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On-column derivatization–capillary electrochromatography with *o*-phthalaldehyde/alkylthiol for assay of biogenic amines

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Abstract

The elution behaviors of the biogenic amines, histamine (HA) and its metabolite methyl histamine (MHA), were evaluated by means of on-column derivatization (OCD)–capillary electrochromatography (CEC) which employed a monolithic octadecylsilica (ODS) capillary column (20 cm of effective length × 50 μm of inner diameter). Five kinds of alkylthiols, e.g., 2- hydroxyethylthiol (or 2-mercaptoethanol (2-ME)), ethanethiol (ET), 1-propanethiol (1-PT), 2-methyl-1-propanethiol (2-MPT) and 1-butanethiol (1-BT) were separately presented at 5 mM each in the OCD–CEC separation run buffer consisting of 60% acetonitrile in 5 mM *o*-phthalaldehyde (OPA)-10 mM borate buffer (pH 10). When 2-ME was present in the run buffer solution, both derivatives corresponding to HA and MHA migrated separately, but closely together through the capillary column. Replacement of 2-ME with 1-BT in the run buffer solution caused a delay in their elution profiles on the electrochromatogram and the separation between those two peaks became remarkably improved. The elution times of HA and MHA followed the increase in alkyl chain length or hydrophobicity of thiol, 1-BT > 2-MPT > 1-PT > ET > 2-ME. Performance of on-line preconcentrations of HA and MHA was also evaluated by varying the electrokinetic injection voltage from 1 kV to 8 kV. The peak area counts corresponding to HA recorded about 50 times higher when 2 kV was applied for 240 s to a 0.1 mM HA solution than when 8 kV was applied for 5 s. This method was next applied to a sample of human urine spiked with HA and MHA at levels of 0.1 μM each. Although HA and MHA peaks were not identifiable among the peaks corresponding to the materials in the urine matrix when OPA/2-ME was employed in a run buffer for the OCD–CEC, the separation and identification of their peaks became possible by replacing 2-ME with 1-BT in the run buffer solution.
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1. Introduction

Micro- or nano-scale separations of biological analytes in an electric field by means of capillary electrophoresis (CE) and capillary electrochromatography (CEC) have become the focus of academic attention in the fields of genomics [1,2], proteomics [3,4] and others [5], because such separation techniques make quick determinations possible even for samples in small volumes, and irregardless of the analyte's complexity or low concentration. In recent decades, numerous methods using CE or CEC techniques [6] have been developed for the determination of many kinds of naturally-occurring analytes. Unfortunately, their application compounds with molecular weight less than around 500 (which are sometimes called "biogenic amines") are very limited. Biogenic amines are involved in wide variety of biological activities and are key compounds in living systems. However, their explicit roles in living systems have not yet been fully clarified. One of the obstacles hampering their elucidation is that biogenic amines are usually found only in very minute quantities within the highly complex matrix of nature, thus, it is very hard to grasp their biological actions in detail. As a consequence, many scientists engaged in this field have been waiting somewhat impatiently for the appearance of a simple, sensitive, selective and useful assay method for biogenic amines. To meet this need, we propose and have been developing

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throughout in-capillary derivatization (ICD)–CE [7–10] as an elegant alternative method for determinations of biogenic amines. The ICD–CE technique is based on the idea that the separation and derivatization of free amines can be performed simultaneously during the electromigration of analytes in a separation capillary tube field with a run buffer containing *o*-phthalaldehyde (OPA) and *N*-acetylcysteine (NAC) as a derivatization reagent for amino compounds. Although this technique requires no-batchwise procedure associated with derivatization to enhance the detection sensitivity for amines, certain problems can occur when the run buffer for the ICD–CE is optimized. For example, the determination of histamine (HA) and other biogenic amines (i.e., spermidine, cadaverine, putrescine, and so on) with ICD–CE can be accomplished using a run buffer consisting of 2 mM OPA/NAC in 20 mM phosphate–borate buffer (pH 10) [9,10]. However, it was expected that the separations and identifications of HA and other substances with a similar chemical structure such as methylhistamine (MHA), which is one of HA metabolites, would be very hard with this method using the run buffer indicated above or any other. One solution for this was to use a different kind of run buffer solution having pH different from 10. Changing the pH of the run buffer is a very powerful way to optimize the separation it, because the elution behaviors of biogenic amines or ionic compounds are remarkably changed depending on the pH of run buffer. However, the pH of a run buffer, in this case, should be around 10, because OPA/NAC can react with amino compounds at pH 10. For this reason, we chose not to develop this method further, but, instead, we subsequently developed an on-capillary column derivatization (OCD)–CEC [11,12] based on the same idea as that of ICD–CE. Using this OCD–CEC, the complete separation of HA and MHA were accomplished, although their peaks were closely spaced. We also expected that another problem would occur when this method is applied to a natural sample such as human urine spiked with HA and MHA. A capillary column packed with silica-based octadecylsilica (ODS) particles should generally be used with a neutral or acidic environment. If the capillary column packed with the alkaline-resistant ODS particles commercially available (Shiseido Capcell Pak C_{18} type UG 120) is used at pH 10, it is recommended that an organic solvent such as acetonitrile should be included in the run buffer solution at a concentration of 50% or more. Accordingly, another approach will be needed for the determination of amines in a natural sample because of the limitation of both pH and concentration of organic solvent of the run buffer solution. One solution is to employ a derivatization reagent other than OPA to modify the elution behaviors of biogenic amines through the capillary column. The derivatizing reagent in this method requires that the reagent itself should be non-active to photometric detectors, such as a UV detector or fluorescence detector, while the analyte–reagent derivative should be active to such detectors. Furthermore, the reaction between analyte and reagent should progress quickly at room temperature. Unfortunately, up until now there has been no suitable reagent other than OPA.

It is well known that OPA reacts with amino compounds in alkaline medium in the presence of a reducing reagent [13,14]. We hypothesized that if different kinds of alkylthiols were used as the reducing reagent instead of NAC, the chemical properties of OPA derivative would be modified and these modifications might have an effect on the elution profiles of biogenic amines under analysis by means of OCD–CEC. For the reasons stated above, in this paper we examine the effect of alkylthiols in the presence OPA on elution behaviors of HA and MHA derivatives during monolithic ODS OCD–CEC, and also our efforts to apply this method to the determination of HA and MHA in human urine.

2. Materials and methods

2.1. Reagents and materials

MHA was obtained form Calbiochem-Novabiochem (CA, USA). OPA was of biochemical, and HA, 2-mercaptoethanol (2-ME), ethanethiol (ET), 1-propanethiol (1-PT), 2-methyl-1-propanethiol (2-MPT) and 1-butanethiol (1-BT) were of superfine reagent grade. Those reagents were purchased from Wako (Tokyo, Japan), and used without further purification. Other reagents used were of the highest or HPLC grade commercially available. All aqueous solutions were prepared using water purified with a Milli-Q purification system (Nippon-Millipore, Tokyo, Japan). The standard amine test solution was prepared by dissolving HA and MHA with water to make concentrations of 100 μM each. The run buffer solutions containing the derivatization reagent were prepared as follows. A mixture of acetonitrile and borate buffer (pH adjusted to10 with 1 M sodium hydroxide) was added to a mixture of 1 ml of 50 mM OPA in acetonitrile and 1 ml of 50 mM alkylthiol in borate buffer (or acetonitrile) to make 10 ml and 60% of final volume and acetonitrile concentration, respectively, in a 10 ml-glass reservoir. The reservoir was placed in a sonication bath (Model UT-52, Sharp, Nara, Japan) under reduced pressure for about 30 s in order to degas. The separation buffers were prepared just before use.

2.2. CEC apparatus

The CEC systems used consisted of a Jasco Model CE-800 (Jasco, Tokyo, Japan) equipped with a fluorescence detector, Model FP-920 (Jasco). The detector was installed with capillary flow cell units instead of the normal cell unit for HPLC. Sample solutions were introduced into the capillary column from the anodic side by electrokinetic voltage injection. The electrochromatograms were produced by monitoring fluorescence at 455 nm excited with 340 nm

wavelength. As the injection and separation voltage for the CEC were usually the same, e.g., 8 kV, when there is no additional description of these voltages, it may be assumed that 8 kV was employed. All data were printed out with an intelligent data processor, model 807-IT (Jasco).

2.3. Monolithic ODS capillary column fabrication

A monolithic ODS capillary column was fabricated using a method slightly modified from the procedure previously described [12] to achieve high density packing of particles in the capillary. The polymer-supported ODS packing particles used here were obtained by taking out the inside of Capcell Pak C_{18} (5 μm particle diameter) type UG 120 (Shiseido, Tokyo, Japan). These particles are tolerant of an alkaline environment of pH 10. One side of the capillary tube (Polymicro Technologies, AZ, USA) of 40 cm length × 50 μm inner diameter (i.d.) was temporally connected to a length a polyethylene tubing 1 cm × 0.58 mm i.d. (Intramedic, MD, USA) of in which a small amount of glass fiber was packed. From the other side of the capillary tube, a suspension of a 300 mg of ODS particles in 0.20 ml of tetraethylorthosilicate (TMOS), 0.73 ml of ethanol and 0.10 ml of 0.12 M hydrochloric acid was introduced by a syringe pressurized with a hand-held vise. During the packing, the capillary was sonicated for 30 s at 5 min intervals. Packing was continued until a column 20 cm long was made and the region beyond the initial stacking point was used to fabricate a window. The window was created within the capillary tube by using hot sulfuric acid (>100 °C) after the column was kept at 120 °C for 1 h to facilitate ethanol evaporation. After removing the temporary polyethylene tubing, the column was pre-conditioned with separation buffer that had been degassed in vacuo by sonication (by pressurizing the column inlet to approximately 200 psi with a hand-held vise; 1 p.s.i. = 6894.76 Pa). Next, the column was further conditioned electrokinetically in the CE instrument by driving the buffer mobile phase through the capillary at an applied voltage of 8 kV until a stable baseline was achieved. This procedure typically takes 5–6 h.

2.4. CEC operations

Two 10 ml reservoirs were placed in the CEC system, one at the anodic site and another at the cathodic site. The anodic and cathodic reservoirs contained 10 ml of run buffer and 10 ml of run buffer lacking the derivatization reagent OPA/alkylthiol, respectively. Before performing CEC, the monolithic capillary column was rinsed with the run buffer containing the derivatization reagent loaded from cathodic site to anodic site of the capillary column by manually pushing a syringe pump. Performing this operation at least once a day is requisite. Both ends of the capillary column were carefully dipped into their respective reservoirs so as to prevent air from entering into the capillary.

2.5. Urine sample preparation

Human urine was collected from a healthy male and the 0.1 mM solutions of HA and MHA were added to it to make 1 μM of each concentration. Equal amounts of the spiked urine (or unspiked urine) solution and aqueous ammonia solution (diluted with water; pH 8.0) were mixed. Two milliliters of the mixture was then passed through an anion exchange cartridge (BondElut-CBA 100MG; Varian, CA, USA). After washing the cartridge with 1 ml water, HA and MHA were eluted with a 1 ml, 0.2 M HCl. The eluant was evaporated to dryness in vacuo and reconstituted with 1 ml water.

3. Results and discussion

3.1. Effect of alkylthiol

OPA, employed with a reducing reagent such as 2-ME, for fluorogenic detection of primary amino compounds, in addition to α-amino acids, peptide, protein and biogenic amines, was first described by Roth [13] and Roth and Hampai [14]. The reaction of OPA/alkylthiol with primary amine produces 1-alkylthio-2-substituted *iso*-indole, which exhibits strong fluorescence [15] as shown in Table 1. In addition, this reaction proceeds very fast in an alkali environment even though the reaction temperature is set at room temperature. The chemical or physical properties of the *iso*-indole derivative partially depend on the 1-alkylthio group in the molecule. If a hydrophilic thiol compound such as 2-ME is used together with OPA for the derivatizing of amino compounds, the R_2 residue, which has hydrophilic properties, is introduced into the *iso*-indole molecule. If a hydrophobic thiol such as ET, 1-PT or 2-MPT, instead of 2-ME is used, the resulted *iso*-indole is hydrophobic property. This idea might help to overcome the problems associated with OCD–CEC, which were mentioned above in Section 1. In this paper, the elution profiles of HA and MHA on OCD–CEC were evaluated by use of combinations of OPA and each of several different types of thiol compounds used as the derivatizing reagent.

Electrochromatograms A, B, C, D and E in Fig. 1 were obtained by the injection of a mixture of HA and MHA at

Table 1
Reaction of OPA/alkylthiol with amino compound

CHO, CHO + R_1–NH_2, R_2–SH → S–R_2, N–R_1

	R_2
2-Mercaptoethanol (2-ME)	HO–CH_2CH_2–
Ethanethiol (ET)	CH_3CH_2–
1-Propanethiol (1-PT)	$CH_3CH_2CH_2$–
2-Methyl-1-propanethiol (2-MPT)	$(CH_3)_2CHCH_2$–
1-Butanethiol (1-BT)	$CH_3CH_2CH_2CH_2$–

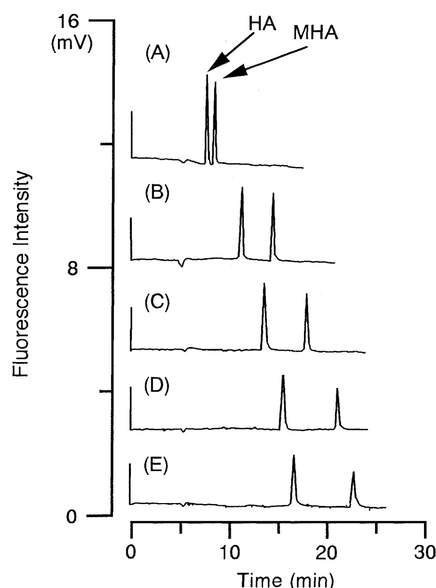

Fig. 1. Electrochromatograms of the separation of histamine (HA) and methylhistamine (MHA). Conditions: run buffer, 60% acetonitorile–5 mM OPA–10 mM borate (pH 10) in the presence of: (A) 5 mM 2-mercaptoethanol (2-ME), (B) ethanethiol (ET), (C) 1-propanethiol (1-PT), (D) 2-methyl-1-propanethiol (2-MPT), and (E) 1-butanethiol (1-BT); sample, 0.1 mM each; injection voltage, 8 kV for 5 s; separation voltage, 8 kV; detection, 455 nm (emission), 340 nm (excitation). For other conditions, refer to Section 2 in the text.

0.1 mM each with 8 kV for 5 s prior to electrochromatography (8 kV) in which 5 mM solutions of 2-ME, ET, 1-PT, 2-MPT and 1-BT were added, in turn, to a run buffer consisting of 60% acetonitrile–5 mM OPA–10 mM borate (pH 10). In the case of 2-ME, two peaks corresponding to HA and MHA appeared very close to each other on the electrochromatogram (Fig. 2(A)), and this separation profile was remarkably improved by using other alkylthiols (Fig. 2(B)–(D)). The retention times of the peaks HA and MHA varied directly with the increase in the length of alkyl chain in the order: 2-ME, ET, 1-PT, 2-MPT, 1-BT, and the resolutions between HA and MHA increased similarly, following this same order. This behavior is due to the partitioning of the analyte between the mobile phase (run buffer) and stationary phase (ODS). Elution time is prolonged owing to the increased attraction of the OPA/thiol derivative to the ODS phase as the hydrophobic property of the OPA/thiol derivative increases. In addition, when an acidic thiol, such as 3-mercaptopropionic acid, was employed, the HA and MHA derivatives could not come out the column because the negatively charged derivatives, in pH 10, traveled toward the anodic site, rather than a detection site or the cathodic site (data not shown). Furthermore, the fluorescence intensity of amine derivatives at 455 nm by excitation with 340 nm wavelengths resulted in almost the same value when the different kinds of alkylthiol compounds, 2-ME, ET, 1-PT, 2-MPT and 1-BT were used under the present conditions.

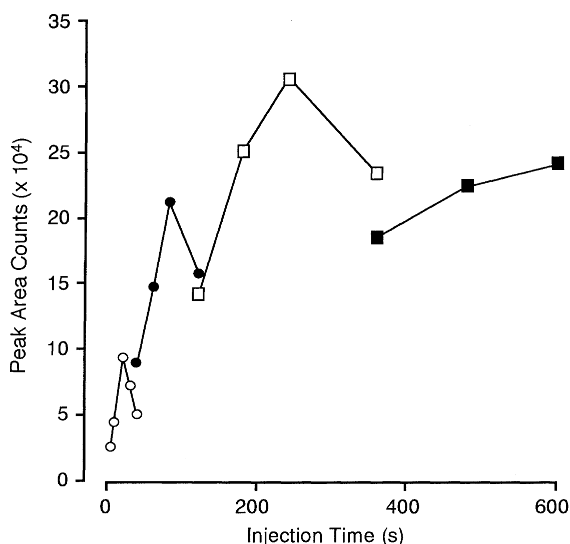

Fig. 2. Effect of the electrokinetic injection voltage of: (■) 1 kV, (□) 2 kV, (●) 4 kV and (○) 8 kV for different times on the peak area counts of histamine (HA). Other conditions as described in Fig. 1.

3.2. On-line preconcentration

Sometimes, biogenic amines are found to exist at nano-mole levels or less in nature. Although CEC is quite applicable not only in cases of small sample volume, but also in cases of highly complex samples, detection sensitivity is inferior to traditional liquid chromatography because of the shortened path-length of light at the photometric detection site. In our previous paper [12], we overcame this problem by means of on-line preconcentration prior to OCD monolithic ODS for the determination of biogenic amines using OPA/2-ME as the derivatization reagent presented in a run buffer. In that study, the effects of electrokinetic injection voltage on the performance of on-line preconcentrations of HA were examined when OPA/1-BT was used for the reagent. Fig. 2 shows the results obtained for different injection times and applications of different injection voltages of 1, 2, 4 and 8 kV. All electrochromatographic experiments were carried out at 8 kV after electrokinetic injection of 0.1 mM HA. In the case of 8 kV, the maximum degree of preconcentration was reached around 30 s after injection, at which time the peak area counts were recorded only 10 times higher than those obtained from a 5 s-injection. In the case of 2 kV for 240 s, the on-line preconcentration of HA measured around 50 times higher in comparison with that of 8 kV applied for 5 s. The maximum of each HA peak area count increased with decreasing injection voltage employed, but required a longer injection to reach the maximum value in each case with the on-line preconcentration OCD–CEC. The lower pre-concentration resulting in the case of 8 kV injection can be attributed to a mixing effect at the boundary between the sample and run buffer solutions. Mixing is caused by the mismatch of velocities (electro-osmotic flow and sample stacking) when the higher electrokinetic injection voltages are applied.

Using 2 kV injection voltage for 240 s, reproducibility and linearity tests were performed with the following results: the relative standard deviation ($n = 5$) values (%) for peak area counts and migration times were <4.3 and 1.0%, respectively. The linearity of response for peak areas corresponding to HA at concentrations of between 0.2 and 100 μM, were calculated by the least squares regression method. The correlation factor was 0.998, and respectively, and detection limit (signal/noise = 3) was recorded at 50 nM (or 50 pM/ml) under the conditions of the present experiment.

In addition, assay method for determination of plasma, serum or urine HA levels requires much higher sensitivity than for its metabolites, because HA released into the blood from mast cells or basophiles disappears rapidly as a result of enzymatic degradation which forms stable MHA and other metabolites, and which are subsequently excreted in urine [16]. Unfortunately, the present method cannot detect those amines presented in human urine because these amines are generally presented at <5 nM in healthy human urine. If a laser-induced fluorescence (LIF) detection system or other equivalent detection system was employed, it would be possible to detect them.

3.3. Application to human urine samples

MHA, which is one of the metabolites of histamine (HA), is often measured to help diagnose allergy. HA released into the blood from mast cells or basophiles is rapidly excreted with urine as a result of enzymatic degradation, which forms stable MHA and other metabolites [16]. In this section, measurements were taken using a human urine sample spiked with HA and MHA at 0.1 μM level each. The urine sample in this experiment was collected from a healthy adult male, and was diluted two times with an aqueous ammonia solution of pH 10, and subsequently passed through the cationic ion exchange cartridge (BondElut CBA) to remove undesired amino acids, peptides, proteins and others. After eluting the amines with 0.1 M HCl, the eluate was evaporated in vacuo to obtain a resulting residue. Next, a sample solution was obtained by reconstituting the residue with water. The recovery of each HA and MHA from the cartridge was calculated and obtained as 85.6%, both of which show the same degree of recovery.

Fig. 3 shows the electrochromatograms obtained for the urine sample spiked with either HA and MHA at 0.1 μM level each (Fig. 3(A)) or not (Fig. 3(B)) after electrokinetic injection with 2 kV for 240 s prior to OCD–CEC using a 60% acetonitrile in 5 mM OPA/2-ME–10 mM borate (pH 10) for the run buffer solution. The peaks corresponding to both HA and MHA were indistinguishable from other peaks corresponding to the components of the urine matrix, hence, they were very difficult to identify. One possibility to improve their separation might be to decrease of electroosmotic flow by using a concentration of borate buffer (pH 10) higher than 10 mM. However, it is very hard to prolong the elution times this way, because if the concentration of

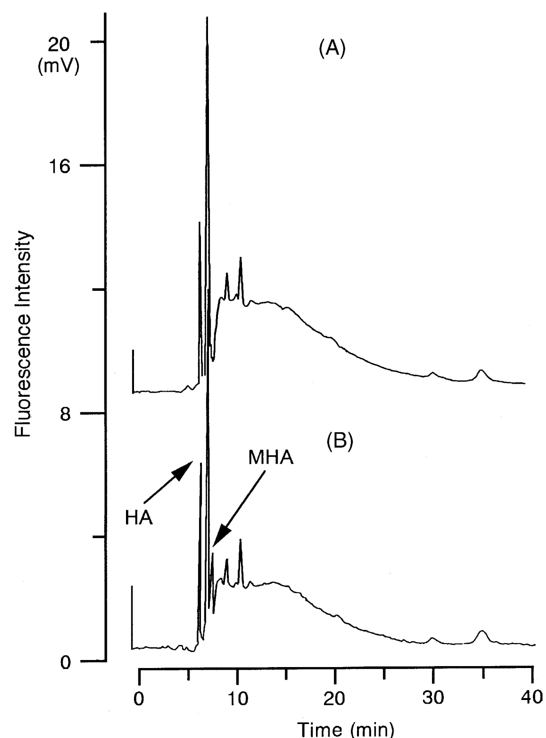

Fig. 3. Electrochromatograms of: (A) non-spiked human urine and (B) urine sample spiked with HA and MHA (0.1 μM each) extracted with BondElut cartridge. Conditions: run buffer, 60% acetonitorile–5 mM OPA–10 mM borate (pH 10) in the presence of 5 mM 2-mercaptoethanol (2-ME); other conditions the same as described in Fig. 1.

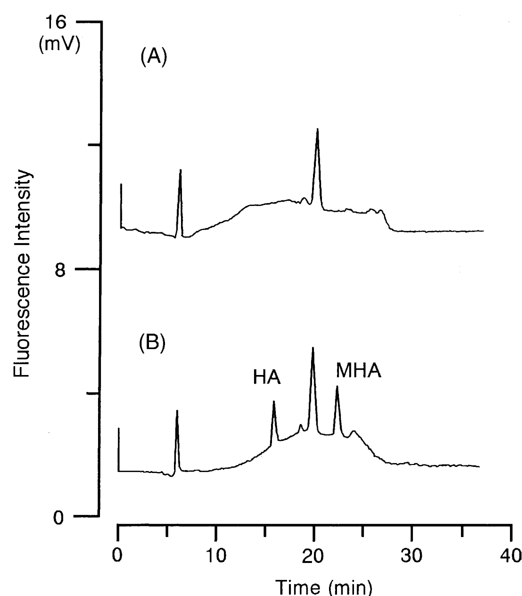

Fig. 4. Electrochromatograms of: (A) non-spiked human urine and (B) urine sample spiked with HA and MHA (0.1 μM each) extracted with BondElut cartridge. Conditions: run buffer, 60% acetonitorile–5 mM OPA–10 mM borate (pH 10) in the presence of 5 mM 1-butanethiol (1-BT); other conditions the same as described in Fig. 3.

the borate buffer is more than 15 or 20 mM, precipitations occur in the run buffer solution. Next, the thiol compound, 2-ME, in the run buffer was replaced with 1-BT, and the urine sample was analyzed again under the same conditions. This time, HA and MHA's peaks were completely separated from each other and were clearly distinguishable among the peaks corresponding to the compounds in urine matrix as shown in Fig. 4. Three other thiols (ET, 1-PT and 2-MPT), were likewise investigated by this method using the same spiked and non-spiked samples. The separation and identification of both HA and MHA was shown to be possible using 2-MPT as well as 1-BT. The electrochromatograms obtained from 2-MPT and 1-BT were almost identical. On the other hand, the identification of the HA and MHA peaks failed in both cases when ET and 1-PT were employed (data not shown).

4. Concluding remarks

In this paper, the elution profiles of HA and MHA within a monolithic ODS capillary column were evaluated when different kinds of alkyl thiols were included together with OPA in a run buffer solution for OCD–CEC. Different elution profiles of HA and MHA resulted when five kinds of thiol compounds (2-ME, ET, 1-PT, 2-MPT and 1-BT) were separately employed. Identification of two peaks corresponding to HA and MHA was shown to be possible for a human urine sample with OPA/1-BT and 2-MPT, but was not possible with OPA/2ME. We believe that the present method is an alternative way to optimize separation conditions using OCD–CEC, thereby overcoming the difficulties of performing assays of certain biogenic amines in natural samples.

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