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Electrophoretically mediated microanalysis for simultaneous on-capillary derivatization of standard amino acids followed by micellar electrokinetic capillary chromatography with laser-induced fluorescence detection☆

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ABSTRACT

Amino acids are crucial compounds involved in most biochemical processes essential for life. Since their dynamic turnover reflects the actual physiology of the cell/organism, a turnover assessment may provide valuable information related to multiple physiological and pathophysiological conditions. The sensitive determination of amino acids is predominantly associated with their derivatization which might be laborious, time-consuming and difficult to standardize. However, capillary electrophoresis offers the automatic injection and mixing of reactants, incubation of the reaction mixture, separation and detection of the reaction products in one on-capillary procedure. Among the on-capillary mixing strategies, electrophoretically mediated microanalysis (EMMA) is superior in terms of mixing efficiency. In this paper, we present an optimization of EMMA for the simultaneous derivatization of standard amino acids by naphthalene-2,3-dicarboxaldehyde/NaCN and its application to targeted human embryo metabolomics. For such a purpose, novel separation conditions were developed involving the background electrolyte, comprised of 73 mM sodium dodecyl sulfate, 6.7 % (v/v) 1-propanol, 0.5 mM (2-hydroxypropyl)-β-cyclodextrin and 135 mM boric acid/sodium hydroxide buffer (pH 9.00). Finally, the optimized EMMA was compared to a fundamentally different mixing strategy, namely the transverse diffusion of laminar flow profiles, and proved to be also suitable for human plasma analysis.

1. Introduction

Amino acids (AAs) are very important compounds for living organisms. They are not only building blocks of proteins with their many functions, but also act as neurotransmitters and precursors of some hormones. Moreover, AAs are involved in cell signaling and gene expression. As a result, the determination of AAs in biological fluids can reveal the physiological state of an organism, and consequently can be used for diagnostic purposes [1]. Our research group recently published two papers covering non-invasive targeted metabolomics of a single human embryo. The first paper was focused on the determination of bioenergetically important compounds, namely pyruvate and lactate [2], whereas the second paper purely aimed at AA determination, and revealed some serious limitations of the diffusion-driven on-capillary mixing of reactants [3].

Research in the field of human embryo metabolomics is driven by the necessity to increase the success rate of the *in vitro* fertilization cycle, thus reducing the emotional and financial burden for patients. The key aspect of the success of *in vitro* fertilization is suggested to be proper selection of the most viable embryo from a batch [4]. Moreover, proper embryo selection may enable the transfer of a single embryo into the uterus of a recipient, while maintaining a desirable success rate of the cycle. Implantation of a single embryo into the uterus decreases the risks for both the mother and child related to multiple pregnancies, such as miscarriage, preterm delivery or infant death [5]. Embryo selection is mostly limited to the embryo's morphological appearance based on a combination of cleavage rates and blastomere fragmentation, while the embryo's functionality remains hidden [6]. Metabolomics may provide insight into the embryo's functionality; however, to overcome several challenges – limited sample volume, low metabolic activity, complexity of the embryo's *in vitro* and *in vivo*

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development – a high-throughput, sensitive and precise analytical method is required. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) may meet such requirements and thus was selected for the determination of AA turnover in an embryo culture medium.

Although most AAs exhibit no native fluorescence, many reactions yielding fluorescent derivatives of AAs have been proved successful (see reviews, *e.g.* [7,8]). One of the disadvantages of classical derivatization in HPLC or CE lies in the number of manual steps required to accomplish this task. However, CE offers the possibility of conducting the enzymatic or chemical reactions on-capillary by the sequential injection of individual reactants followed by reactant mixing either by diffusion or electromigration [9]. The fundamental on-capillary mixing strategies are diffusion-driven *at-inlet reaction* [10] and *transverse diffusion of laminar flow profiles* (TDLFP) [11], and electromigration-driven *electrophoretically mediated microanalysis* (EMMA) [12,13]. In order to fully harness the benefits of the on-capillary conducted derivatization, the suitable fluorogenic reagent/fluorescent label should yield fluorescent derivatives at the separation temperature within a few minutes and should be stable in solution at the carrousel temperature for the purpose of sequential automatic analyses. From this perspective, naphthalene-2,3-dicarboxaldehyde (NDA) stands out among other fluorescent labels. NDA is a non-fluorescent compound which reacts rapidly with the primary amino group in the presence of a cyanide anion and yields highly fluorescent *N*-substituted 1-cyanobenz[*f*]isoindoles (CBIs) [14]. Moreover, NDA is stable in water-based solution at laboratory temperature, as it does not undergo hydrolysis. The reaction scheme of the reaction of NDA with AA is shown in Fig. 1. The resulting AA-CBIs are characterized by three excitation maxima at 250, 420 and 440 nm [15]; however, efficient excitation was also reported at 488 nm with a commonly used argon-ion laser [16].

In this paper, a new on-capillary derivatization of all standard AAs (except for proline) by means of EMMA was developed. Moreover, the separation conditions described in our recently published paper [3] were critically evaluated and the disadvantages that were noticed initiated the development of novel separation conditions that are superior in terms of instrumental practicality and shorter analysis time. The applicability of the developed method was demonstrated on the non-invasive targeted metabolomics of human embryos as a response to a plea for enhancement of embryo selection accuracy.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade purity and were used as received. L-Alanine (Ala), L-alanyl-L-glutamine (Ala-Gln), L-arginine monohydrochloride (Arg), L-asparagine (Asn), L-aspartic acid (Asp), H_3BO_3, L-cystine (Cyn), L-glutamine (Gln), L-glutamic acid (Glu), glycine (Gly), L-histidine monohydrochloride monohydrate (His), (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), HCl (37%), isobutanol, L-isoleucine (Ile), L-leucine (Leu), L-lysine monohydrochloride (Lys), methanol (MeOH), L-methionine (Met), NDA, L-norvaline (nVal), L-phenylalanine (Phe), 1-propanol (1P), 2-propanol (IPA), NaCN, sodium dodecyl sulfate (SDS), L-serine (Ser), NaOH, H_2SO_4 (95%), taurine (Tau), L-threonine (Thr), L-tryptophan (Trp), Tween® 20, L-tyrosine (Tyr) and L-valine (Val) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized (DI) water (18.2 MΩ at 25 °C) was obtained by Direct-Q® 3 UV water purification system (Merck Millipore, Billerica, MA, USA).

2.2. Reagent solutions

The stock solution of 8 mM NDA was prepared in a mixture of MeOH and IPA (4:1) dried over molecular sieves (3 Å, Sigma-Aldrich) and stored in an amber microtube. The stock solution of 250 mM NaCN was prepared in DI water. Stock solutions (20 mM) of individual AAs and Ala-Gln dipeptide were prepared in DI water except for Tyr and Cyn, which were prepared in 0.1 M HCl and 0.1 M NaOH, respectively, to facilitate their dissolution. The stock solution of the reaction buffer was 200 mM H_3BO_3 titrated with 1 M NaOH to pH 9.8 and was used for the preparation of working solutions of NDA, NaCN, the AA model and real samples. The working concentration of the reaction buffer – 100 mM H_3BO_3/NaOH – was kept constant during all experiments. Stock solutions were prepared fresh on a weekly basis and were stored in a refrigerator (+4 °C), except for the stock solution of the reaction buffer (stored at laboratory temperature). Working solutions were prepared fresh daily by dilution of the stock solutions.

2.3. AA model and real samples

A model sample was prepared daily from frozen aliquots (−20 °C) of a mixture of 21 AAs and Ala-Gln dipeptide. Ala-Gln is a substitute for Gln in embryo culture media, as Gln is unstable in solution, forming pyroglutamate and toxic ammonium. On the day of use, an aliquot was thawed at laboratory temperature and diluted in the stock solution of the reaction buffer, resulting in the AA model sample comprised of 2 μM nVal (internal standard, IS), 5 μM Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Ser, Tau, Thr, Trp, Tyr, Val, 15 μM Ala-Gln, 20 μM Cyn, Lys and 100 mM H_3BO_3/NaOH, pH 9.8.

Pooled EDTA-plasma was kindly provided by eight volunteers and aliquots of it were stored frozen in a refrigerator at −70 °C. Before analysis, an aliquot was thawed at laboratory temperature and plasma proteins were precipitated by the addition of 8 μl of the mixture of MeOH and IPA (4:1) to 2 μl of the plasma sample. The proteins were left to precipitate for 5 min at laboratory temperature. The precipitate was removed from the solution by centrifugation at 12,000 × *g* for 10 min using a MiniSpin® plus (Eppendorf, Hamburg, Germany). The supernatant was collected and diluted in the stock solution of the reaction buffer enriched with nVal (IS), resulting in a 100 × diluted plasma sample in the reaction buffer (100 mM H_3BO_3/NaOH, pH 9.8) containing 2 μM nVal.

Both fresh and spent Sydney IVF Cleavage Medium (SICM; Cook Ireland, Limerick, Ireland) culture media designed for the *in vitro* cultivation of human embryos were analyzed. The spent culture media were collected after the cultivation of embryos originating from intracytoplasmic sperm injection to avoid contamination of the medium with sperms. Embryos were cultivated individually in 25 μl drops of SICM culture medium under a layer of Ovoil™ paraffin oil (Vitrolife, Göteborg, Sweden) at 37 °C in an atmosphere comprised of 20% O_2 and 5.6% CO_2. Sample contamination with residual paraffin oil was avoided by short-spin centrifugation forming a clear oil-medium phase interface. The fresh and spent culture media were stored at −20 °C and thawed at laboratory temperature prior to analysis. The subsequent sample treatment was the same as for the plasma sample described in the previous paragraph. The samples of the spent culture media were kindly provided by the Center of Assisted Reproduction at University Hospital Brno.

2.4. Capillary treatment

Bare fused-silica capillaries of 50 μm I.D. and 375 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 66.0 cm, and the polyimide layer was removed with boiling H_2SO_4 45.0 cm from the inlet capillary