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Development of a capillary electrophoresis method for analyzing adenosine deaminase and purine nucleoside phosphorylase and its application in inhibitor screening

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ABSTRACT

A novel capillary electrophoresis (CE) method was developed for simultaneous analysis of adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) in red blood cells (RBCs). The developed method considered and took advantage of the natural conversion from the ADA product, inosine to hypoxanthine. The transformation ratio was introduced for ADA and PNP analysis to obtain more reliable results. After optimizing the enzymatic incubation and electrophoresis separation conditions, the determined activities of ADA and PNP in 12 human RBCs were 0.237–0.833 U/ml and 9.013–10.453 U/ml packed cells, respectively. The analysis of ADA in mice RBCs indicated that there was an apparent activity difference between healthy and hepatoma mice. In addition, the proposed method was also successfully applied in the inhibitor screening from nine traditional Chinese medicines, and data showed that ADA activities were strongly inhibited by Rhizoma Chuanxiong and *Angelica sinensis*. The inhibition effect of *Angelica sinensis* on ADA is first reported here and could also inhibit PNP activity.

Adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP), ubiquitous and abundant in human tissues, play important roles in nucleotide metabolism and activation of nucleoside prodrugs [1–4]. ADA and PNP activities are essential for cell survival and function. Their abnormal activities in tissues or blood are involved in various diseases [3,4]. On the one hand, the deficiency of ADA or/and PNP can result in immune dysfunction [5–7]. For example, ADA activity was down to 1% of the normal level in human red blood cells (RBCs) of severe combined immunodeficiency disease (SCID) patients [5]. On the other hand, the two enzymes' activities increase in some diseases such as diabetes [8], tuberculosis [9], and liver disorders [10]. What is more, the elevated ADA and/or PNP activities were also found in some cancerous tissues such as bladder cancer, colon carcinoma, and breast cancer [11–15]. The reasons may be that high concentrations of adenosine can inhibit cell division, and the cancer cells need to maintain their reproducibility by keeping high ADA and PNP activities to metabolize adenosine [4,11–15]. ADA and PNP in blood mainly exist in RBCs, and they decide the metabolism of adenosine in whole blood [16,17]. The elevated activities of ADA and/or PNP were also observed in the RBCs of some cancer patients such as renal adenocarcinoma [17] and myeloid leukemia [18]. The relationship between ADA and/or PNP and various diseases is being further investigated. The current research results indicate that the determination, especially the joint determination [4,19,20], of the two enzymes' activities may be beneficial for disease diagnosis and treatment monitoring.

There are some available methods for quantitatively analyzing ADA and PNP such as ultraviolet (UV) spectroscopy [21,22], colorimetry [23–25], aptasensor [26,27], radiative method [28], and high-performance liquid chromatography (HPLC) [20,29–31]. In the UV method, ADA and PNP activities are calculated by measuring the decrease of the substrate at 265 nm or the increase of the product at 235 nm. However, this spectrophotometric method

Abbreviations used: ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; RBC, red blood cell; SCID, severe combined immunodeficiency disease; UV, ultraviolet; HPLC, high-performance liquid chromatography; NADPH, nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt; CE, capillary electrophoresis; CZE, capillary zone electrophoresis; MEKC, micellar electrokinetic chromatography; EOF, electroosmotic flow; TCM, traditional Chinese medicine; *T*%, transformation ratio; AMP, adenosine monophosphate; 5′-NT, 5-nucleotidase; AK, adenosine kinase; IMP, inosine monophosphate; SDS, sodium dodecyl sulfate; Rs, resolution; RSD, relative standard deviation; LOD, limit of detection; LOQ, limit of quantitation; S/N, signal/noise.

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without a separation function can only determine the total absorbance at a certain wavelength so that it is challenging to exactly quantify substrate and product because of the existing interference from analytes or complex biological matrix. Colorimetry is the most common method for analyzing ADA and PNP activities in clinical and pharmacological research. The method detecting specific visible or fluorescence absorption can determine amounts of certain produced substances such as nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) or colored quinones from the hypoxanthine or ammonia through multi-enzyme reactions to calculate enzyme activities. However, this method is time-consuming, complicated, and low sensitive. The radioactive method is effective, but it is inconvenient and environmentally toxic. Currently, the application of aptamers is attracting wide attention, and some fluorescent aptasensors and electrochemical aptasensors have been designed to analyze ADA. This method was highly sensitive and could detect ADA activity of 0.005 U/ml. However, human tissues have abundant ADA and PNP, and the very low detection limit is not so much necessary. Furthermore, the method needs many labeled molecules and the label has high costs. HPLC is also employed in ADA and PNP analysis based on the separated substrates and product. This method has superior reproducibility, but inevitably it needs intensive sample pretreatment, has long retention times, and has high costs. Capillary electrophoresis (CE), with high separation efficiency, short analysis times, low costs, and small sample volumes, has been widely applied in enzyme analysis [32]. To our best knowledge, there are few applications in ADA and PNP analysis using CE. In the limited reports, CE displayed excellent analysis ability. Pei and coworkers [33] researched the conversion of adenosine and nucleoside prodrugs catalyzed by ADA under different CE modes, including immobilized enzymatic reactor (IER), electrophoretically mediated microanalysis (EMMA), capillary zone electrophoresis (CZE), and micellar electrokinetic chromatography (MEKC). The CZE and MEKC methods were fast and reproducible, and MEKC was recommended to separate nucleoside prodrugs and corresponding products catalyzed by ADA. Iqbal and Müller [34] monitored ADA and PNP in membrane preparations of human 1539 melanoma cells with reversed electrode polarity switching mode—micellar electrokinetic chromatography (REPSM—MEKC). The sensitivity of the analytes was improved approximately 10-fold by large-volume stacking with switching polarity and electroosmotic flow (EOF). Carlucci and coworkers [5] determined ADA and PNP activities to diagnose and monitor SCID through comparing ADA and PNP activities in RBCs of healthy individuals and patients. ADA and PNP activities were analyzed individually based on the separated substrates and products with MEKC. However, from the aspect of enzymatic reactions, adenosine and inosine are the common substrates of ADA and PNP, respectively. The product of ADA is naturally converted by PNP existing in human RBCs and other tissues. If it was not taken into account, ADA activity that was estimated only by the formed inosine needed further verification [22,34]. For example, Paul and coworkers [22] studied ADA reaction in Hut-78, T cell lymphoma, and cell line lysates by HPLC, but the further decomposition of inosine was not considered. In Ref. [33], ADA was standard enzyme samples purified from calf spleen, whereas the conversion of ADA in the actual sample was different because of the coexistence of PNP or another metabolic pathway. Although actual sample was analyzed in Ref. [34], inosine was still thought to be the only product of ADA. The results needed to be further verified. As for Ref. [5], ADA and PNP were analyzed individually. The incubation conditions, such as incubation buffer, time, and substrate concentration, were improper to quantitatively determine ADA and PNP activities. It can only qualitatively compare ADA and PNP activities between healthy individuals and patients. All in all, it is necessary to develop a rapid, low-cost, effective, and accurate method for ADA and PNP analysis.

The inhibitors of ADA and/or PNP are also a central interest for their potential antitumor and anti-inflammation activities because they can be further developed into new drugs. For example, deoxycoformicin (DCF), an inhibitor of ADA, has been used to treat hairy cell leukemia and chronic lymphocytic leukemia [3,4,35,36]. Moreover, traditional Chinese medicines (TCMs) are important sources of new drugs due to their low costs, low toxicity, and good performance. Therefore, screening inhibitors of ADA and PNP from natural extracts is also attracting more and more attention [26,37,38]. In the reported inhibitor screening, pure ADA or PNP from calf intestine and *Escherichia coli* with diverse properties were used, and the screening results may be different with human enzymes. Although the enzymes can also be purified from human RBCs, the interaction of drugs with other molecules in cells would be ignored. Therefore, the enzymes in real human tissues as the direct targets for inhibitor screening are more significant.

Here a novel, simple, and efficient CE approach was developed for simultaneous analysis of adenosine deaminase and purine nucleoside phosphorylase in human RBCs. Compared with the reported studies of ADA and PNP activity analysis, ADA and PNP were analyzed in simple CZE mode using cis—diol borate complexes with analytes. In addition, we considered and took advantage of the effect of natural conversion from the ADA product, inosine to hypoxanthine, and introduced the transformation ratio based on the separated substrate and product to obtain precise quantitative analysis results. The enzyme activities were analyzed under the optimized enzymatic conditions. Meanwhile, the developed method was applied in inhibitor screening of ADA and PNP from nine traditional Chinese medicines using the enzymes in RBCs as the direct screening targets.

Materials and methods

Reagents and chemicals

Unless otherwise stated, all chemicals and reagents were analytical grade or better. Adenosine and inosine were purchased from Beijing Solarbio Science and Technology (Beijing, China). Hypoxanthine and perchloric acid (70%) were obtained from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Adenine was obtained from Shanghai Lanji Science and Technology Development (Shanghai, China). Boric acid, disodium tetraborate decahydrate, dipotassium phosphate, monopotassium phosphate, sodium hydroxide, potassium hydroxide, dimethyl formamide, and sodium dodecyl sulfate were purchased from Tianjin Jiangtian Chemical (Tianjin, China). Heparin was purchased from Tianjin Biochem Pharmaceutical (Tianjin, China). *Hedyotis diffusa*, Orange peel, *Angelica sinensis*, *Scutellaria baicalensis* Georgi, *Solanum nigrum* L, Rhizoma Chuanxiong, *Prunella vulgaris* L, *Dendranthema indicum*, and Paclitaxel were purchased from Tianjin Tongrentang Pharmacy (Tianjin, China). Distilled water was obtained from Yongyuan Distilled Water Manufacturing Centre (Tianjin, China).

Blood was contributed by healthy human individuals from Affiliated Hospital of Tianjin Institute of Chinese Medicine (Tianjin, China). The work involving humans described in this article was carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki). The contributors were informed and consentaneous, and their privacy rights were protected. Human hepatocellular cancer HepG2 cell lines were purchased from Cancer Hospital Chinese Academy of Medical Sciences (Beijing, China). Nude mice (4—5 weeks old) were purchased from Beijing HFK Bioscience (Beijing, China). The mice were housed