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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CATECHOLAMINES IN RAT BRAIN USING A LASER FLUORIMETRIC DETECTION SYSTEM

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SUMMARY

A sensitive high-performance liquid chromatographic method for the determination of catecholamines in rat brains has been developed using a fluorescence detector equipped with a continuous wave laser as an excitation light source. A new pre-purification and derivatization method was established and confirmed to be useful for the determination of catecholamines in biological samples. This pre-treatment method was simple, reproducible and specific. About 1 mg of the rat brain tissue was enough to determine catecholamines levels. The levels of dopamine and norepinephrine in rat brain were 0.40 and 0.87 ng, respectively, which agree with the findings of other workers.

INTRODUCTION

The measurements of micro amounts of catecholamines (CA) in brain are of obvious importance in neurochemistry. Various methods for the determination of CA have been reported, including high-performance liquid chromatography (HPLC) with fluorimetric [1—4] or electrochemical detection [5—9], gas chromatography [10] and gas chromatography—mass spectrometry [11, 12]. Of these, HPLC was suitable for the precise, sensitive and rapid determination of CA in biological samples. In particular, HPLC with fluorescence detection (HPLC—FD) is a sensitive and economic method for determining CA.

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In HPLC—FD analysis, both pre- and post-column derivatization methods have been widely used. Mell et al. [13] described the post-column chromatographic detection of biogenic amines, which necessitated the use of complex post-column derivatization components. Refshauge et al. [5] also reported that the trihydroxyindole fluorescence technique, which is a post-column method, has disadvantages, in that it requires substantial investment in equipment and the assay is complex and not easy to perform. In this work, the pre-column derivatization method was employed for the highly sensitive detection of CA using a laser as an excitation light source because the post-column method involves a seriously large background compared with the pre-column method.

Some papers have already been published on the pre-column method for CA using various kinds of fluorescence reagents. Davis et al. [14] derivatized various amines in biological samples with *o*-phthalaldehyde (OPA)—2-mercaptoethanol, and the fluorescent derivatives were immediately extracted with ethyl acetate prior to HPLC analysis. Mell et al. [13] examined CA in urine by means of OPA—2-mercaptoethanol derivatization, and an aliquot of reaction mixture was injected directly on to a column. Imai and Tamura [15] derivatized CA in urine with fluorescamine, and the fluorophore mixture was injected on to a TSK LS-160 column. Schwedt and Bussemas [16] found that CA derivatized with dansyl chloride could be separated and analysed by means of a Zorbax-ODS column.

In a previous paper [17], we stated that the sensitivity of a detector for HPLC could be substantially increased by use of a continuous wave argon laser as an excitation source combined with an optical fibre as a wave guide. The sensitivity obtained with this system was about 20 times higher than that with conventional fluorescence detection systems. The laser fluorimetric detection system was applied to the determination of norepinephrine (NE) and dopamine (DA) in rat brain by use of a newly developed pre-treatment method. The pre-column derivatization methods mentioned above were employed in HPLC analysis using the laser fluorimetric detection system. However, interfering peaks derived from the large excess of reagents appeared on the chromatogram. This paper describes a new pre-treatment method for the highly sensitive HPLC determination of CA, and also reports the determination of CA in rat brain tissue.

EXPERIMENTAL

Apparatus

A high-performance liquid chromatograph (Model TRI-ROTOR I; Japan Spectroscopic Co., Tokyo, Japan) equipped with a laser fluorimetric detection system constructed as shown in Fig. 1 was used. The HPLC separation was carried out with a 250 × 4 mm I.D. stainless-steel column packed with LiChrosorb RP-18 (5 μm) using the balanced slurry packing method. The column temperature was kept at 50°C by circulating water. The eluate from the column was introduced into the laser fluorimetric detector, which was constructed from a Model FP-110 spectrofluorimeter (Japan Spectroscopic Co.), optical fibre (Machida Endoscope Co., Tokyo, Japan) and a Model 165-05

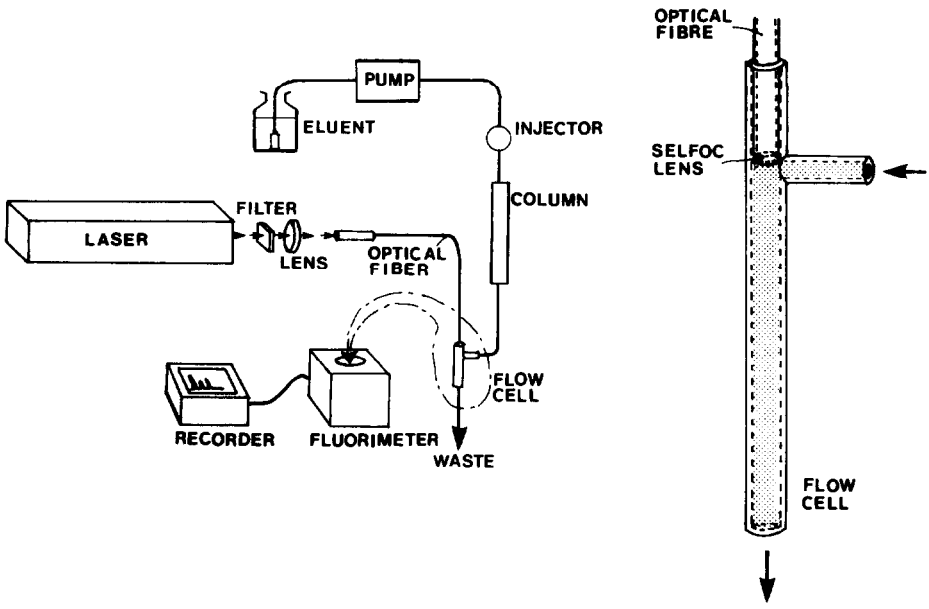

Fig. 1. Schematic diagram of the experimental system for HPLC with laser fluorimetric detection. The argon laser beam transmits along an optical fibre which is axially introduced into a flow cell system to excite the fluorophores of a flowing sample in a capillary cell, without irradiating the cell walls and/or the liquid surface.

argon ion laser (Spectra Physics, Santa Clara, CA, U.S.A.). The flow cell used in the detector was made by Kyowa Seimitsu Co. (Tokyo, Japan) according to our specification. The structure of the flow cell is also shown in Fig. 1. The chromatogram was recorded with a Model 3066 recorder (Yokogawa Electronics Works, Tokyo, Japan).

Reagents

Norepinephrine hydrochloride (NE · HCl) and dopamine hydrochloride (DA · HCl) were purchased from Nakarai Chemicals (Kyoto, Japan) and 3,4-dihydroxybenzylamine hydrobromide (DHBA · HBr) was obtained from Aldrich (Milwaukee, WI, U.S.A.). *o*-Phthalaldehyde (OPA) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and sublimed under reduced pressure (heated to 45—50°C before use). Ethanethiol was obtained from Wako (Osaka, Japan). Alumina (Woelm neutral activity grade I) was purified according to Anton and Sayre [18].

Preparation of reagent solutions

OPA solution was prepared daily by dissolving 5 mg of OPA and 100 μl of ethanethiol and 1 ml of ethanol in 10 ml of 0.1 *M* phosphate buffer (pH 8.60) solution containing sodium metabisulphate (0.05 mg/ml). A standard mixture of CA was prepared by dissolution in and dilution with 0.04 *N* perchloric acid solution so that it contained 80 and 160 ng/ml of NE and DA, respectively. The solution of DHBA which was used as an internal standard was prepared in a similar manner (25 ng/ml). The 0.4 *N* perchloric acid solution

used for the deproteinization contained 0.1 *N* EDTA disodium salt (20 μl/ml). Tris—HCl buffer (pH 8.60) solution (3.0 *M*) was prepared by dissolving 0.4 mg/ml of EDTA disodium salt.

Procedure

Rat brain tissue was homogenized in 0.4 *N* perchloric acid solution in a Potter homogenizer. After centrifugation (10,000 *g* for 10 min at 4°C), 10 μl of the supernatant (corresponding to 1 mg of rat brain tissue) were transferred into an Eppendorf micro tube with 40 μl of the internal standard solution, 15 mg of acid-washed alumina and 50 μl of 3.0 *M* Tris—HCl buffer solution (pH 8.60). The tube was rotated on the rotary rod for 5 min to allow the adsorption of CA on the alumina as shown in Fig. 2. Then, the alumina was allowed to fall to the bottom of the tube and the supernatant was aspirated off. The alumina was washed with about 10 ml of water as shown in Fig. 2.

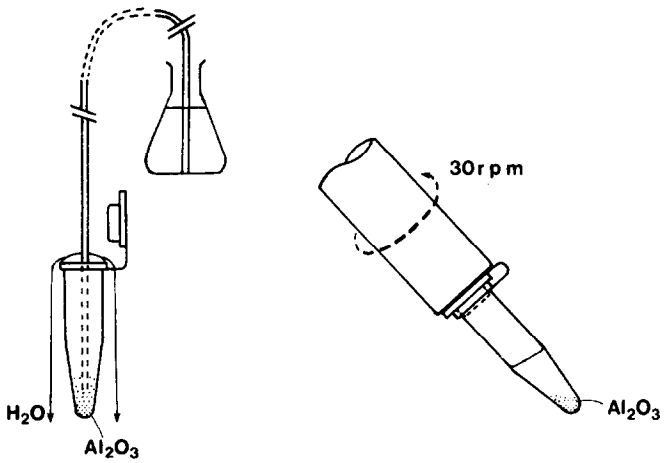

Fig. 2. Schematic diagram of the CA pre-treatment method. Right: to mix the alumina with Tris—HCl buffer or OPA solution, the Eppendorf tube attached on the rotary rod was rotated at a constant rate (30 rpm). Left: to wash the alumina with water, the water, which was continuously flowing through the PTFE tube, was circulated in the Eppendorf tube. The water, but not the alumina, overflowed from the tube and fresh water was applied constantly to the tube from the water reservoir.

After the washings had been aspirated off, 500 μl of OPA reagent solution were added to the tube for the derivatization. To complete the reaction, the mixture was rotated for 10 min in a manner similar to that of adsorption of CA on to alumina. After the removal of the excess of reagent solution by aspiration, the alumina was washed with about 10 ml of water as shown in Fig. 2. After the last washing, the derivatives of CA on alumina were eluted by gentle rotation for 30 sec with 50 μl of 0.4 *N* acetic acid in ethanol (Fig. 2). Then, 20 μl of the supernatant were injected on to the HPLC column. HPLC separation and detection were carried out under the conditions described in the legend of Fig. 4.

RESULTS AND DISCUSSION

Both pre- and post-column derivatization using fluorescent labelling reagents have been widely used. For sensitive analysis, post-column derivatization for HPLC—FD generally seems to be unsuitable for highly sensitive analyses because of the large excess of fluorescence reagent in the mobile phase, which causes a large background on the chromatogram. The pre-column derivatization method, on the other hand, may provide a low background level on the chromatogram. Therefore, the pre-column derivatization method should be applicable to highly sensitive analyses.

With the CA pre-treatment method of Mell et al. [13] and Davis et al. [14], the background derived from the large excess of reagent appeared on the chromatogram in our detection system. To overcome this problem, we investigated a new CA pre-treatment method. An outline of the method is given in Fig. 3.

The procedure for the pretreatment of CA consists of three steps.

In step (1), the adsorption of CA on to the alumina can be performed by adjusting the pH of the sample solution to be slightly alkaline. For this purpose, 50 μl of 3.0 *M* Tris·HCl buffer were added to the mixture of sample solution and alumina. Further adjustment was not necessary. Interfering substances were washed out from the alumina with water. The adsorption of CA on to the alumina and washing were performed by a simplified method shown in Fig. 2. Higa et al. [19] reported that the use of boric acid gel for the pre-purification

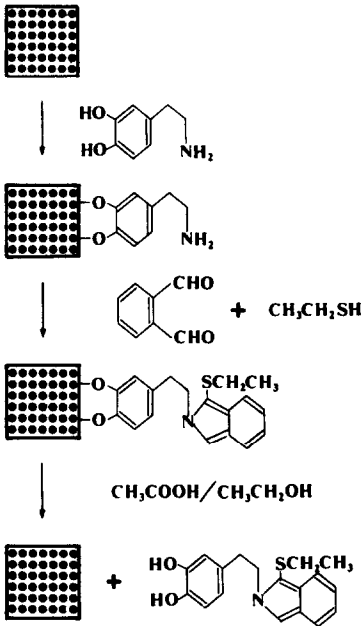

Fig. 3. Procedure for the pre-treatment of CA (for example, DA) consists of three steps. (1) The CA selectively forms a complex with alumina in a weakly alkaline solution. The alumina was washed with water. (2) The adsorbed CA on alumina was derivatized to the fluorophore with OPA—ethanethiol, and the alumina was washed with water. (3) The fluorophore was desorbed from the alumina using a small amount of acid, and the acidic solution was then injected directly on to the column.

provided better recoveries of CA than those with alumina. However, the background level derived from boric acid gel was very high, making it impossible to use boric acid gel in the pre-treatment of CA for the laser fluorimetric HPLC detection. On the other hand, virtually no increase was found in the background level derived from the alumina.

In step (2), the CA on the alumina was derivatized with OPA—ethanethiol into a fluorophore. During the derivatization, the CA should be stable. It is generally considered that the free molecule of CA is unstable and decomposes rapidly under alkaline conditions, probably because of autoxidation of catecholic groups [20]. However, a certain amount of CA adsorbed on the alumina was allowed to stand overnight in weakly alkaline solution, then derivatized with OPA—ethanethiol into a fluorophore and analysed chromatographically. The result was almost the same as with that derivatized immediately after the treatment with alumina. These results suggested that CA adsorbed on to alumina is stable at least overnight in a weakly alkaline solution. 2-Mercaptoethanol is generally used for the derivatization of compounds with a primary amino group into fluorescent products with OPA. It is well known, however, that such fluorescent products are not very stable.

Simons and Johnson [21] reported that the products produced using ethanethiol are more stable than those obtained using 2-mercaptoethanol. Therefore, ethanethiol was used with OPA for the derivatization of CA into a fluorescent compound. However, some decomposition of the fluorescent compound was found in the period necessary for the procedure. Therefore, it is important that the operation after the addition of the reagent solution is carried out in a constant time.

Chen et al. [22] reported that the reaction of CA and OPA was essentially complete within a few seconds. However, we adopted a reaction time of 10 min, because the reactivity between the adsorbed CA molecule on the alumina and the reagents might be lower than that in solution. In order to complete the reaction in a short time, the test tube was rotated on a rotary rod.

An advantage in step (2) is that the interfering excess of fluorescence reagents can be removed. After the derivatization, this was carried out by washing with water. Even after washing with water, the adsorbed CA fluorophore remained on the alumina, and the excess of fluorescence reagents was almost totally eluted. With the procedure of Davis et al. [14] and Mell et al. [13], it was difficult to separate OPA-labelled CA from the peaks of the excess of reagent. In our procedure, only small front peaks on the chromatogram were found, as shown in Fig. 4.

In step (3), the fluorophore on the alumina was desorbed with 0.4 *N* ethanolic acetic acid. When the tube was vigorously rotated, it became impossible to obtain reproducible results, probably because the fluorophore was decomposed by mixing with air. The rotation of the tube was carried out in the same manner as described earlier.

Fig. 4 shows the chromatogram of an authentic mixture of CA. Each CA was effectively separated with isocratic elution. The laser fluorimetric HPLC detection system was compared with a conventional fluorimetric HPLC detector (in this instance, the JASCO FP-110) equipped with a high-pressure mercury lamp (excited at 365 nm). Both fluorimetric measurements were per-

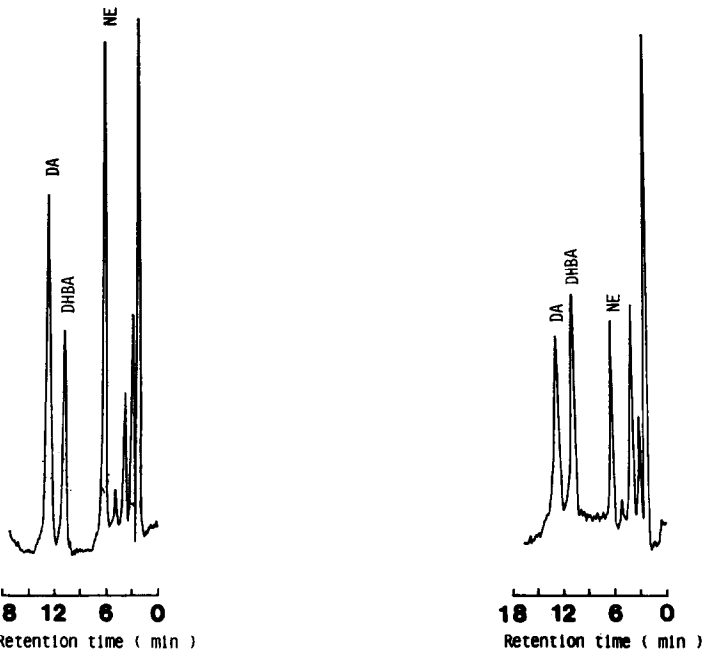

Fig. 4. High-performance liquid chromatogram using the laser fluorimetric detection system obtained from a standard mixture of catecholamines. Operating conditions: column, 250 mm × 4.0 mm I.D., LiChrosorb RP-18 (5 μm); column temperature, 50°C; mobile phase, acetonitrile—0.05 *M* monochloroacetate buffer, pH 2.50 (35:65); flow-rate, 1.0 ml/min; detector, fluorescence spectrophotometer (detection at 450 nm, excitation at 351 and 363 nm of excitation lines of argon laser, laser power ca. 15 mW).

Fig. 5. High-performance liquid chromatogram using the laser fluorimetric detection system obtained from an aliquot of whole rat brain sample (corresponding to 1 mg): DA, 0.87; NE, 0.40; DHBA, 1.0 ng per sample.

TABLE I

TEST OF RECOVERY OF CATECHOLAMINE FROM RAT BRAIN

Sample	Sample No.	NE (ng per sample)	DA (ng per sample)
Added brain	1	1.04	1.93
	2	0.95	1.91
	3	0.99	2.02
	4	1.04	2.09
	5	1.00	2.12
	6	0.92	2.03
	Average	0.99	2.02
	C.V.* (%)	4.9	4.2
Brain		0.40	0.87
Added		0.60	1.20
Recovery (%)		98.3	95.8

*C.V. = coefficient of variation.

formed with the same detector except for the light source. With a laser, the signal-to-noise ratio for 0.8 ng of NE per sample was about 300, for 1.6 ng of DA per sample it was about 200 and for 1.0 ng of DHBA per sample it was about 120. On the other hand, when the light source was a mercury lamp, the signal-to-noise ratio for 0.8 ng of NE was 15 and for 1.6 ng of DA it was about 10. These results suggest that the sensitivity of the laser fluorimetric HPLC detection system is 20 times higher than that with the conventional fluorimetric HPLC detection system.

Linear relationships between the peak height ratio of both NE and DA to DHBA and the amount present were obtained over ranges of at least 0.2–1.6 ng for NE and 0.4—3.2 ng for DA.

The results of the precision and recovery test with this method are shown in Table I; a series of determinations were made on the same rat brain sample. Good results were obtained, as shown by the precision and recovery of CA.

The rat whole brain sample prepared as described above provided the chromatogram shown in Fig. 5. The values obtained by the present method were in good agreement with those reported by other workers [23].

Some investigators [24—27] have reported that the sensitivity of fluorescence detection in HPLC could be increased by the use of a laser as the excitation light source. Diebold and Zare [24] reported a high sensitivity of a laser fluorimetric detector with a wall-less flow cell in the determination of picogram levels of aflatoxins. In a previous paper [17], we reported that the detection limit of dansylalanine using the present detection system with a laser was 2 pg (signal-to-noise ratio = 2), which was more sensitive by an order of magnitude than that obtained with the same fluorimeter with a mercury lamp.

It is interesting to compare the sensitivity of the present method with that of conventional HPLC methods for CA. An exact comparison of the practical sensitivity of the methods is not easy, however, because the limits of quantitation were reported in different terms. The detection limits of CA in HPLC methods reported so far are shown in Table II.

A comparison of these values shows that the detection limits of CA in biological samples using our method exceed those using the OPA pre-column derivatization method [14] and the native fluorescence method [30, 31]

TABLE II

COMPARISON OF DETECTION LIMITS OF CATECHOLAMINE IN BIOLOGICAL SAMPLES USING VARIOUS HPLC DETECTION SYSTEMS

Method	Detection limit (compound analysed)	Sample	Ref.
Laser fluorescence	5 pg (NE), 16 pg (DA)	Rat brain	This work
Trihydroxyindole	7 pg (NE)	Plasma	[28]
Trihydroxyindole	20 pg (NE, DA)	Plasma	[29]
OPA pre-column	50—100 pg (NE, DA)	Plasma, tissue	[14]
Native fluorescence	100—500 pg (NE, DA)	Urine	[30]
Native fluorescence	100 pg (NE), 300 pg (DA)	Rat brain	[31]
Electrochemical detection	10 pg (NE, DA)	Rat brain	[32]
Electrochemical detection	100 pg (NE, DA)	Rat brain	[33]

by more than an order of magnitude. Also, the sensitivity of our method was slightly better than with the trihydroxyindole method [28, 29] and the electrochemical detection method [32, 33].

To use this laser fluorimetric HPLC for the sensitive determination of CA in biological samples, it is very important to reduce background signals caused mainly by an excess of fluorescent reagents. Although a new pre-column derivatization method has been established in this work to reduce the background, the HPLC system could not be used with such a large range of sensitivity. Therefore, if better derivatization methods for CA could be developed, it might be possible to determine CA in biological samples with greater sensitivity using this laser fluorimetric HPLC system. Moreover, if a laser providing a stable high output power in the UV region can be developed, highly sensitive quantitation might be achieved with the fluorescence method.

CONCLUSION

A highly sensitive HPLC method for the determination of CA using new pre-purification and derivatization methods has been developed. The pretreatment method is simple and effective not only for the clean-up of CA from the sample but also for the removal of excess of reagents from the reaction mixture. As a result, a highly sensitive fluorescence detection system using a laser as the excitation light source was used for the determination of trace amounts of CA in rat brain tissue.

Although derivatization with OPA and ethanethiol as fluorescent labelling reagents was used in this work, other reagents such as dansyl chloride and fluorescamine could also be used.

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