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Short communication

Determination of urinary succinylacetone by capillary electrophoresis for the diagnosis of tyrosinemia type I

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Abstract

The presence of succinylacetone in urine or blood or amniotic fluid is pathognomonic of an inherited metabolic disorder, named tyrosinemia type I. We developed a capillary electrophoretic method for the fast analysis of succinylacetone in urine samples. The separation was performed at reversed polarity mode using either a cationic surfactant as the buffer additive, or a capillary coated with a positively charged polyelectrolyte. Under these conditions, urine samples were directly injected to the capillary without any pretreatment step. The utility of the method was demonstrated by the identification of succinyacetone in urine from patients with hereditary tyrosinemia type I. For all patients, diagnostic peaks at the expected migration times were detected. The developed method is rapid, simple, inexpensive, and suitable for the determination of succinylacetone in clinical urine samples.

Keywords: Hereditary tyrosinemia type I; Succinylacetone; Capillary electrophoresis

1. Introduction

Tyrosinemia type l is an inherited metabolic disorder, which is caused by reduced activity of fumarylacetoacetate hydrolase (FAH), the final enzyme in the degradation pathway of tyrosine. The deficiency of FAH leads to an accumulation of the metabolites fumarylacetoacetate and maleylacetoacetate, which together form succinylacetone. Since succinylacetone (4,6-dioxoheptanoic acid) is not found in normal body fluids, the presence of succinylacetone in urine or blood or amniotic fluid is pathognomonic of tyrosinemia [1,2]. The only effective therapy for tyrosinemia type l has been liver transplantation. However, Lindstedt et al. reported in 1992 the successful use of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) as an alternative to liver transplantation in hereditary tyrosinemia [3].

Though it is very important to diagnose newborns within a short period of time, there are only a few analysis methods reported so far. Many clinical laboratories employ capillary gas chromatography/mass spectrometry (GC/MS) to detect succinylacetone in urine and serum [4–7]. One of the drawbacks of this technique is the relatively long time required for complex sample pretreatment, derivatization, and analysis.

More recently capillary electrophoresis (CE) has become a complementary technique to the chromatographic methods for clinical analysis. The advantage of the capillary electrophoretic methods is the considerable diminution in the sample preparation and analysis times, as well as in the reagent consumption. CE is particularly suitable in the analysis of complex natural matrices, owing to its higher resolving power. Moreover, fused silica capillaries are much less expensive than chromatographic columns, easily washed between runs, and free of irreversible contamination of the matrix, unlike the packed columns. Thereby, in many capillary electrophoretic analysis methods, body fluids can be directly injected to the capillary without any pretreatment step or after a

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simple process of sample pretreatment like deproteinization by precipitation or ultrafiltration.

In this paper, we propose a rapid and easy method using capillary zone electrophoresis in order to determine succinylacetone in urine without any pretreatment of the urine sample.

2. Materials and methods

2.1. Materials

Succinylacetone was purchased from Sigma (St. Louis, MO, USA). Polyethyleneimine (PEI) (molecular mass range 6×10^5 to 1×10^6) and cetyltrimethylammonium bromide (CTAB) were purchased from Fluka (Buchs, Switzerland). Sodium tetraborate and sodium chloride were from Merck (Darmstadt, Germany). All solutions were prepared with deionized water purified in an Elgacan C114 (Elga, England) filtration system.

Urine diluted solutions were daily prepared by diluting fresh human urine with purified water (1/5). Succinylacetone stock solution was preapared by dissolving the succinylacetone in water. The stock solution was stored at $-4\,^{\circ}$C. The injected solutions were prepared daily prior to use. Before use, all solutions were filtered using a microfilter with a pore size of 0.45 μm.

2.2. Aparatus and operating conditions

Separations were performed with an Agilient capillary electrophoresis system equipped with a diode-array detector. The data processing was carried out with the Agilient ChemStation software. The wavelength was set at 295 nm. The separation was performed at −28 kV (reversed polarity mode). Injections were made at 5.10^{-3} MPa for 5 s.

The fused silica capillaries used for separation experiments were 50 or 75 μm i.d. (for coated capillaries) and were obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 48 cm and the length to the detector was 40 cm. The fused silica capillary was conditioned prior to use by rinsing with 1 mol/l NaOH for 30 min, water for 10 min, and buffer (at pH 9.2) with 10 min. Capillary was washed with buffer solution for 1 min between the runs. The coating procedure with PEI was reported before [8]. Briefly, the coating procedure involves the following: The fused silica capillary was first etched by flushing the capillary with a solution of 1 mol/l sodium hydroxide for 30 min at 1×10^{-1} MPa and with water for 15 min at the same pressure. Then, the capillary was flushed with a solution of 10% PEI in water at 1.5×10^{-1} MPa for 10 min and the PEI solution was left in the capillary for 1 h. Next, the polymer solution was pressed out of the capillary with air at 1.5×10^{-1} MPa. Finally, the capillary was rinsed with water and the running buffer for 15 min.

A Metrohm 654 Digital pH Meter (Switzerland) with combined pH glass electrode (Metrohm) was used for pH measurements.

3. Results and discussion

3.1. Identification of succinylacetone

Determination of small anions by capillary electrophoretic methods depends on the slowing down or reversal of the electroosmotic flow (EOF) in the capillary. This can be achieved by using buffer additives like cationic surfactants or coating the capillary wall by a positively charged polymer. Since in both cases the capillary surface acquires a positive charge, the electroosmotic flow within the capillary is reversed towards the anode. Since the electrophoretic mobility of small anions and the EOF are in the same direction, the rapid migration and detection of the anions is possible.

In this work, the necessary separation conditions for succinylacetone have been determined both with the addition of the cationic surfactant CTAB and with the use of PEI coated capillary.

Preliminary experiments showed that the succinylacetone standard sample can be determined in 2.17 min with a good sensitivity when using a 50 mmol/l borate buffer containing 20 mmol/l NaCl and 0.05 mmol/l CTAB as the cationic surfactant in a fused silica capillary. Buffer pH was kept at pH 9.2. NaCl addition to the borate buffer improved the peak shapes. It is known that succinylacetone does not exist in healthy human urine. Freshly collected healthy urine was diluted (1:5), filtered, and injected to the same buffer. No interfering peaks appear in healthy urine under the present conditions. Fig. 1a shows the electropherogram of a diluted healthy urine (1:5) and Fig. 1b shows the electropherogram of a diluted healthy urine (1:5) spiked with succinylacetone.

The utility of the method was demonstrated by the detection of succinylacetone in five samples of urine from ill ba-

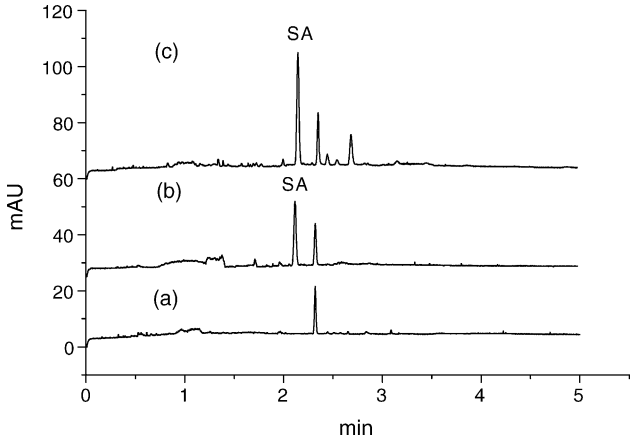

Fig. 1. (a) An urine sample (1:5) from a healthy person. (b) An urine sample (1:5) spiked with succinylacetone. (c) An urine sample (1:5) from a patient with tyrosinemia type l. Buffer: 50 mmol/l borate, 20 mmol/l NaCl, 0.05 mmol/l CTAB in uncoated capillary, pH 9.2.

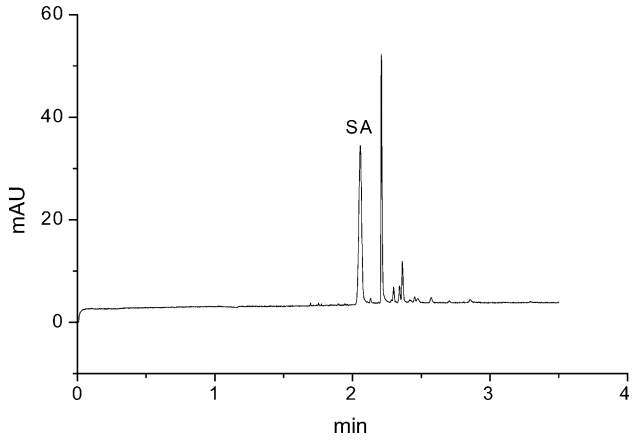

Fig. 2. An urine sample spiked with succinylacetone in PEI-coated capillary. Buffer: 50 mmol/l borate, pH 9.2.

bies, as confirmed by GC–MS. Before analysis, the samples were diluted (1 volume of sample plus 4 volumes of water) with water and filtered. For all patients, the succinylacetone peak at the expected retention time was detected and verified with diode array detector and standard addition. Fig. 1c shows the electropherogram of urine (1:5) from a patient with tyrosinemia type l.

Secondly, we used a non-covalent PEI coating in the analysis of succinylacetone without the addition of modifier to the buffer. The polymer layer of the coating has a positive charge over a wide pH range, which results in an electroosmotic flow towards the anode. Because of this feature, the PEI coating is suitable for the fast separation of small organic anions [9,10]. Here, a 50 mmol/l borate buffer was used and the addition of NaCl did not change the peak shapes. Fig. 2 shows the electropherogram of diluted urine spiked with succinylacetone in the PEI-coated capillary.

3.2. Quantitative aspects

3.2.1. Linearity range and calibration curve

Though the presence of succinylacetone is sufficient to the detection of metabolic disorder, the quantification of succinylacetone may be necessary to follow the babies treated by NTBC.

The linearity was checked by injecting diluted normal human urine (urine–water, 1:5) solution spiked with succinylacetone in the concentration range from 0.0625 to 2.0 mmol/l. Parallel experiments were performed for uncoated and coated capillaries in the experimental optimal conditions expressed before. The regression equations were $y = 2.98465 \times 10^{-1}x - 1.07010 \times 10^{-3}$ ($r = 0.99996$) and $y = 3.060011 \times 10^{-1}x + 8.64116 \times 10^{-3}$ ($r = 0.99590$), respectively.

3.2.2. Limits of detection

The limit of detection (LOD) was obtained as the SA concentration that caused a peak with a height three times the baseline noise level. LOD was calculated as 0.042 mmol/l in uncoated capillary and as 0.027 mmol/l in coated capillary. LOQ was 0.08 and 0.06 mmol/l, respectively. Since the internal diameter of the coated capillary is 75 μm, the sensitivity is better than in 50 μm i.d. capillaries.

3.2.3. Precision

The precision of method was calculated as the coefficient of variation (CV) of migration times and peak areas for seven successive injections of spiked (0.25 mmol/l) urine samples. Repeatability of migration times for uncoated and coated capillaries were 1.6 and 0.7%, respectively, and the corrected peak areas (A/t) were 10.9 and 7.6%, respectively.

4. Conclusion

The CE method described for the succinylacetone assay in urine is rapid, simple, and inexpensive. It does not require any sample pretreatment. A very small volume of urine samples is directly injected to the capillary and the presence of metabolite is detected in only 2.17 min. Since succinylacetone is not found in normal urine, the presence in urine is pathognomonic of tyrosinemia and, thereby, the method is suitable for use as a quick metabolic assay of newborn babies in whom there is a possibility of this inborn error.

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