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Determination of urinary free noradrenaline by reversed-phase high-performance liquid chromatography with on-line extraction and fluorescence derivatization

OSAMU NOZAKI* and YASUHIRO OHBA
Department of Clinical Pathology, Kinki University School of Medicine, 377–2, Ohno-Higashi, Osaka-Sayama, Osaka 589 (Japan)

ABSTRACT

A fully automated reversed-phase high-performance liquid chromatographic method with fluorimetric detection for the determination of urinary free noradrenaline was developed. Urine samples, diluted with buffer were injected into a boric acid gel column (12 μm, TSK: 10 × 4.6 mm I.D.) without prior extraction. Urinary noradrenaline was simultaneously extracted and derivatized with an alkaline mobile phase of pH 11, containing *o*-phthalaldehyde and 2-mercaptoethanol, in a boric acid gel column. After switching columns, the fluorescent derivatized catecholamines were separated with an ODS-4PW column (TSK) and a mobile phase of pH 2 and the fluorescence was monitored with excitation at 340 and emission at 440 nm. The retention times of noradrenaline and dopamine were 11.0 and 14.2 min, respectively. The detection limits for noadrenaline and dopamine were 0.2 and 20 ng, respectively.

This method has the advantages of not requiring preliminary extraction of urinary catecholamines, high sensitivity and stability of *o*-phthalaldehyde-derivatized catecholamines.

INTRODUCTION

Urinary catecholamines are determined as markers of the adrenal medulla and sympathetic nervous system by high-performance liquid chromatography (HPLC). The method normally requires prior isolation of catecholamines by chromatography on alumina[1,2], an ion-exchange gel[3,4] or a boric acid gel[5–8]. These procedures are time consuming and become impractical when large numbers of samples need to be analysed. The method described here dose not require the extraction of catecholamines, but permits their highly sensitive detection and rapid analysis.

If a hard boric acid gel[9] is used as a precolumn, together with an alkaline mobile phase containing *o*-phthalaldehyde (OPA) and 2-mercaptoethanol (2-ME), extraction and OPA derivatization of catecholamines may be performed simultaneously. Fig. 1 shows the principle of the method. The reaction between catecholamines and a boric

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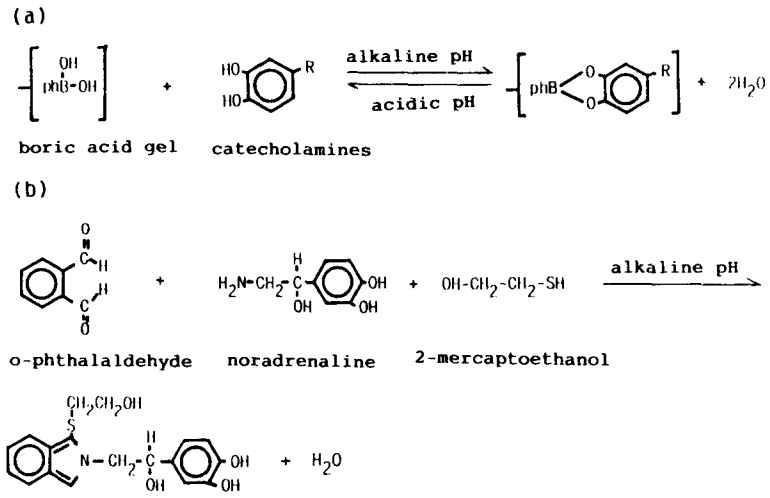

Fig. 1. Principle of the proposed HPLC method for urinary catecholamines (a) Scheme of the adsorption of catecholamines on boric acid gel. (b) Scheme of the fluorescence derivatization of noradrenaline with *o*-phthalaldehyde and 2-mercaptoethanol.

acid gel[7] and the reaction between catecholamines, OPA and 2-ME[10,11] are commonly carried out in alkaline solution and, after switching columns[12], OPA-derivatized catecholamines are separated with an acidic eluent on an ODS column and monitored by fluorimetry. A fully automated HPLC system was developed, based on a column-switching technique. As a result, urinary free noradrenaline in samples from patients can be determined.

EXPERIMENTAL

Reagents

Noradrenaline and dopamine were obtained from Sigma (St. Louis, MO, U.S.A.), potassium biphthalate from Tokyo Kasei (Tokyo, Japan), disodium hydrogenphosphate, tetrahydrofuran and phosphoric acid from Wako (Osaka, Japan) and 2-mercaptoethanol, acetonitrile, ethanol, 4 *M* sodium hydroxide solution and 0.1 and 1 *M* hydrochloric acid from Kantokagaku (Tokyo, Japan). All reagents were of analytical-reagent grade and used without further purification. Distilled water was used after being passed through an ion-exchange column (Milli-Q; Millipore, Bedford, MA, U.S.A.).

Stock standard solutions of noradrenaline and dopamine (1.0 mg/ml in 1 *M* hydrochloric acid were placed in polypropylene sample cups with caps and stored at −20°C. Working standard solutions of noradrenaline and dopamine were prepared by dilution of the stock standard solutions with 0.1 *M* hydrochloric acid, stored at 4°C and used within 1 day. The OPA solution (3.0 mg/ml in ethanol) was stored at 4°C.

The alkaline mobile phase (pH 11.0) (S_1) for the boric acid gel precolumn was 5 m*M* disodium hydrogenphosphate–acetonitrile–4 *M* NaOH–3 mg/ml OPA–2-ME (160:40:0.36:0.40:0.10, v/v) and was prepared just before use. The acidic mobile phase

(pH 2.0) (S_2) for the ODS-4PW analytical column was 10 m*M* potassium biphthalate–acetonitrile–tetrahydrofuran–phosphoric acid (280:120:20:0.33, v/v).

Sample collection

Samples of 0.90 ml were collected from patients in polypropylene sample cups containing 0.1 ml of 1 *M* hydrochloric acid, capped, stored at 4°C and analysed within 1 day.

Chromatographic system

Fig. 2 shows a block diagram of the HPLC system. This system consisted of two HPLC pumps (880-PU; JASCO, Tokyo, Japan), an autosampler (850-AS, JASCO), a high-pressure switching valve (892-01, JASCO), a fluorimonitor (FS-8000, TSK, Tokyo, Japan), an integrator (805-GI, JASCO), a precolumn (boric acid gel, 12 μm, TSK; 10 × 4.6 mm I.D.), a guard column (ODS-4PW, 8 μm, TSK; 10 × 4.6 mm I.D.) and an analytical column (ODS-4PW, 8 μm, TSK; 250 × 4.6 mm I.D.). The two HPLC pumps, the high-pressure switching valve, the autosampler and the integrator were automatically regulated with a system controller (801-SC, JASCO). All HPLC columns were packed by Tosoh (Yamaguchi, Japan) and were used at room temperature.

Chromatographic separation and fluorimetric determination

Samples of urine (200 μl), diluted with the same volume of 100 m*M* disodium hydrogenphosphate (pH 8.4) and kept at 4°C, were injected by an autosampler every 25 min, and 3.5 min after sample injection the alkaline mobile phase containing OPA and 2-ME was passed through the boric acid gel precolumn at a flow-rate of 1.3 ml/min to extract and derivatize urinary catecholamines almost simultaneously. Then the high-pressure switching valve was turned and the fluorescent OPA-derivatized catecholamines were eluted from the precolumn, transferred to the ODS-4PW column and separated with the acidic mobile phase at a flow-rate of 1.0 ml/min. The eluate was monitored with a fluorimeter with excitation at 340 nm and emission at 440 nm.

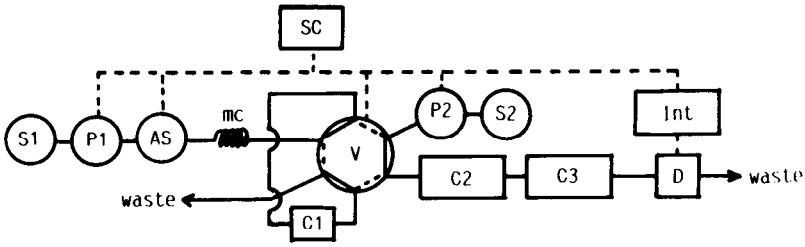

Fig. 2. Block diagram of the HPLC system with column switching. S_1 and S_2 = solvents; P_1 and P_2 = HPLC pumps; AS = autosampler; mc = mixing coil (2 m × 0.5 mm I.D.); V = high-pressure switching valve; D = fluorescence monitor; Int = integrator; SC = system controller; C_1 = precolumn (boric acid gel, 12 μm, TSK; 10 × 4.6 mm I.D.); C_2 = guard column (ODS-4PW, 8 μm, TSK; 10 × 4.6 mm I.D.); C_3 = analytical column (ODS-4PW, 8 μm, TSK; 250 × 4.6 mm I.D.). The flow-rate of solvent S_1 was 1.3 ml/min and that of solvent S_2 1.0 ml/min both before and after the column switching. Solvents S_1 and S_2 passed through the solid line of the high-pressure switching valve (V) before turning the valve, and then the broken line of V after turning the valve.

RESULTS

Influence of alkaline mobile phase (S_1) pH on simultaneous extraction and OPA derivatization of noradrenaline and dopamine using a boric acid gel column

Fig. 3 shows changes in the relative peak areas of OPA-derivatized noradrenaline and dopamine standards corresponding to pH 9.27, 10.0, 11.2, 11.7, 11.9 of the mobile phase (S_1). The maximum peak areas of the derivatized noradrenaline and dopamine were both obtained in the pH range 10–11 and a mobile phase (S_1) pH of 11 was adopted.

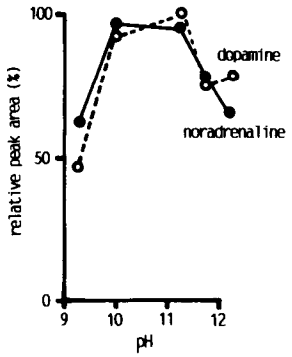

Fig. 3. Influence of mobile phase pH on adsorption and fluorescence derivatization of catecholamines in the boric acid gel column.

Influence of 2-mercaptoethanol concentration in the mobile phase (S_1) on OPA derivatization of noradrenaline and dopamine using a boric acid gel column

Fig. 4 shows changes in the relative peak areas of OPA-derivatized noradrenaline and dopamine standards with variation in the concentration of 2-ME (0.02, 0.04, 0.05, 0.06 and 0.10%) in the mobile phase (S_1). The maximum peak areas were obtained at 2-ME concentrations of 0.04–0.06% and 0.05% was adopted.

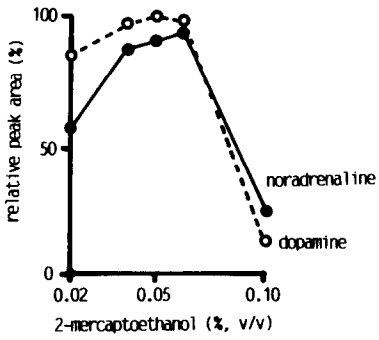

Fig. 4. Influence of 2-mercaptoethanol in the mobile phase on fluorescence derivatization of catecholamines in the boric acid gel column.

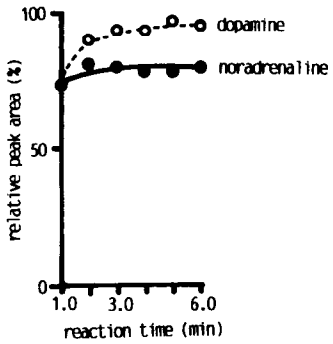

Fig. 5. Reaction time of fluorescence derivatization of catecholamines with *o*-phthalaldehyde and 2-mercaptoethanol in the boric acid gel column.

Column switching time for the maximum peak areas of OPA-derivatized noradrenaline and dopamine for the boric acid gel column

The column switching time that provided both maximum OPA derivatization and maximum extraction yields of noradrenaline and dopamine on the boric acid gel column was determined between sample injection and the time of column switching at a flow-rate of 1.3 ml/min of the mobile phase (S_1) and at room temperature. Fig. 5 shows the changes in the relative peak areas of OPA-derivatized noradrenaline and dopamine standards each minute from 1.0 to 6.0 min. The optimum column switching time was determined to be 3.5 min, because the maximum relative peak area of OPA-derivatized noradrenaline was observed between 2.0 and 6.0 min and that of OPA-derivatized dopamine between 3.0 and 6.0 min.

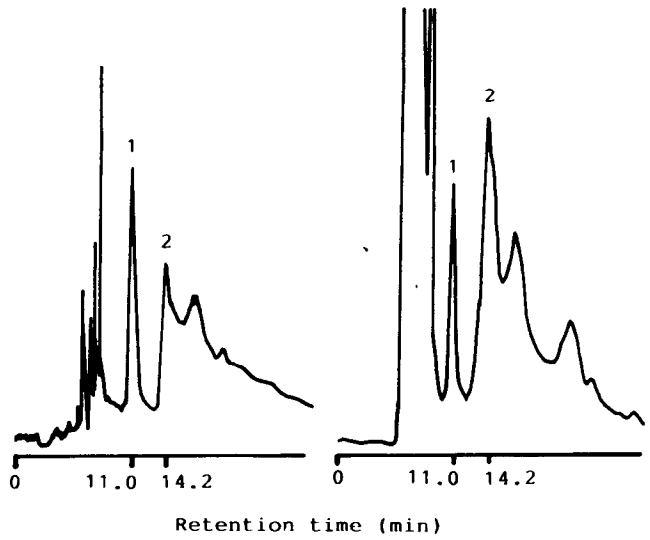

Fig. 6. Chromatograms obtained: left, standard solution containing 40 ng of noradrenaline and dopamine; right, 100 μl of urine from a patient. Peaks: 1 = noradrenaline; 2 = dopamine.

Chromatograms

Fig. 6 shows chromatograms of standard solutions containing 40 ng of noradrenaline and dopamine and a 100-μl urine sample which was diluted to 200 μl with 100 m*M* disodium hydrogenphosphate. The retention times of OPA-derivatized noradrenaline and dopamine were 11.0 and 14.2 min, respectively. Nevertheless, the peak corresponding to noradrenaline was well separated from the other peaks, even in urine samples. The peaks corresponding to dopamine were sometimes overlapped with other unknown peaks in the standard solutions and in urine samples. The detection limits were about 0.2 and 20 ng for noradrenaline and dopamine, respectively (signal-to-noise ratio = 2).

The intra- and inter-assay relative standard deviations for noradrenaline (120 ng) in urine were 3.4 and 4.8%, respectively (n = 5).

Identification of the peaks corresponding to noradrenaline and dopamine in chromatograms of urine samples was based on coincidence of both the retention times and the maximum fluorescent excitation and emission wavelengths in comparison with noradrenaline and dopamine standards.

DISCUSSION

It is known that boric acid gel can react with the diol groups of catecholamines under alkaline conditions and the reaction is reversible under acidic conditions[5–8]. The soft boric acid gels normally used in the preliminary extraction of urinary catecholamines cannot be packed into HPLC columns. However, the boric acid gel (TSK) used in this study is hard and can withstand high column pressures such as 300 kg/cm^2, so there were no problems in using it in a short column of 10 × 4.6 mm I.D.

The proposed method was successful in extracting urinary catecholamines with the boric acid gel column and an alkaline mobile phase (S_1) of pH 11 (Fig. 3).

Although boric acid gel is also known to have affinities for sugars, nucleosides and nucleotides[13,14], these substances are not thought to interfere in the present detection method, because the OPA derivatives were monitored at their maximum excitation and emission wavelengths of 340 and 440 nm, respectively.

The boric acid gel column used in this study tolerated hundreds of injections of crude urine samples, and there was no need to replace the column during this study.

It is well known that a primary amino group reacts rapidly with OPA in the presence of 2-ME and under alkaline conditions at room temperature[10,11]. The derivatization reactions of noradrenaline and dopamine with OPA and 2-ME are thought, from the results in Fig. 5, to have occurred in part in the mixing coil, but mainly in the boric acid gel column, because the peak areas of these catecholamines increased 2 and 3 min after sample injection, respectively. Hence, the boric acid gel column played two roles, as an affinity column and as a solid-phase reactor for OPA derivatization of noradrenaline and dopamine.

There is another advantage of this on-line OPA derivatization method for catecholamines. The usual instability of OPA derivatives of catecholamines after derivatization was eliminated because the derivatives were produced on-line and analysed within 20 min after sample injection.

The pH of the mobile phase (S_2) for the ODS-4PW column needed to elute OPA-derivatized catecholamines from the boric acid gel column was below 4. The

peak shapes of the OPA-derivatized catecholamines became sharper, corresponding to changes in the pH of the mobile phase (S_2) from 4 to 2.

We used an ODS-4PW gel column (TSK) for the separation of OPA derivatives of catecholamines because this gel, which is prepared by introducing octadecyl groups into a hydrophyric resin (TSK gel 4000 PW), can be used with mobile phase solutions in the pH range 2–12. ODS-4PW gel can tolerate alkaline solutions eluted from a boric acid gel precolumn to an analytical column when the columns are switched.

Under these conditions, good separation of noradrenaline OPA derivative peaks from other peaks in chromatograms of urine samples was obtained. On the other hand, peaks of OPA-derivatized dopamine often showed interference from peaks of other unknown substances (Fig. 6)[15]. Presumably the boric acid binding does not interfere with the OPA reaction and *vice versa*. We are now making efforts to solve this problem.

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