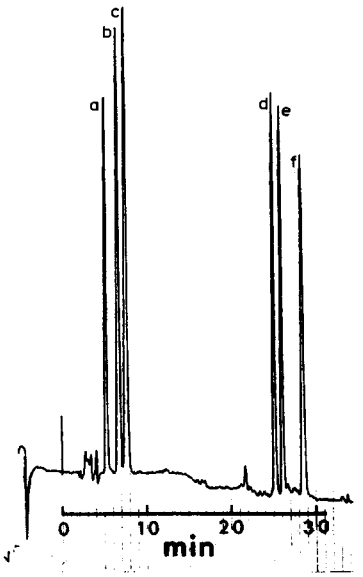

Fig. 2. HPLC chromatogram of enkephalins, their metabolites, and precursors. The peaks are Tyr (a), Tyr-Gly-Gly (b), Tyr-Gly (c), Arg^0-Met-enkephalin (d), Met-enkephalin (e), and Leu-enkephalin (f). For details of the chromatography see the Experimental section. The gradient is generated by the Biograd system with 5-sec cycles and the run time is 30 min. The gradient profile is as follows:

Run time (%)	*Solvent B (%)*
0	0
20	0
25	45
100	50

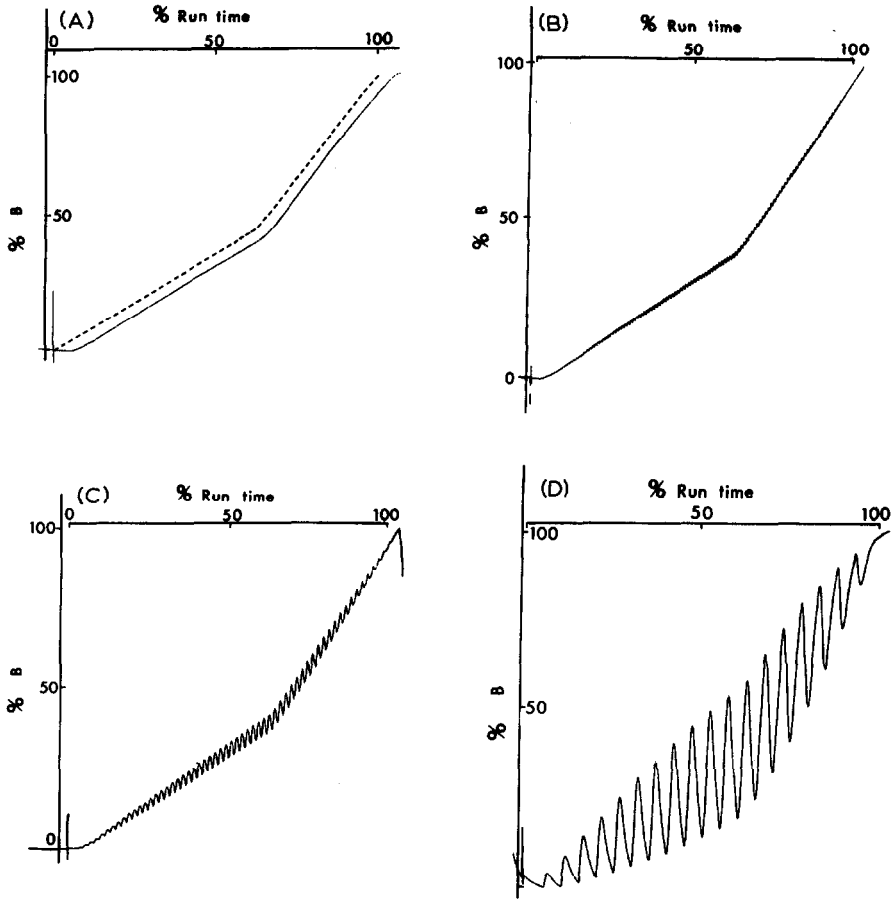

Fig. 3. Gradient processor test. Comparison of the electronic trace of the signal sent to the valve with the actual UV trace obtained for the analogous tyrosine solution gradient in water. For details see the Experimental section. (A) The electronic trace (broken line) and the UV trace (solid line) of the gradient with 1-sec cycles; (B) UV trace of the gradient with 5-sec cycles; (C) UV trace of the gradient with 10-sec cycles; (D) UV trace of the gradient with 30-sec cycles.

25.2 min, respectively (Fig. 2). With the gradient system, these amino acid and peptides were chromatographed five times with identical elution times (±2%).

The accuracy of the gradient depends largely on the length of the on/off cycles. When the "cycle time" is 1 sec, practical no step can be seen in the UV tracing of the mixture (Fig. 3A). If the "cycle time" is 5 sec or longer, the steps can be seen in the solvent (Fig. 3B–D). The mobile phase profile is well reproduced with good transition and little rounding or corner (Figs. 3A and 4). It illustrates the fidility of the gradient system, and no mixing chamber is necessary to put on line. Fig. 4 shows the reproducibility of the gradients. Ten or more consecutive gradients maintained the same high quality.

The gradient system provides the maximum resolution per unit time in HPLC, liquid chromatography, density centrifugation, gel electrophoresis, and electrofocus-

Fig. 4. Gradient reproducibility test. The UV trace obtained for the analogous tyrosine solution gradient in water. The sensitivity of the lower tracing is 1/10 of the upper one. For details see the Experimental section. The time of each run is 10 min and the program of each run is as follows:

Run time (%)	*Solvent B (%)*
0	0
20	0
35	10
45	50
55	55
70	0
80	25
90	80
100	100

ing. In liquid chromatography, flow-rate and temperature of the mobile phase have a minor effect on reproducibility; a 1% change in flow-rate produces a 1% change in retention, and 1°C change in temperature produces a change in retention of about 2%. The major contribution to changes in retention time and peak area (or height) comes from the mobile phase composition. The inaccuracy of the gradient program is the greatest source of error in the reproducibility experienced in the gradient elution.

There are two types of gradient formers according to whether the mixing of the solvent takes place at low (atmospheric) pressure or at high pressure. A high-pressure system requires two relatively sophisticated pumps (speeds variable by remote control) and a high-pressure mixing chamber. The method, however, has the disadvantage of being more expensive, it suffers further from problems due to its inability to pump very small volumes (<0.04 ml/min) and to flow inaccuracies at very low and very high flow-rates. Although the low-pressure gradient former uses only one pump, most of the commercial instruments for such applications are expensive and beyond the reach of many research laboratories. Based on a 10-step timer, a gradient programming system was designed to produce a linear gradient[6]. This system suffers from several drawbacks; the gradient step is clearly distinguishable when it is without a mixing chamber. At higher flow-rates (1.5 ml/min), the gradient forms steps even with a mixing chamber on line. The linear gradients are reproduced with the running time varied from 0.5 to 2 h. At the start of each run, the gradient program must be re-entered, which is tedious and time-consuming. Furthermore, it can not produce a gradient with 100% solvent B.

By taking the advantage of the recent advent of the microcomputers, the present system precisely and repeatedly generates different kinds of gradients. The performances of the system matches the dual pumps one. In addition, it can control up to four valves simultaneously; one can run four different independent gradients, or use the extra three controls to turn on or off the components of the liquid chromatography, namely pumps, recorders, or activate automatic injectors. The high-

speed valve can be converted to a stream splitter by reversing the flow of the liquid and provides any proportioning ratio, which is very useful in post-column derivatization[7]. It can also serve as a liquid divertor which directs the eluate, for example, from the collector to the waste or *vice versa.*

Because of its excellent versatility, convenience, accuracy, and reproducibility, the present gradient system is now in use with a HPLC column for the isolation of the cytoskeletal proteins from brain and the method will be published elsewhere. It has been adapted for the preparation of gradient polyacrylamide gels for the electrophoresis of the neurofilament proteins using different gradient profiles. The present gradient system can also be used in conventional chromatography at low temperatures for purification of peptidases that participate in the enkephalin inactivation[8].

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