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pH gradient-liquid chromatography tandem mass spectrometric assay for determination of underivatized polyamines in cancer cells

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ABSTRACT

Altered levels of polyamines in biological specimens have been suggested as potential biomarkers for cancer. Difluoromethylornithine (DFMO, an irreversible inhibitor of ornithine decarboxylase) is reported to modulate polyamines to potentially attenuate proliferation of neuroblastoma cells. A clinical trial is being conducted to evaluate DFMO in various cancers. To determine the pharmacodynamics effect of DFMO, an analytical assay is needed to accurately measure the changes in polyamines in cancer cells. In this study, a novel pH gradient LC-ESI-MS/MS method was developed and validated for the quantitation of polyamines (putrescine, spermidine and spermine) in cancer cells. To separate polar and basic polyamines, a multi-mode column composed of ODS and weak ionic ligands was used. The pH gradient was generated from pH 5.3 to pH 2.7 with 2 mM ammonium acetate and 0.4% acetic acid in 10% acetonitrile as mobile phase. The detection of polyamines was performed utilizing multiple reaction monitoring on electrospray ionization mass spectrometry operated in positive ion mode. A pH gradient method increased resolution and decreased peak width of conventional analytical assays, resulting in 10–250-fold higher detection limits. Mobile phases without ion-pairing reagents were LC-MS compatible and eliminated possible signal suppression and MS contamination. The developed method was successfully applied to the analysis of polyamines in neuroblastoma and leukemia cells treated with DFMO. Putrescine levels were significantly ($p < 0.001$) decreased in CCRF-CEM (3.68 vs 1.23 ng/mg protein), SK-N-BE (2) (1.98 vs 1.31 ng/mg protein) and CHLA-20 (2.06 vs 0.90 ng/mg protein) cells treated with DFMO relative to vehicle control. The assay will provide a useful tool in determining the pharmacodynamic effect of DFMO in cancer clinical trials.

1. Introduction

Polyamines are simple alkylamines with low molecular weight naturally derived from arginine in cells to interact electrostatically with and stabilize negatively charged moieties such as DNA, RNA, proteins and phospholipids [1,2]. The three major polyamines, putrescine, spermidine, and spermine, are frequently found in prokaryotic as well as eukaryotic cell [3], and their intracellular concentrations are tightly regulated at nanomolar to micromolar concentrations [4]. Studies have shown that polyamine levels are abnormally increased in biological specimens of patients with various types of solid tumors [5,6]. Altered levels of polyamines reflect the enhanced activity of the enzymes involved in polyamine synthesis and cellular uptake in cancer cells [7]. Ornithine decarboxylase (ODC) catalyzes decarboxylation of L-ornithine to produce diamine putrescine (Fig. 1). Putrescine is catalyzed into spermidine and then into spermine by spermidine synthase and spermine synthase. Because ODC is the key enzyme regulating polyamine biosynthesis and is rate limiting with a short half-life (20 min), ODC has been targeted in cancer chemotherapy and prevention [8] using difluoromethylornithine (DFMO), an irreversible inhibitor of ODC [9]. DFMO depleted putrescine and spermidine with varying effect on spermine in cells [10,11]. Although DFMO as a single agent has shown modest therapeutic effects in early trials [12–14], a significant synergy has been reported for the combination of DFMO and other anticancer agents in preclinical studies [15,16]. DFMO is currently in clinical trials of neuroblastoma (clinicaltrials.gov identifier: NCT02679144, NCT02139397, NCT02559778) [16,17], and thus an assay to quantitate the changes in polyamines in samples from laboratory and clinical studies is needed to determine the efficacy of DFMO as an ODC inhibitor.

Various analytical methods, including thin-layer chromatography (TLC), ion-exchange chromatography (IEC), high-performance liquid

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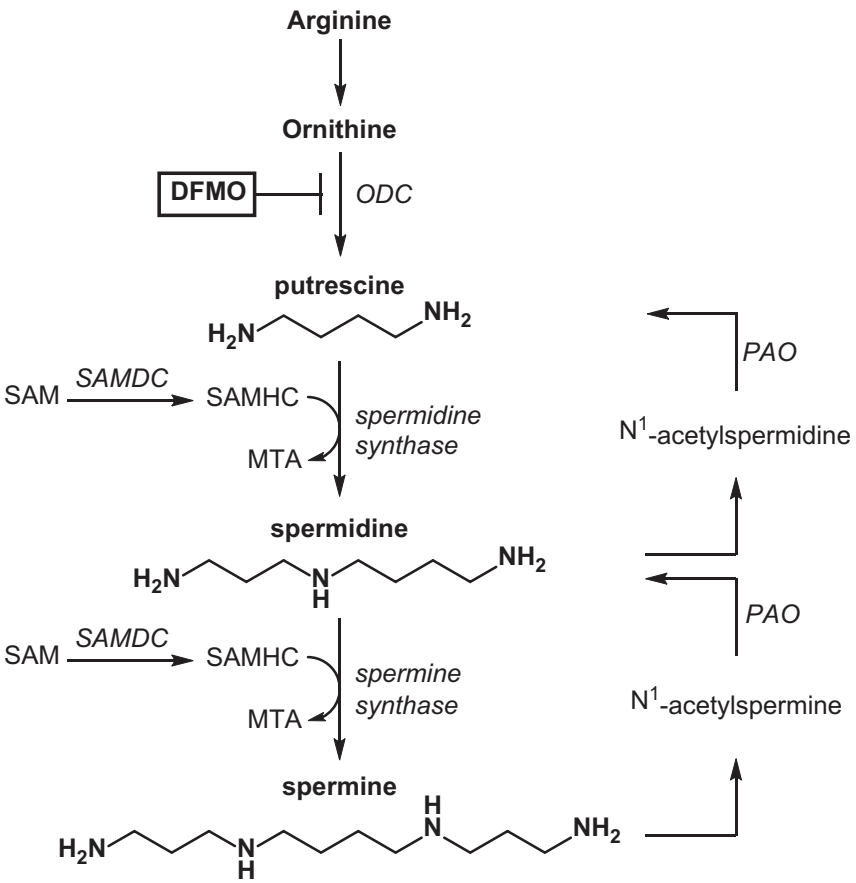

Fig. 1. Polyamine biosynthesis pathway. ODC, ornithine decarboxylase; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase; SAMHC, S-adenosylmethionine homocysteamine; MTA, 5′-deoxy-5′-methylthioadenosine; PAO, N^1-acetylpolyamine oxidase.

chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE) and immunoassays (ELISA), have been widely applied for the determination of polyamines in biological samples [18–20]. HPLC with pre- or post-column derivatization has been a preferred method due to the absence of chromophore or fluorophore groups in polyamines. However, the derivatization procedures have several disadvantages: time-consuming, low recovery, unstable products, and interfering by-products. HPLC coupled with mass spectrometry (MS) may overcome these issues and provide improved detection limits and specificity. Recently, several studies have detected underivatized polyamines by liquid chromatography–tandem mass spectrometry (LC–MS/MS), using volatile ion-pairing reagents such as trifluoroacetic acid (TFA), pentafluoropropanoic acid (PFPA) and heptafluorobutyric acid (HFBA) [21,22]. The ion-pairing reagents improved chromatographic separation and retention for polar compounds in reverse-phase HPLC. However, they significantly suppressed signal under the electrospray ionization (ESI) [23]. Furthermore, high conductivity and surface tension of the solvents for ion-pairing reagents may result in large spray droplets which will contaminate the MS ion source. The post-column addition of propionic acid and isopropanol (75:25, v/v) was tried, which increased the signal intensity by approximately 10-fold, but the analysis system is overly complicated and the limitations of ion-pairing remain [24]. More recently hydrophilic interaction liquid chromatography (HILIC) was attempted for the analysis of polar amines [25], but this method required high concentrations of buffer (> 20 mM) to decrease the peak tailing resulted from the secondary interaction with a residual silanol group of the stationary phase. Especially, the eluted peak of spermine was distorted even at higher concentrations of buffer.

A pH gradient ion-exchange chromatography has been applied in protein mixtures to determine ionic or ionizable compounds in the field of proteomics [26]. A separation of highly polar amines with cationic groups could also be adapted via ionic interactions with ligand exchange columns. Since the charge density on amines is pH-dependent, amines could be eluted from a column by increasing or decreasing the pH of elution buffer [26,27]. The pH gradient ion-exchange chromatography provides better chromatographic resolution compared with other methods by allowing the use of volatile elution buffer with acid supplemented with salt at lower concentrations.

In the present study, a sensitive and reproducible LC–MS/MS method using pH gradient was developed and fully validated for the determination of polyamines in cancer cells. The pH gradient from neutral to low pH was obtained in a multi-mode ODS column composed of weak ionic ligands and ODS ligands. The validated method was successfully applied to the study of polyamines in cancer cells treated with DFMO or vehicle control.

2. Experimental

2.1. Chemicals and reagents

Putrescine, spermidine and spermine were obtained from Sigma-Aldrich (St. Louis, MO, USA). 1, 6-diaminohexane (internal standard) was supplied by Acros Organics (Geel, Belgium). Ammonium acetate, ammonium formate and ethyl ether were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetic acid, formic acid, trichloroacetic acid, dimethyl sulfoxide (DMSO) and LC-MS-grade acetonitrile were purchased from Sigma-Aldrich (St Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Thermo Fisher Scientific (Richardson, TX, USA). Deionized water was prepared by using a Synergy UV water purification system (Millipore, Bedford, MA, USA).