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AUTOMATED PRECOLUMN DERIVATIZATION DEVICE TO DETERMINE NEUROTRANSMITTER AND OTHER AMINO ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A device to derivatize amino acids with *o*-phthaldialdehyde, which is directly connected to high-performance liquid chromatographic equipment is described. Its principle is that a sample (10–500 μl) is mixed with a reagent (containing *o*-phthaldialdehyde, 2-mercaptoethanol and sodium hydrogen carbonate buffer), using a peristaltic pump. This mixture is pumped into a loop of a pneumatically controlled injection valve at atmospheric pressure. When the derivatization is complete the valve switches, so that the sample is applied to a column and the amino acid derivatives are separated with a gradient of methanol–phosphate buffers. The reproducibility is such that brain perfusates or tissue extracts can be analyzed for the amino acid transmitter content and no internal standard is necessary. The major advantages of the present device are that it produces thorough mixing of reagent and sample, so that a high and constant degree of derivatization occurs (thus producing high sensitivity; less than 0.1 pmol can be detected) and its low cost.

INTRODUCTION

In recent years several methods to assay endogenous amino acids in biological material have been described. Among the most sensitive assays are those based on gas–liquid chromatography, in combination with mass spectrometry, and on high-performance liquid chromatography (HPLC), in combination with fluorimetry[1–5].

In the earlier methods the amino acids were rendered fluorescent after separation by ion-exchange chromatography[4–6], but more recently, fluorescing derivatives of the amino acids have been prepared before HPLC. Fluorescing amino acid derivatives have been made with, *e.g.*, *o*-phthaldialdehyde[7,8], Dns chloride[9] or dimethylaminoazobenzenesulphonyl chloride (Dabs)[10] and they were separated by reversed-phase HPLC.

Derivatization with *o*-phthaldialdehyde is especially attractive because the fluorophore is formed rapidly in aqueous solution and no laborious purification procedures preceding HPLC are required[7,8,11–13]. A disadvantage of this approach

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is that the fluorescent derivatives of the various amino acids are not of similar stability[14,15], implying that the derivatization reaction time has to be fixed. This is impracticable when, in the case of automation, the samples have to be prepared several hours in advance.

Here we describe a device to derivatize automatically amino acids with *o*-phthaldialdehyde for a fixed time interval before chromatography. The principle of the device is similar to that of the fluorimetric assay of γ-aminobutyric acid (GABA) with a continuous flow system, developed previously in our laboratory[16]. The present device is sufficiently versatile for application to other precolumn derivatization procedures.

MATERIALS AND METHODS

Amino acids and reagents

All solutions were made in water doubly distilled from quartz. Stock solutions of mixtures of the amino acids (1 g l^{-1}, analytical grade) were diluted to concentrations of 0.5 or 0.1 μg ml^{-1} and approximately 10 or 50 μl were used for HPLC. The fluorophores were produced with a reagent prepared as folows: 25 mg *o*-phthaldialdehyde [Cat. No. 79760 (Fluka, Buchs, Switzerland) or Cat. No. 44462 (BDH, Poole, Great Britain)] were dissolved in a drop of methanol and added to 25 ml 0.5 *M* $NaHCO_3$ (pH adjusted to 9.5 with sodium hydroxide solution) containing 25 μl 2-mercaptoethanol (Cat. No. 805740; E. Merck, Darmstadt, G.F.R.).

HPLC and detection

Chromatography of the amino acid derivatives was performed at room temperature on a column (20 cm × 4.6 mm I.D.) packed with LiChrosorb RP-18 (particle size 5 μm, Merck Cat. No. 9333) with a guard column (7.5 cm × 2.1 mm I.D.) of pellicular reversed phase, particle size 30 μm (Chrompack Cat. No. 28623) on top. The amino acid derivatives were eluted with a concave gradient of the following buffers: A, methanol–tetrahydrofuran (Merck, Cat. No. 9731)–0.05 *M* sodium phosphate buffer, pH 6.5 (adjusted with H_3PO_4 in the complete mixture) (15:0.5:85, v/v/v); B, methanol–0.05 *M* sodium phosphate (final pH 6.5) (80:20, v/v). Two Waters HPLC pumps (6000A), controlled by a solvent programmer (Waters 660), produced the concave gradient of 3–35% B in 40 min, at a flow-rate of 2 ml min^{-1}. Prior to use, the buffers were passed through a membrane filter OE 67 (maximum particle size 0.45 μm, Schleicher and Schüll Ref. No. 360111). This elution procedure is a slight modification of that of Jones *et al.*[17].

The fluorophores were detected by a Kontron spectrofluorometer (SFM 23) equipped with a 100-μl flow-through quartz cell and with two monochromators (holographic concave gratings) and a Xenon high-pressure lamp (150 W) as a light source. The excitation wavelength was 340 nm and emission was recorded at 460 nm or with a Kodak-Wratten 2A filter (cut-off at 450 nm).

Precolumn derivatization

Principle. Using a peristaltic pump a sample of approximately 100 μl is taken from a mixture of several amino acids in a small plastic cup, placed in a sampler and mixed with the *o*-phthaldialdehyde reagent obtained from a bulk solution. This mix-

ture is led through a loop (of about 10 or 50 μl) of a standard pneumatically controlled injection valve for HPLC. After completion of sampling an air-bubble is introduced.

Thus, in front of each sample an air-bubble from the previous sample moves through the injection loop and connecting tubing to the bubble detector. On arrival of this bubble in the detector the position of the injection valve is changed from the load-sample to the inject-wash position, but the sampler and the magnets are still in the position described in the upper part of Fig. 1. The latter are moved when the air-bubble has passed the detector and the remainder of the sample and reagent —not applied to the column— arrives. As a consequence, the positions of both injection valve and sampler are now as shown in Fig. 1, lower part.

In Fig. 1 only two of four possible configurations of the combined positions of the valve and the sampler are shown.

After the sample has been applied to the column and the analysis is almost complete, the positions of the valve, the magnets, the sampler and the stainless-steel sample needle are changed, and the cycle is repeated, implying that the positions described in Fig. 1 (upper part) are reached again.

During the switchings the peristaltic pump gives a constant flow. Any change in the flow-rate does not alter the mixing ratio between sample and reagent (which is usually 4), but does alter the reaction time, as more or less time is required before the air-bubble reaches the air-bubble detector. An alternative to air-bubble detection is

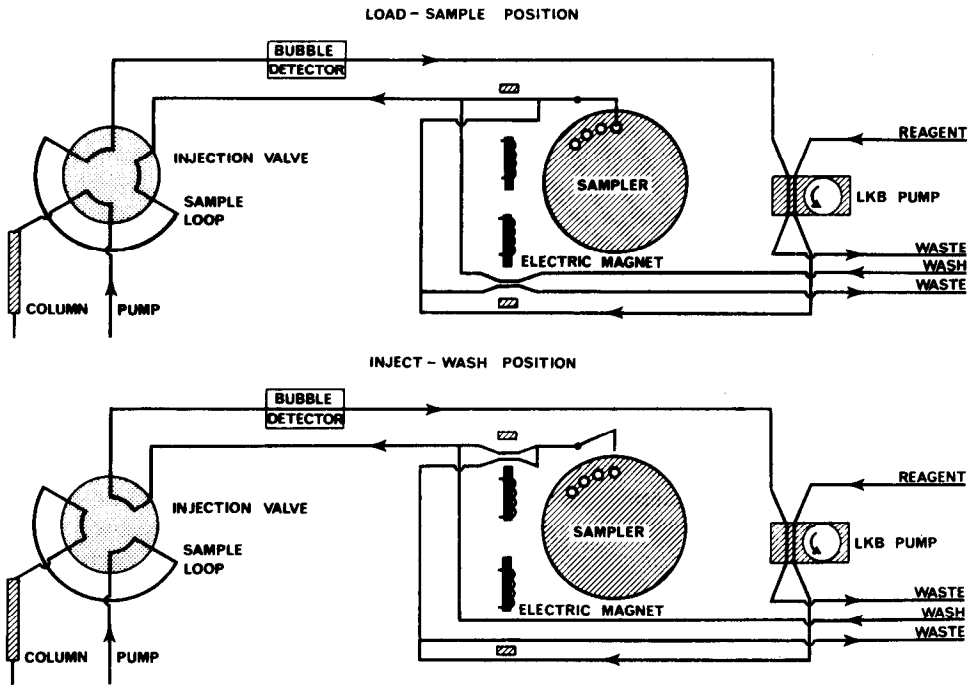

Fig. 1. Schematic representation of the precolumn derivatization device. Two of the four positions are shown. The principle and the components are described in detail in the text (Materials and methods).

the use of a timer, which controls the various switches, sampler and injection valve. In this case the reaction time is also dependent on the flow-rate of the peristaltic pump.

Device. The precolumn derivatization device consists of the following components:

A peristaltic pump (LKB 12000, Varioperpex) with two Tygon Autoanalyzer tubes with internal diameters of approximately 0.04 and 0.09 mm for reagent and sample, respectively.

A sampler made from a fraction collector for gas chromatography (Packard Model 852) by a simple change of the control of the sample inlet. Samples were kept in plastic cups (having a maximum volume of approximately 0.75 ml, a conical tip and a cap). Usually the volumes of the samples are less than 0.1 ml, so that at the end of the sampling air is introduced, which is necessary to provide a signal for the bubble detector. Other sample volumes are allowed, but then the volume of the tubing of the device has to be adapted. When a timer is used, any sample volume can be employed.

Solenoid valves, that control the connections of the samples to the injection valves, are derived from an automatic sample changer (Unicam SP 92) used with a flame spectrophotometer. Each electric magnet closes two tubes, made of flexible material (silicone, 0.60 mm I.D.).

The injection valve, an air-actuated sample injection valve (Valco, Model AH-CV-6-UM Pa-N60) usually equipped with sample loops of 10 or 50 μl, but other volumes are also applicable. The valve is controlled by a four-way air switch (Chrompack, Cat. No. 12717, manufactured by Humphrey Air Kalamazoo).

The bubble detector, consisting of a reflective object sensor (optocoupler, 1C OPB 703, Optron) placed near a glass tube (1.5 mm I.D., 2.3 mm O.D.). When air-bubbles pass through the glass tube the IR light emitted by the reflective object sensor is diffracted differently compared to when the glass tube is filled with aqueous solutions. The signal from the bubble detector is recorded by a microprocessor.

Microprocessor. All switches of the derivatization device and the sampler are controlled by a microprocessor (Apple I.T.T. 2020 microcomputer). The electrical circuits from the microprocessor to the device are separated by optocouplers. The microprocessor is programmed either to react to signals from the bubble detector or to function as a timer. In any case the injection valve and the sampler plus solenoid valves can be controlled separately.

RESULTS AND DISCUSSION

An example of a chromatogram is shown in Fig. 2, upper part.

As seen in Fig. 1, with the exception of the sample needle and adjacent tubes, all parts of the system are extensively washed between sampling. The needle, however, is washed only by the first part of a sample and this part passes through the loop until it reaches the bubble detector and is therefore not chromatographed. The carry-over of samples was determined and the lower part of Fig. 2 represents a record, when only water is applied, following the sample shown in the upper part. Carry-over of much less than 1 % is found, which is negligible for our research purposes.

In preliminary experiments the reagent was optimized. The search to obtain a high ratio of sample to reagent volumes resulted in a practical ratio of 4. The reproducibility of the procedure is high (less than 1 % variation, when the same sample was analyzed repeatedly) and the sensitivity is such that less than 1 pmol can be analyzed reproducibly. The method is linear between 1 pmol and at least 10 nmol of the amino acids per sample, and also when volumes ranging from 10 to 500 μl are analyzed.

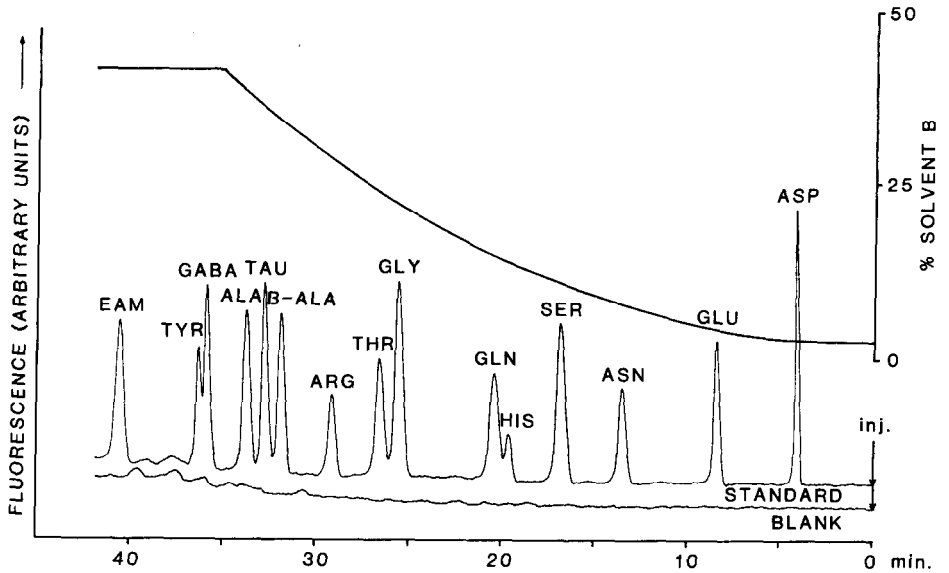

Fig. 2. Recordings of a standard mixture of fourteen amino acids and ethanolamine (EAM), applied amounts 50 pmoles, and a consecutive blank at the same sensitivity to demonstrate the carry-over of sample. Also shown is the composition of the eluent. Abbreviations of the amino acids are as recommended by the International Union of Biochemistry.

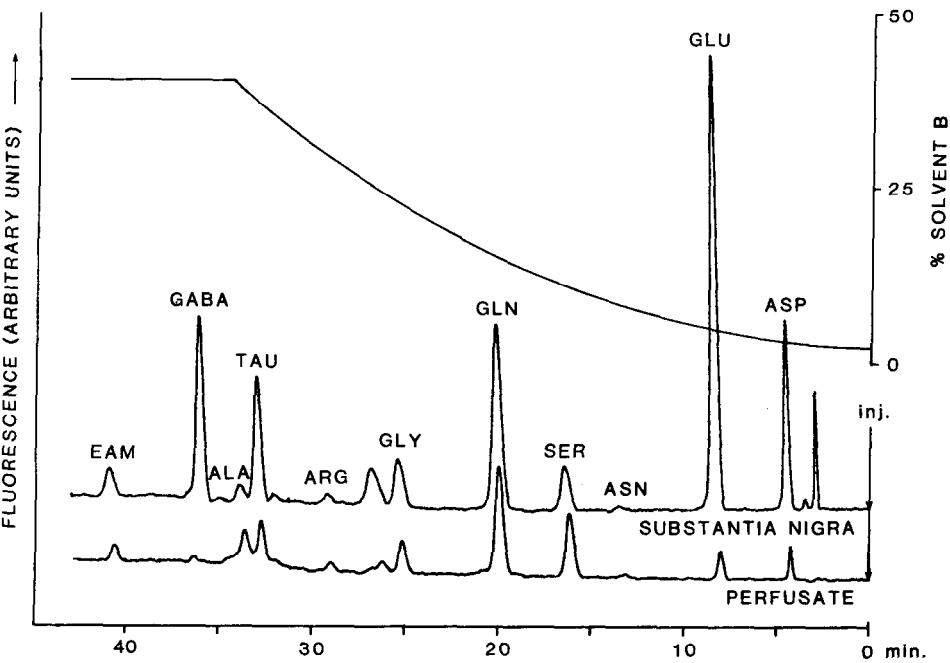

Fig. 3. Recordings of a tissue extract of 1 mg of brain (the *substantia nigra* of a rat) and a brain perfusate (from the rat thalamus). The composition of the eluent is also shown. Further details as in Fig. 2.

Recently, alternative equipment for automatic precolumn derivatization became commercially available (Waters HPLC Automation System, Model 720 System Control and a Waters TAG TM). At present we are unable to compare the two equipments, in terms of cost, sensitivity, reproducibility and sample volumes that can be handled.

Between April 1981 and the submission of this paper more than 1000 samples of diluted brain tissue extracts in 5 % trichloroacetic acid or perfusates of various brain areas, without purification, have been succesfully analyzed (methods and results in refs. 18, 19). Examples of recordings of brain perfusate (from the thalamus of an anaesthetized rat) and brain tissue extracts (from the *substantia nigra* of a rat brain) are shown in Fig. 3. The application of the equipment described has been predominantly applied to the analysis of amino acid transmitters of the brain (including glutamate, aspartate, glycine and γ-aminobutyric acid), but in principle all amino acids can be analyzed, when other HPLC conditions are used (examples are described in refs. 7, 8 and 11–13). The disadvantage of the *o*-phthaldialdehyde reagent is that proline and hydroxyproline are not rendered fluorescent. With our device other reagents, such as Dns chloride or Dabs, which require increased temperature to produce quantitatively fluorescent products, can also be used. A part of the tubing has to be heated, but owing to the ease of accessibility of the various components no major problems are expected to arise with alternative precolumn derivatization procedures. When no precolumn derivatization procedure is necessary the described device can, of course, also be used as an automated sampler.

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