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Determination of urinary biogenic amines' biomarker profile in neuroblastoma and pheochromocytoma patients by MEKC method with preceding dispersive liquid–liquid microextraction

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ABSTRACT

The unbalanced secretion of biogenic amines (BAs) is considered to be a relevant biochemical biomarker in the screening for neuroendocrine tumors, such as: neuroblastoma and pheochromocytoma. However, there is still a need to improve the bioanalytical procedures for BA determination in biological samples due to their instability (photo- and thermosensitivity, easy oxidation) and low concentration in the body fluids.

In this study, the primary analytical challenge was to optimize the method of extraction of seven compounds from among BAs and their precursors from urine samples. Several methods based on liquid–liquid extraction (LLE) or solid phase extraction (SPE) techniques were tested. By optimization of the extraction and data analysis using chemometric tool, the dispersive liquid–liquid microextraction (DLLME) has been chosen due to its low solvents consumption, high efficiency of isolation, preconcentration and suitable clean-up of biological matrix. Further, α-cyclodextrin-modified micellar electrokinetic chromatography (MEKC) with ultraviolet detection (UV) has been applied for quantification of the analyzed biologically active compounds with limits of detection (LOD) and limits of quantification (LOQ) at 0.15 and 0.5 μg mL^{-1}, respectively. Finally, the optimized and validated DLLME-MEKC-UV method has been employed for the analysis of real urine samples, obtained from 6 children with neuroendocrine tumors and 6 healthy children. It was stated that concentrations of BA could serve to differentiate between the patients and healthy children. This pilot study indicates that the elaborated fast and sensitive DLLME-MEKC-UV method for determination of panel of biomarkers could be successfully applied in everyday clinical practice to help to confirm the clinical diagnosis of neuroendocrine tumors in children.

1. Introduction

Recently, numerous factors responsible for the development, growth and progression of cancers have been discovered. They include specific genes and markers (like proteins, lipids and small molecules) which create the complex signaling pathways within cancer cells [1,2]. Currently it is well known that these signaling pathways communicate between each other creating specific networks. Their complexity, resulting from a large number of interacting molecules, cause great difficulties to predict the role and biochemical relevance of particular substances secreted by the tumor [1–3]. Therefore, the simultaneous determination of several molecules rather than examining of each compound and/or pathway alone should be implemented. Such approach could help to discover novel cancer biomarkers or possible molecular targets for new therapeutic modalities. Especially for cancers which are rare and difficult to be detected in early stages of disease, it is of utmost importance to search for new biomarkers useful in early diagnosis, prognostication and treatment monitoring.

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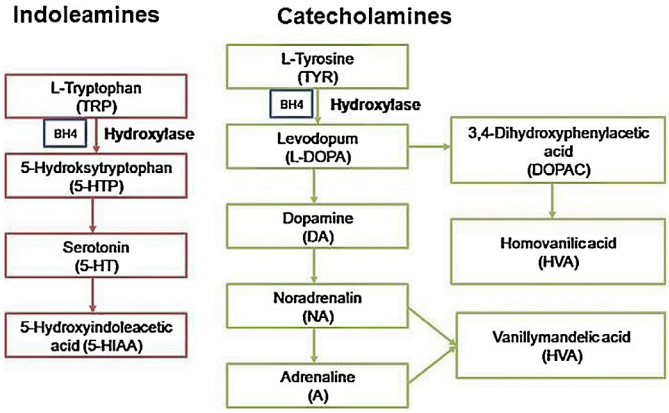

Fig. 1. The monoamine synthesis and metabolism pathway.

Neuroendocrine tumors (NETs) are a group of neoplasms, which are ideal candidates for investigation of released biomarkers. NETs are rare tumors, originating from neuroectoderm, which can arise in almost every part of the body. They are usually asymptomatic or can present with symptoms related to the biomarkers secreted by the cancer cells. However, not all NETs release biogenic amines (BAs) in sufficient quantities to cause clinically visible symptoms. Also, the secretion of the biomarkers varies with time and maybe obscured by many unspecific factors [4].

The most common NET, which develops in infancy and early childhood, is *neuroblastoma* (NBL). It is the second most frequent extracranial malignant solid tumor in children, constituting the second cause of childhood mortality in developed countries [5]. Most NBL are located within the abdomen, arising from adrenal glands or prevertebral spinal sympathetic ganglia. Neuroendocrine cells of adrenal medulla receive sympathetic input and release catecholamine neurotransmitters to the systemic circulation and the symptoms of the overproduction of these neurotransmitters are observed in a proportion of cases. They include: hypertension, sweating, weight loss, however these symptoms are not specific for NBL [6]. Diagnostics of NBL is complex and requires not only biochemical, but most importantly histopathological and genetic tests to determine the precise tumor type and its biological aggressiveness.

It should be noticed that the ideal procedure for the tumor detection should be low cost, not invasive, easy accessible and straightforward to limit the time needed for the diagnosis [7]. Moreover, screening for various molecules (called biomarkers) secreted by NBL and released to the body fluids could serve as such not invasive tool. However, for many malignancies, such as NETs, the available biomarkers are often not sufficiently sensitive and/or specific for the detection of the disease in its early stage. Also the determination of only one compound may not reflect the status of the cancer. More information could be gained from simultaneous screening of several differentially secreted compounds – a panel of biomarkers [4,8,9]. In case of NBL, the biomarkers recommended by the diagnostic protocols include: vanillylmandelic acid (VMA) and homovanilic acid (HVA). Also other components of the catecholamines pathway, such as: dopamine (DA), levodopa (L-DOPA), noradrenaline (NA) and adrenaline (A) might be of value as NBL biomarkers (Fig. 1). This is due to the fact that the pathway of synthesis of catecholamines may be incomplete, especially in the less mature tumor cells [10,11]. In such conditions intermediate products of catecholamines synthesis, such as L-DOPA, are released. In turn, the mature cells of NBL often produce and release the final products of synthesis of catecholamines: A and NA. Moreover, the correlations between selected catecholamines and their metabolites, such as: the VMA to HVA and DA to VMA ratios are of great importance. Moreover, also increased excretion of A, NA, DA, L-tyrosine (L-Tyr), and L-DOPA have been found in NBL [12]. Also L-tryptophan (L-Tryp) might be a biomarker of NBL. There is a significant connection between L-Tyr and L-Tryp: and there are great similarities of the rate-limiting enzymes of those amino acids (AA) important for neuroendocrine tumors' metabolic pathways conversion of tyrosine to catecholamines and L-Tryp to serotonin (5-HT). With few exceptions, the mechanism of action of tyrosine hydroxylase and tryptophan hydroxylase and their tertiary structure of the catalytic domains are nearly identical. Moreover, tyrosine hydroxylase is able to catalyze the hydroxylation of tryptophan in addition to tyrosine [13,14].

It has been confirmed that BAs determination in body fluids allows to identify the NETs. In 90% of NBL cases, the elevated catecholamines and/or their metabolites levels are detected in urine and serum samples [10]. The amount of particular analyzed compound secreted to the urine depends on the NBL stage, its differentiation and the efficiency of the catecholamine storage mechanism within tumor cells.

Therefore, the simultaneous determination of BAs and their main metabolites in body fluids might be of major usefulness in clinical diagnosis and monitoring of NBL course [15]. Urine samples are nowadays the main source of BAs available for analysis due to the BAs' longer biological half-life in comparison to their plasma half-lives [16].

The wide dynamic range and low urine concentration of BAs require the use of highly sensitive and selective detection and separation techniques. Several studies were focused on capillary electrophoresis (CE) and liquid chromatography (LC) separation of the BAs with the use of various detectors, such as: mass spectrometry (MS), spectrophotometry, as well as electrochemical and fluorescence detectors [8,17–21]. Recently, CE has become a good alternative to LC because of CE's simplicity, higher efficiency, lower expenditure of samples and chemicals, and better matrix tolerance [22]. In terms of neurotransmitters detection, the electrochemical detector has been usually employed to LC, but this method depends on the mobile phase composition, circumscribed ion-paring reagents content and needs extensive cleaning between analyses of complex matrices [15]. To the contrary, the UV detector, which is widely available in laboratories, could be applied successfully for BAs analyses. Therefore our previously published optimized micellar electrokinetic chromatography (MEKC) with UV detector method was applied in this study for the separation of chosen BAs: A, NA, DA, 5-HT, L-DOPA and their precursors: L-Tryp and L-Tyr.

We had previously reported that this method allowed for efficient separation of all chosen BAs and their main acidic metabolites: HVA, VMA and 3,4-dihydroxyphenylacetic acid (DOPAC), but extraction of only acidic metabolites was carried out [23]. Therefore, the main goal of the present study was to optimize the effective method for extraction of other BAs and their precursors from urine samples. For this propose, several new and already published BAs' sample preparation methods based on liquid–liquid extraction (LLE), solid phase extraction (SPE) techniques and the dispersive liquid–liquid microextraction (DLLME) were tested. In this paper, for the first time we propose the DLLME procedure in combination with MEKC-UV as the most effective approach for the quantification of seven selected BAs in urine samples. Further, the unquestionable advantages of the DLLME over LLE or SPE for the isolation of BAs for MEKC-UV detection have been confirmed. Moreover, the elaborated DLLME-MEKC-UV method has been validated for each of the compound of interest and applied for the analysis of urine samples obtained from the patients suffering from NBL and other NETs and from healthy volunteers participating in the study.