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Polyamines in biological samples: Rapid and robust quantification by solid-phase extraction online-coupled to liquid chromatography–tandem mass spectrometry

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ABSTRACT

Polyamines are ubiquitous active biogenic amines which contribute to basic cellular functions. Hence, their quantification in samples of diverse biological origins is essential for understanding how they function, especially in disease-relevant conditions. We present here a robust, high-throughput solid-phase extraction online coupled to a liquid chromatography–tandem mass spectrometry (SPE–LC/MS/MS) approach for the simultaneous quantification of eight polyamines in various biological samples. The polyamines include 1,3-diaminopropane, putrescine, cadaverin, *N*-acetyl-putrescine, spermidine, spermine, N^1-acetyl-spermine, and L-ornithine. The novelty of the work is the use of two SPE columns online coupled to LC/MS/MS, which minimizes the sample pretreatment to a single derivatization step. The analysis is complete within 4 min, making the method highly suitable for routine clinical analysis and high throughput screenings. The method was fully validated with serum samples. Dynamic ranges were 0.03 to 15 μg/ml for ornithine and 1 to 500 ng/ml for other polyamines, which cover physiological concentrations in serum samples. Lower limits of quantification (LLoQ) were found to be between 0.1 and 5 ng/ml. As a proof of concept, we investigated gender differences in polyamine levels by analyzing the serum levels of 102 subjects.

1. Introduction

Polyamines are ubiquitous polycationic compounds associated with a variety of biological processes [1,2]. These active biogenic amines contribute to basic cellular functions, including cell growth, proliferation and differentiation, as well as regulating cellular stress-response and survival mechanisms. Spermidine and spermine are two polyamines reported to have anti-inflammatory as well as anti-oxidant activities [3], and have thus been implicated in cellular and organismal stress defense during aging and disease [4,5]. For instance, spermine was shown to provide protection from lethal sepsis in mice when applied intraperitoneally [6], and from paraquat-induced death in the fruit fly *Drosophila melanogaster* when added to ordinary food [7]. Indeed, spermidine supplementation extends life span and reduces markers of cell stress in various model organisms, including yeast, flies, worms and human immune cells [8], while polyamine-enriched food has been shown to reduce mortality and prolong the health span of aging mice [8,9]. The longevity-promoting effects of spermidine in lower model organisms requires the induction of autophagy, a degradation pathway that contributes to cellular self-renewal and cell protection [8,10,11]. In fact, we recently revealed spermidine to be a novel, conserved, and potent inducer of autophagy in model organisms, in human cell cultures, and in vivo in various tissues of mice [8,12]. On the other hand, polyamines can also be toxic to cells at high levels, and can facilitate cell death, mainly by oxidative mechanisms [13,14]. In this regard, polyamines are important clinical biochemical markers for malignancy [15]. This was first indicated in the urine of cancer patients, where polyamines were present at higher levels than in healthy humans [15].

To understand the relevance of changes in polyamine profiles detected in a given tissue, cell population or organism (obtained from, for instance, patient samples or basic research models), the biosynthetic activities of the polyamines, as well as their catabolic pathways, need to be determined. Hence, metabolic profiling of polyamines and their close derivatives is prerequisite for

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understanding the potentially complex changes in polyamine content that can be induced by disease-relevant conditions. For such metabolic profiling, an analytical method to accurately identify and quantify a broader group of polyamines and their derivatives in a single analysis would be most useful. Such an advance would also be significant for the potential clinical use of polyamines as biomarkers or pharmacological interventions.

The widespread interest in polyamines has led to the development of several analytical methods for polyamine determination [2,16]. In general, analysis of polyamines obtained from various biological samples is performed by chromatographic separation prior to detection with selective detectors or mass spectrometers [17–23]. Hyphenated techniques, such as LC/MS, GC/MS or capillary electrophoreses coupled to MS are among the most powerful methods for the analysis of metabolites [19–24]. Nonetheless, many published analytical methods suffer from at least one of the following drawbacks: they have long chromatographic run times; they are optimized for determination of polyamines only in one specific matrix; they have higher limits of detection; or they require time-consuming sample pretreatment steps [1,2,16,21,22,24–32].

Hence, there is a need for a more versatile technique that can be used across a range of matrices, so that levels in different tissues or fluids from a single person can be directly compared. Such comparisons between different tissue samples are important, because polyamines are not evenly distributed throughout the human body. For example, a number of polyamines have been shown to be raised in the serum of breast cancer patients, even when the urine shows normal levels [21].

The present study was undertaken to develop a robust and high-throughput analytical method for the simultaneous determination of various polyamines, at levels ranging from trace amounts to high concentrations. In particular, we aimed to design a universal method for quantification of polyamines in a broad range of biological matrices. We report here a novel analytical method applying two SPE columns on-line coupled to a highly sensitive and selective LC/MS/MS that allows the separation and unambiguous determination of eight polyamines in a broad variety of biological samples. Using two SPE columns in parallel means that sample preparation can be performed simultaneously with chromatographic separation and analyte detection, reducing the time for a whole single analysis to just 4 min. Since SPE sample preparation was performed online, sample pre-treatment was significantly accelerated by minimizing it to a single derivatization step before measurement. No further extraction or manual clean-up was necessary. The method is fully validated for human serum samples and could easily be extended to a higher number of polyamines if required.

2. Materials and methods

2.1. Chemicals

Biogenic amines, i.e. 1,3-diaminopropane (1,3-dap, ≥99%), putrescine (put, ≥99%), cadaverine (cad, ≥99%), *N*-acetylputrescine (*N*-actput, ≥98%), spermidine (spd, ≥98%), spermine (sp, ≥99%), N^1-acetyl-spermine (N^1-actsp, ≥97%), and L-ornithine (orn, ≥99%), along with four isotopically labeled internal standards d_8-putrescine (d_8-put, ≥99%), $^{13}C_5$-ornithine ($^{13}C_5$-orn, 95%), d_8-spermin (d_8-sp, 95%), and $^{13}C_4$-spermidine ($^{13}C_4$-spd, ≥95%), as well as acetonitrile and methanol (both HPLC gradient grade), trichloroacetic acid (TCA, ≥99%), sodium carbonate (≥99%), hydrochloric acid (fuming, 37%), acetic acid (99.8–100.5%), isobutyl chloroformate (≥99%), and human serum from clotted human male whole blood, sterile-filtered (mycoplasma tested, virus tested), were purchased from Sigma-Aldrich (Vienna, Austria). Phosphate buffered saline (PBS buffer, pH 7.2–7.3) was purchased from Apotheke LKH–Univ. Klinikum Graz, Austria. Ultra-pure water purified by a Milli-Q Gradient system (resistivity >18 MΩ cm; Millipore, Bedford, USA) was used for all experiments.

2.2. Preparation of standard solutions

Stock solutions of sp, *N*-actput, N^1-act, sp, orn, 1,3-dap, spd, put, and internal standard d_8-sp were prepared by dissolving the appropriate amount in water/methanol (7:3, v/v) to yield a concentration of 1 mg/ml for sp, *N*-actput, N^1-actsp, orn, 1,3-dap, 5 mg/ml for spd, and 0.5 mg/ml for put and d_8-sp. Stock solutions of internal standards (ISTD) d_8-put, $^{13}C_5$-orn, and $^{13}C_4$-spd were prepared in water/methanol (1:1, v/v) to achieve a concentration of 1 mg/ml. A stock solution of cad was prepared by dissolving the appropriate amount in water/acetonitrile (7:3, v/v) to give a concentration of 1 mg/ml. All stock solutions were stored at −80 °C.

The concentration of the calibration solution was generally varied from 1 to 500 ng/ml in order to cover as broad a physiological range as possible. Five non-zero calibration standards and one zero sample were used for calibration in each batch. In serum samples, however, the concentration of orn is typically higher than 500 ng/ml, so calibration solutions for orn were 0.03 to 15 μg/ml. The calibration solutions for serum samples were prepared by diluting all stock solutions, except orn, with water/methanol (7:3, v/v) to achieve an intermediate working concentration of 10 μg/ml. The stock solutions of orn and the working standard solutions were further diluted with water (double-distilled) to the desired concentration for the calibration solutions and three quality control (QC) samples. Five non-zero calibration solutions and one zero calibration solution were used for calibration in each batch. The QC samples were prepared from a different set of working stock than the calibration solution. QC concentration levels for orn were 0.5, 5, and 10 μg/ml and for the other polyamines 5, 50, and 100 ng/ml. ISTD stock solution was also diluted with water (double-distilled)/methanol (7:3, v/v) to achieve a working solution with a concentration of 6 μg/ml, and was further diluted with water (double-distilled) to prepare an ISTD mix solution with a concentration of 200 ng/ml. The isotopically labeled d_8-put was used as internal standard for *N*-actput, 1,3-dap, cad and put to calculate peak/area ratios. For sp and N^1-actsp, d_8-sp was used as internal standard. For Spd and orn, the internal standards $^{13}C_4$-spd and $^{13}C_5$-orn, respectively, were used.

2.3. Extraction and derivatization protocols

Polyamine extraction from biological sources is commonly performed by acid extraction with cold TCA or perchloric acid (PCA) with subsequent neutralization for analysis of cultured cells [33] and animal and plant tissue [34]. In general, homogenization is required for tissue, and a variety of standard techniques can be used (for a set of references see [34]). For tissues that are difficult to homogenize (e.g. plant tissues or chitin-rich insects), a protocol involving repeated freeze–thaw cycles may be beneficial, especially when larger sample numbers need to be processed [34]. Based on these methods [33,34], the following protocols were developed for the polyamine analyses described in this paper. Extraction protocols for serum, tissue, cell and whole-blood samples are summarized in Fig. 1. For tissue, cell or whole-blood samples, an individual dilution step was required before sample preparation, which enabled polyamine concentrations to fall within the linear calibration range. Table 1 shows the expected concentrations of polyamines in various tissues, organism or cells. Based on this concentration range, samples were individually diluted by using the dilution factor ***D*** specific for tissue, cell or whole blood. This dilution factor ***D*** was calculated for the analytes put, spd and sp. Sample