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Development of a fast and simple gas chromatographic protocol based on the combined use of alkyl chloroformate and solid phase microextraction for the assay of polyamines in human urine

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ABSTRACT

Polyamines are aliphatic amines with low molecular weight that are widely recognized as one of the most important cancer biomarkers for early diagnosis and treatment. The goal of the work herein presented is the development of a rapid and simple method for the quantification of free polyamines (i.e., putrescine, cadaverine, spermidine, spermine) and *N*-monoacetylated polyamines (i.e., N^1-Acetylspermidine, N^8-Acetylspermidine, and N^1-Acetylspermine) in human urine. A preliminary derivatization with propyl chloroformate combined with the use of solid phase microextraction (SPME) allowed for an easy and automatable protocol involving minimal sample handling and no consumption of organic solvents. The affinity of the analytes toward five commercial SPME coatings was evaluated in univariate mode, and the best result in terms of analyte extraction was achieved using the divinylbenzene/carboxen/polydimethylsiloxane fiber. The variables affecting the performance of SPME analysis were optimized by the multivariate approach of experimental design and, in particular, using a central composite design (CCD). The optimal working conditions in terms of response values are the following: extraction temperature 40 °C, extraction time of 15 min and no addition of NaCl. Analyses were carried out by gas chromatography-triple quadrupole mass spectrometry (GC-QqQ-MS) in selected reaction monitoring (SRM) acquisition mode. The developed method was validated according to the guidelines issued by the Food and Drug Administration (FDA). The satisfactory performances reached in terms of linearity, sensitivity (LOQs between 0.01 and 0.1 μg/mL), matrix effect (68–121%), accuracy, and precision (inter-day values between −24% and +16% and in the range 3.3–28.4%, respectively) make the proposed protocol suitable to be adopted for quantification of these important biomarkers in urine samples.

1. Introduction

One of the crucial aspects to fighting cancer in an effective manner is early diagnosis and rapid treatment of this disease. In this respect, the development of new, rapid, and cheap tumor diagnostic techniques represents one of the main targets of recent clinical research. Several studies have been focusing on the discovery of cancer biomarkers because their identification can be of crucial importance in early detection programs. Indeed, a high concentration of these molecules or in some cases just their detection provides information on tumor occurrence, type of cancer, and disease progression at very early stages [1].

Polyamines are aliphatic amines with low molecular weight, which are essential for normal growth and cellular differentiation [2]. The marked increase of the biosynthesis of polyamines has been associated with rapid tumor growth, resulting in the increase of their level in urine and plasma [3]. Because of these findings, it is widely recognized that polyamines are among the most important cancer biomarkers for early diagnosis and treatment [4]. Putrescine, cadaverine, spermidine, and spermine are the major polyamines in cells and are present in almost all living organisms. Polyamines can also exist as conjugate forms in cells and biofluids, in particular as acetylated forms. These compounds including *N*-acetylputrescine, *N*-acetylspermine, and *N*-acetylspermidine were supposed to be

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the products of polyamine metabolic pathway catalyzed by acetyl-CoA and spermidine/spermine *N*-acetyltransferase [5].

The extensive interest for polyamines has led to the development of several analytical methods mainly based on chromatographic separation [6–8]. Typical analytical approach relies on high-performance liquid chromatography (HPLC) with optical [9–12] or mass spectrometric detection (MS) [13–19] as well as gas chromatography (GC) with MS [20–25] or traditional detectors [26–30]. Gas chromatography is an established analytical approach in several analytical fields because of its robustness, high capability in peak separation, low running costs, and relative ease of operation. However, for some classes of compounds such as amines, a prior derivatization step with suitable reagents is mandatory before gas chromatographic analysis so as to improve chromatographic elution and resolution by decreasing volatility and polarity of analytes. Various derivatization methods have been developed, such as perfluoroacylation [3], alkylsilylation [22], and alkoxycarbonylation [20,21,24]. Unlike perfluoroacylation and alkylsilylation that require multiple tedious and time consuming steps for sample purification, alkoxycarbonylation allows for derivatization of amines directly in aqueous matrices without the requirement of heating the solution, thereby simplifying the sample pretreatment and the derivatization procedure [31]. Our research group has long been using alkoxycarbonylation in method development and have recently demonstrated the compatibility of this derivatization approach with the use of solid phase microextraction (SPME) in environmental and clinical studies [32].

Urine is one of the most commonly used matrices in bioanalysis because it is relatively easy to collect and its sampling is non-invasive. However, when urine samples have to be analyzed, a suitable sample preparation is required because matrix compounds may interfere with the analysis, especially when the target compounds are present at trace levels. Several strategies have been proposed to extract polyamines from urine specimens including solid phase extraction (SPE) [12,28], liquid–liquid extraction (LLE) [3,11,15–17,19,20,22], single hollow fiber supported liquid membrane (SLM) extraction [9], and ion exchange resin procedure [13]. Most of these approaches require many operation tasks, which make sample preparation very time and labor consuming. Furthermore, they are not optimal for the routine clinical usage because of a number of issues such as those related to organic solvent usage, waste management, low automation, and throughput possibilities. In recent years, many strategies in the simplification and miniaturization of the extraction techniques were developed to overcome these concerns in sample preparation. SPME is a well-known microextraction technique that allows for the simultaneous extraction and preconcentration of the analytes in a single step, thus minimizing operational errors. When SPME is employed in association with gas chromatography, the whole procedure can be carried out in the absence of organic solvents because the analytes are directly desorbed into the injector port of the gas chromatograph. Moreover, the relative ease of online coupling to chromatographic system enables the complete analysis process to be automated [33].

The normal range of polyamine concentration is analyte dependent and in some cases it is quite broad. For instance, according to literature, normal values for spermidine are 0.37 μg/mL [8], 0.10 μg/mL [34] and 0.14 μg/mL [22]. However, a quite thorough normal range of concentration for the investigated polyamine is between 0.1 and 1 μg/mL.

The goal of the work herein presented was the development of an easy and rapid method for the analysis of polyamines in human urine by SPME-GC–MS/MS after a derivatization step with alkyl chloroformate. To the best of our knowledge, this is the first study based on SPME for the quantification of polyamines in a biological matrix. The combined use of alkyl chloroformate as derivatizing reagent and SPME for analyte extraction was chosen to develop a simple protocol involving minimal sample handling and no consumption of toxic organic solvents. The variables affecting the derivatization reaction and the SPME analysis were optimized by the multivariate approach of experimental design. Moreover, this work proposes for the first time the use of GC-QqQ-MS for the determination of polyamines. Tandem mass spectrometry provides enhanced selectivity and sensitivity compared to the mass spectrometry with single quadrupole analyzer and, at the same time, maintains the capability of analyte identification unchanged. These features were exploited to achieve a high specific protocol capable of unambiguous identification of the analytes and their reliable assay.

2. Experimental

2.1. Chemicals and materials

Putrescine (Put), cadaverine (Cad), spermidine (Spd), spermine (Spm), and N^1-Acetylspermine trihydrochloride (N^1-AcSpm) were purchased from Fluka (Milan, Italy). N^8-Acetylspermidine dihydrochloride (N^8-AcSpd), and N^1-Acetylspermidine hydrochloride (N^1-AcSpd) were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA) and Cayman Chemical Company (Ann Arbor, Michigan, USA), respectively. 1,6-diaminohexane dihydrochloride (1,6-DAH) and spermidine-(butyl-d8) trihydrochloride (Spd-d_8), used as internal standards, were bought from Sigma Aldrich (Milan, Italy). Propyl chloroformate, sodium phosphate dibasic, and sodium phosphate tribasic dodecahydrate were purchased from Sigma-Aldrich (Milan, Italy). The surveyed solid phase microextraction fibers, namely polyacrylate 85 μm (PA), carboxen/polydimethylsiloxane 85 μm (Car/PDMS), divinylbenzene/carboxen/polydimethylsiloxane 50/30 μm (DVB/Car/PDMS), polydimethylsiloxane/divinylbenzene 65 μm (PDMS/DVB), and polydimethylsiloxane 100 μm (PDMS), were purchased from Supelco (Bellefonte, PA, USA) and conditioned as recommended by the manufacturer. Aqueous solutions were prepared using ultrapure water obtained from a Milli-Q plus system (Millipore, Bedford, MA). Synthetic urine (negative urine control) produced from Cerilliant (proprietary composition; Round Rock, TE, USA) was commercialized by Sigma-Aldrich.

2.2. Instrumentation and apparatus

GC–MS analyses were carried out using a TSQ Quantum GC (Thermo Fischer Scientific) system constituted by a triple quadrupole mass spectrometer (QqQ) Quantum and a TRACE GC Ultra equipped with a TriPlus autosampler. Chromatographic separation of the analytes was performed using a Restek Rxi-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, 95% polydimethylsiloxane, 5% polydiphenylsiloxane). The GC oven temperature was initially held at 70 °C for 3 min, then ramped at 15 °C/min to 300 °C, and held at this temperature for 10 min. The carrier gas was helium (purity 99.999%) at 1 mL/min, whereas argon (purity 99.999%) at a pressure of 2.3 mTorr was used as collision gas. A Thermo PTV straight Liner 0.75 × 2.75 × 105 mm was used in the GC injector. Analyses were performed in splitless mode with the injector temperature set at 270 °C. The QqQ mass spectrometer was operated in electron ionization (EI). The preliminary analyses for the identification of the derivatized analytes were performed in full scan mode, whereas analyte quantification was carried out in selected reaction monitoring (SRM) mode. The transfer line and ionization source temperatures were set at 280 °C and 250 °C, respectively. The emission current was set at 25 μA. The scan width and peak width of Q1 were set at *m*/*z* 1 and 0.7 amu for

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