

Separation and Detection of Thiols Derivatized with 5,5'-dithiobis (2-nitrobenzoic acid) by Capillary Electrophoresis

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Abstract: The determination and detection of low molecular weight thiols by capillary electrophoresis (CE) system has been studied. Though electrophoresis of thiols has to be carried out at low pH due to their instability, each migration time of thiols was fairly long and the separation of thiols wasn't completely. We examined to derivatize thiols with 5,5'-dithiobis (2-nitrobenzoic acid) which has been used as the photometric reagent for the determination of thiols, usually called as Ellman's reagent. In this study, ethanethiol, 1-propanethiol and 1-butanethiol were investigated to be separated by CE with UV detection. Running buffer was prepared to be pH 7.4 containing 10mM disodium hydrogenphosphate and 80 μ M tetradecyltrimethylammonium bromide. Ethanethiol, 1-propanethiol and 1-butanethiol were prepared as a 0.5mM solution in 10mM sodium phosphate buffer (pH=7.4). The applied voltage was set at +30kV. The detection of derivatized thiols was measured at 357nm. These thiols were separated each other in 18 min.

Key words: thiols, capillary electrophoresis, Ellman's reagent, derivatized thiol

Introduction

Low molecular weight thiols occur widely in nature. They are easily oxidized to disulfide, this redox system have a important role in human bodies. Although they are known as flavor component of food, but on the other hand they have a strong peculiar odor. They are one of the volatile organosulfur compounds released into the atmosphere by the decomposition of organisms with bacterial reactions. It is required to determine low molecular weight thiols in the environmental and biological studies. For the determination of glutathione which is important in biological systems, many different determination methods have been studied¹⁾. The analysis of other thiols has been further achieved by several techniques, principally by HPLC with UV/Visible²⁾ or amperometric³⁾ detection, gas chromatography, including the application of UV derivatization and electrochemical detection. But not so many studies for the determination of low molecular weight thiols have been investigated

because of their instability and peculiar odor. Therefore the rapid method is required to separate and determine low molecular weight thiols. Only a few studies deal with low molecular weight thiols using amperometry in a flow-injection system⁴⁾. Capillary electrophoresis (CE) has been applied to separate and determine thiols in biological samples^{5, 6)}. CE has the advantages of excellent resolving power and fast analysis. CE separation of thiols has to be carried out in low pH solution in which they are relatively stable. But in low pH solution, mixed thiols are not separated completely and each migration times of thiols are rather long. The primary purpose of this study is to achieve rapid separation of low molecular weight thiols by CE with photodiodearray detection. For a more rapid and clear separation, the authors examined to derivatize thiols with 5,5'-dithiobis (2-nitrobenzoic acid) which has been used as the photometric reagent for the determination of thiols, usually called Ellman's reagent. Ellman's reagent reacts with thiols to form UV-absorbing mixed disulfides which allow detection at a wavelength of 357 nm. Good separation and rapid migration of thiols were

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achieved for the Ellman's derivatized thiols using a detection wavelength of 357 nm^{7, 8)}.

Experimental

Reagent

5,5'-dithiobis (2-nitrobenzoic acid) from Wako Pure Chemical Industries was analytical grade. Tetradecyltrimethylammonium bromide (TTAB) was obtained from Waters Co. as CIA-Pak™ OFM Anion-BT. Ionexchanged water was further purified by a Milli-Q system for reagent grade water.

Apparatus

Thiol capillary electrophoresis was carried out by using Capillary Electrophoresis system CAP-3200 (Otsuka Electronics). The column used was polyimide coated fused-silica capillary of total length 500mm (378mm to detector window) and 0.075mm in diameter.

Procedure

Capillary electrophoresis of underivatized thiols

Ethanethiol and 1-propanethiol were prepared as a 5 mM solution in 2 mM hydrochloric acid. A running buffer was a 100mM solution of disodium hydrogen-phosphate adjusted to pH 2.3 with hydrochloric acid. All sample solutions were injected into the hydrodynamic mode where the capillary was immersed in the sample at a height 10mm above the running buffer level for 30s. The temperature was maintained at 25°C with an air coolant control system. The applied voltage was set at 15kV. The detection of underivatized thiols was measured at 200nm.

Capillary electrophoresis of thiols after derivatization with Ellman's reagent

A running buffer was a 10mM disodium hydrogen-phosphate of pH 7.4 with sodium hydroxide. Ethanethiol, 1-propanethiol and 1-butanethiol were prepared as 0.5mM solutions in 10mM sodium phosphate running buffer. Ellman's reagent was prepared as a 3 mM solution in 10mM sodium phosphate running buffer. One hundred microlitres of the thiol-containing sample solution was added to 100 μ l of Ellman's reagent in a Eppendorf tube, and after mixing, the reaction mixture was left at room temperature for 20min until equilibrium was achieved. Applied voltage was set at +30kV and detection wavelength was 357nm throughout electrophoresis. Other conditions were the same as described previously.

Separation of low molecular weight thiols

Ethanethiol, 1-propanethiol and 1-butanethiol were prepared as a 0.5mM solution in sodium phosphate buffer (pH=7.4). They reacted with Ellman's reagent as described previously. A running buffer was a 10mM solution containing 0.08mM tetradecyltrimethylammonium as CIA-Pak™ OFM Anion-BT. Electrophoresis was carried out in identical conditions as described previously.

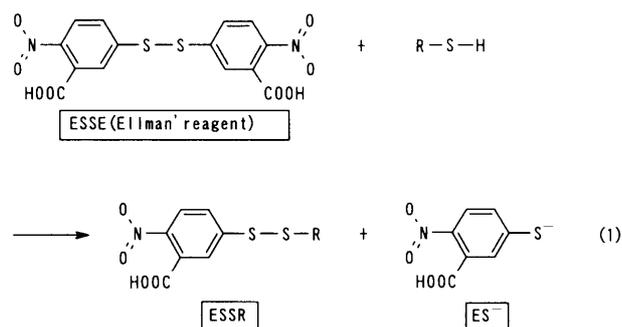
Results and discussion

Electropherogram of underivatized thiols

Though thiols are easily oxidized, the oxidation was controlled in a low pH solution due to protonation of the thiolate species. After capillary electrophoresis of ethanethiol and 1-propanethiol in low pH running buffer and sample solution, electropherograms of ethanethiol and 1-propanethiol were observed for 55 min, and they weren't separated. As thiols are electrically neutral molecules in this low pH solution, they migrated only by electroosmotic flow (EOF). In spite of long time of migration, they couldn't be separated completely.

Capillary electrophoresis of thiols after derivatization with Ellman's reagent

To separate thiols, they were derivatized with Ellman's reagent, a thiols-specific reagent which can be detected at 357nm. Ellman's reagent has been used to study the separation of thiol-containing compounds in biological samples. The reaction of Ellman's reagent (ESSE) with the thiol is given in Eq. (1).



ESSE (Ellman's reagent) reacts with RSH to form disulfide ESSR and thiolate anion (ES⁻). Ellman's reagent is in excess, the reaction reaches equilibrium as Eq. (1). The major products of the reaction are ES⁻ which has a absorption maximum at 412nm, ESSR which has a absorption maximum at 357nm, and excess Ellman's reagent which has a absorption maximum at

325 nm. The absorption bands of ESSR and excess ESSE are broad and overlap. Therefore absorbance of thiolate anion band was measured to determine thiols. But the formation of thiolate anion irrespective of the structure of the thiol, makes the Ellman's reagent ideal for the measurement of the total level of thiols in mixture. CE was applied for the separation of these absorption bands. When the absorption band of ESSR was separated from other absorption bands, a difference of migration time based on each R group was observed. The products of the reaction of Ellman's reagent with thiols are sensitive to decompose at high pH and are insoluble at pH less than 7.4, therefore a pH 7.4 sodium phosphate running buffer was used. Fig. 1 shows

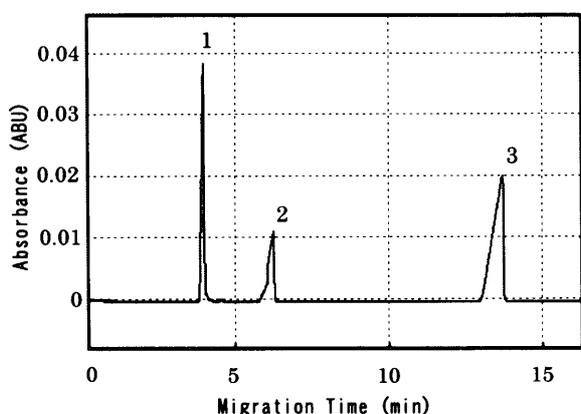


Fig. 1 Electropherogram of the products of the reaction between Ellman's reagent and ethanethiol: 1=mixed disulfide (ESSR), 2=thiolate anion (ES⁻), 3=excess Ellman's reagent (ESSE)

the electropherogram of ethanethiol with Ellman's reagent measured at 357 nm. The migration time of ESSR was under 5 min. The migration time of ethanethiol was decreased by reacting thiol with Ellman's reagent compared with that of undervivatized ethanethiol. As shown in Fig. 1, peaks of ESSR, ES⁻ and excess Ellman's reagent (ESSE) were separated clearly.

Complete baseline separation of thiols

Good separations were investigated as a function of the applied voltage between +10 kV and +30 kV. In CE, better separation is generally obtained by applying high voltage. But efficiency of mobility is decreased due to the change in heat generated by applying high voltage during the electrophoresis process. It is required to dissipate the heat generated. However, applying high voltage leads to the best efficiency of migration in this study. As shown in Fig. 2, the shortest time of migration could be achieved by applying voltage as high as possible. In the same applied voltage range, Fig. 3 shows the peak heights were not so much difference. Electrophoresis of thiols were carried out at +30 kV of applied voltage. But in this electrophoresis, migration peaks of mixed thiols overlapped.

For complete baseline separation of the mixed thiols, TTAB solution was added to the running buffer solution to modify the capillary wall. Fig. 4 shows the relationship between the migration time of derivatized thiols and the concentration of TTAB in the running buffer solution. Under the concentration of 60 μM of

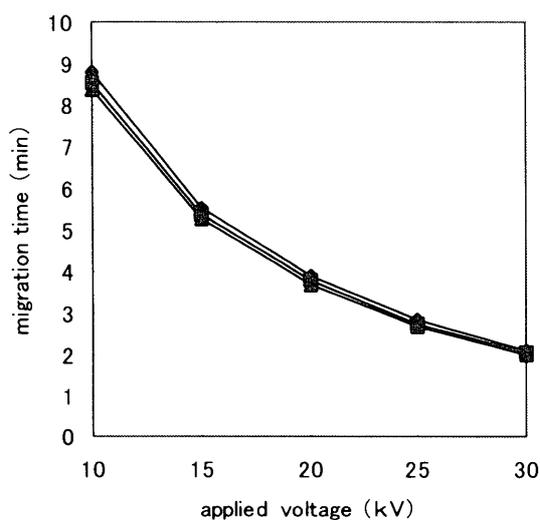


Fig. 2 Relationship between applied voltage and migration time: (◆) ethanethiol, (■) 1-propanethiol, (▲) 1-butanethiol

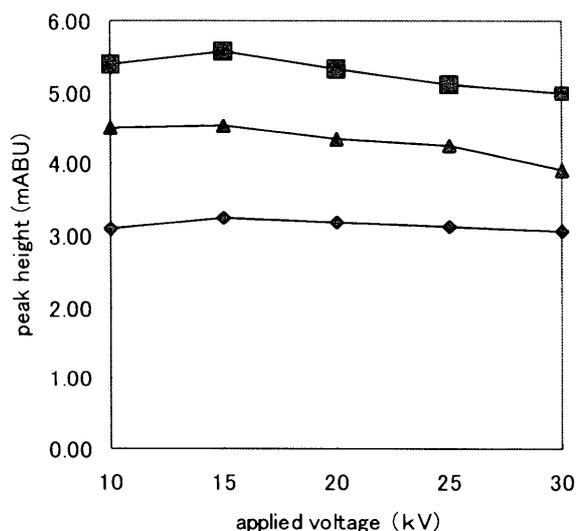


Fig. 3 Relationship between applied voltage and peak height: (◆) ethanethiol, (■) 1-propanethiol, (▲) 1-butanethiol

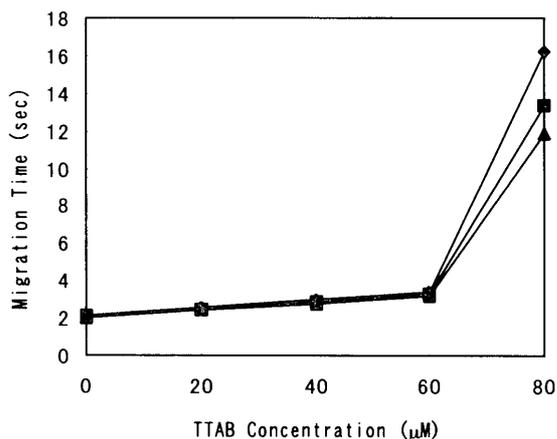


Fig. 4 Relationship between TTAB concentration and migration time: (◆) ethanethiol, (■) 1-propanethiol, (▲) 1-butanethiol

TTAB in the running buffer solution, the mobility of ethanethiol, 1-propanethiol and 1-butanethiol were not different. At a TTAB concentration of $80\mu\text{M}$ in running buffer solution, each peak was separated clearly. The EOF affects the mobility of all thiols strongly. Then each mobility caused by a difference of R of RSH did not appear. By the addition of TTAB to the running buffer, it modified the capillary wall and the EOF was decreased. The characteristic mobility of each thiol based on the difference of R appeared. Therefore thiols can be separated by the difference each group. As shown in Fig. 5, the complete separation of the mixed thiols was finally obtained with the running buffer containing $80\mu\text{M}$ solution of TTAB. Ethanethiol, 1-propanethiol and 1-butanethiol were separated each other in 18min.

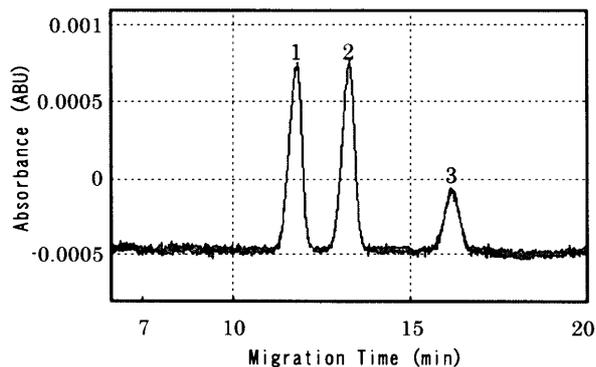


Fig. 5 Electropherogram of low molecular weight thiols: 1=1-butanethiol, 2=1-propanethiol, 3=ethanethiol CE conditions: running buffer 10mM sodium phosphate, $80\mu\text{M}$ TTAB (pH=7.4); applied voltage +30kV; sample injection 10mm, 30s; detection wavelength 357nm

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キャピラリー電気泳動による誘導体化した チオール類の分離と検出

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要旨 低分子のチオール類は揮発性が高くまた空气中で酸化されやすい。そこで 5,5'-ジチオビス (2-ニトロ安息香酸; Ellman 試薬) を用いてチオールを誘導体化したのち, キャピラリー電気泳動によりチオール類の分離・検出を行った。SH 基を有する化合物に Ellman 試薬を過剰に加えると, 混合ジスルフィドとチオレートアニオンが生成するが, 電気泳動を行うことにより, 一部重複していた各成分のピークの分離が可能となった。泳動液は $80 \mu\text{M}$ テトラデシルアンモニウムブロミド (TTAB) を含む 10mM リン酸二水素ナトリウム溶液を用いた。TTAB 溶液を添加することで, キャピラリー内壁の電荷を制御し電気浸透流を減少させることにより各成分の持つ構造の違いによる泳動時間の差が明確になり分離が完全になった。キャピラリーは内径 0.075 mm , 全長 500 mm (有効長 378 mm) の溶融シリカ管を用いた。試料は落差 10 mm で 30 秒間キャピラリー内に注入し, 印加電圧 $+30 \text{ kV}$ にて泳動を行った。キャピラリー周囲は 25°C に保った。検出には Ellman 試薬を用いて誘導体化したチオール類の吸収極大波長である 357 nm を用いた。エタンチオール, 1-プロパンチオール, 1-ブタンチオールを短時間で分離・検出できた。

キーワード: チオール類, キャピラリー電気泳動, Ellman 試薬, 誘導体化