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Development of a new HPLC method using fluorescence detection without derivatization for determining purine nucleoside phosphorylase activity in human plasma

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ABSTRACT

Purine nucleoside phosphorylase (PNP) activity is involved in cell survival and function, since PNP is a key enzyme in the purine metabolic pathway where it catalyzes the phosphorolysis of the nucleosides to the corresponding nucleobases. Its dysfunction has been found in relevant pathological conditions (such as inflammation and cancer), so the detection of PNP activity in plasma could represent an attractive marker for early diagnosis or assessment of disease progression. Thus the aim of this study was to develop a simple, fast and sensitive HPLC method for the determination of PNP activity in plasma. The separation was achieved on a Phenomenex Kinetex PFP column using 0.1% formic acid in water and methanol as mobile phases in gradient elution mode at a flow rate of 1 ml/min and purine compounds were detected using UV absorption and fluorescence. The analysis was fast since the run was achieved within 13 min. This method improved the separation of the different purines, allowing the UV-based quantification of the natural PNP substrates (inosine and guanosine) or products (hypoxanthine and guanine) and its subsequent metabolic products (xanthine and uric acid) with a good precision and accuracy. The most interesting innovation is the simultaneous use of a fluorescence detector (excitation/emission wavelength of 260/375 nm) that allowed the quantification of guanosine and guanine without derivatization. Compared with UV, the fluorescence detection improved the sensitivity for guanine detection by about 10-fold and abolished almost completely the baseline noise due to the presence of plasma in the enzymatic reaction mixture. Thus, the validated method allowed an excellent evaluation of PNP activity in plasma which could be useful as an indicator of several pathological conditions.

1. Introduction

Purine nucleoside phosphorylase (PNP- EC 2.4.2.1) is a ubiquitous enzyme that plays a key role in the metabolism of purines. PNP catalyzes the phosphorolytic cleavage of the glycosidic bond of purine nucleosides to generate the corresponding nucleobases and ribose-1-phosphates [1] (Fig. 1). Structurally, PNP can be divided in two main classes: trimeric and hexameric. The homotrimeric forms are specific for 6-oxopurine nucleosides namely inosine (INO) and guanosine (GUO) and are present in mammalian cells and in some microorganisms. The homohexameric forms are mainly present in prokaryotes, and are able to cleave both 6-oxopurine and 6-aminopurine nucleosides (such as adenosine) but with some differences [1–6].

Abbreviations: PNP, purine nucleoside phosphorylase; Pi, inorganic phosphate; INO, inosine; GUO, guanosine; UA, uric acid; HYPO, hypoxanthine; GUA, guanine; XAN, xanthine; NaOH, sodium hydroxide; K_2HPO_4, dipotassium phosphate; KH_2PO_4, monopotassium phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; DAD, diode array detector; PFP, pentafluorophenyl; QC, quality control; LOD, limit of detection; LOQ, limit of quantification; *r*, correlation coefficient.

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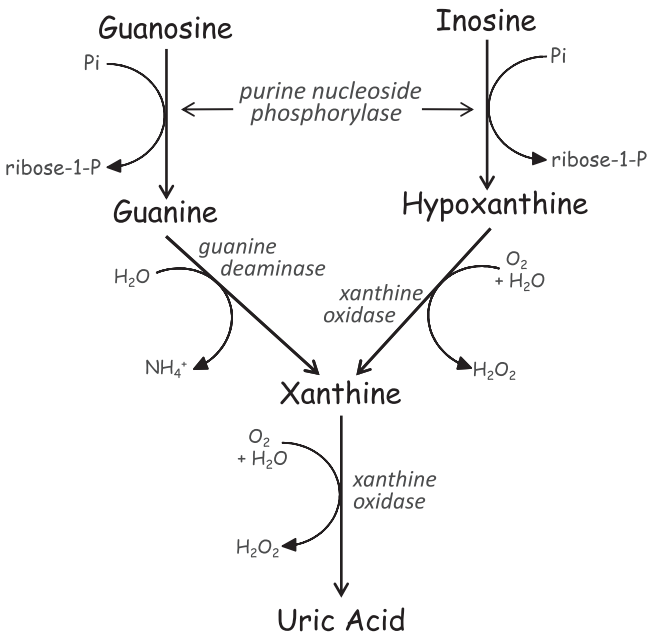

Fig. 1. Schematic presentation of the enzymatic pathways involved in degradation of purine nucleosides.

PNP activity is crucial in the purine salvage pathway, since it allows the cells to utilize purine bases to produce nucleotides thus avoiding the ex-novo synthesis, which is energetically expensive. Thereby, PNP activity is essential for cell survival and function while its abnormal activity is involved in different diseases. Indeed, PNP defect in humans leads to a selective T cell immunodeficiency associated with neurological abnormalities and/or autoimmune diseases [7,8]. On the contrary, PNP mRNA expression levels are significantly upregulated in several tumor cells [9–11]. Furthermore, Sanfilippo et al. [12] have found a relationship between tissue PNP and biological aggressiveness in human colon carcinoma.

Therefore, altered PNP activity in biological fluids could be considered an attractive indicator of various pathological conditions either to facilitate an early diagnosis or to follow the disease progression. Among the biological matrices, plasma and serum are commonly used for clinical studies. Roberts et al. [13] showed that plasma PNP activity was higher in patients with different types of tumors. Vareed et al. [14] found elevated levels of PNP in pancreatic ductal adenocarcinoma and altered serum levels of some purines, such as GUO, that could be used as markers to monitor the progression from inflammation to pancreatic adenocarcinoma. Furthermore, PNP activity may add information to the current assessment of liver toxicity in studies of compounds in development [15].

However, in spite of these good premises, studies evaluating PNP activity from blood are still few. The most frequent analytical methods used include radiochemical [16,17], colorimetric and spectrophotometric methods [18,19], HPLC [20], and capillary electrophoresis [21]. There are several limitations on the use of these methods, especially for their sensitivity. Indeed, Lopez-Cruz et al. [22], using a colorimetric assay, found that PNP activity in human plasma samples was below the detection limits. One of the most frequently used method is the spectrophotometric one. There are two main variants of this assay. The first is a coupled assay in which, xanthine (XAN) oxidase is added to the reaction mixture to convert the hypoxanthine (HYPO) formed by the PNP reaction to uric acid (UA) (Fig. 1), that is easily detected at 293 nm, bypassing the small differences in spectral properties between INO and HYPO [19]. However, this assay can generate several problems as discussed by Yamamoto et al. [20] who considered it insufficient to measure the low PNP activity in plasma, especially turbid plasma. Moreover, due to its low solubility, the formation of high amounts of UA in the reaction mixture can modify the limpidity of the medium interfering with the spectrophotometric measurement. The second method is a direct assay in which GUO is used as substrate and the formation of guanine (GUA) is directly monitored by the absorption at 252 nm [19], but also in this case spectral properties of these two compounds are very similar. To overcome these problems, HPLC assays have been developed to directly assess the amount of newly formed products (HYPO or GUA). UV detection is a very commonly used detector for HPLC analysis, but it is not very sensitive, especially when purine nucleosides and nucleobases are determined in biological samples [23]. Hence, to improve the sensibility and to reduce interferences, a fluorescence detector can be used. Until now, the fluorescence-based method for detecting purine compounds requires their conversion into fluorescent derivatives. The most widely used fluorogenic reagent is chloroacetaldehyde which forms fluorescent 1,N^6-ethenoderivatives of the adenylate compounds [24,25], but is unable to react with INO [26] thus resulting unsuitable to monitor the activity of PNP. Since GUA, GUO and their nucleotides can be converted into fluorescent derivatives by reaction with phenylglyoxal, PNP activity can be evaluate by an HPLC-based fluorometric assay using GUO as substrate. However, the disadvantage of many derivatization reactions is that the fluorescent products are often unstable. Indeed, Maes et al. [27] prefer to use the native fluorescence of acyclovir and ganciclovir, two nucleoside analogues derived from GUA, rather than to take advantage of their derivatization with phenylglyoxal, which did not yield linear calibration curves. Many years ago, Udenfriend and Zaltzman [28] demonstrated that numerous purine compounds do emit appreciable natural fluorescence but with some differences: the fluorescence of adenine and its derivatives is relatively weak, while GUA, its nucleoside and nucleotides and some of its methylated derivatives represent the most intensely fluorescing compounds among purines.

Thus, this fluorescence could be used to develop new methods for the assay of guanine-based purines, as here described. In particular, we have developed a fast, simple and sensitive method to evaluate PNP activity, combining the advantage of HPLC with that of the fluorescence-based detection of GUO and GUA, the natural substrate and product of PNP reaction, respectively. To demonstrate its suitability for biological samples, we used human plasma from a pool of healty donors to avoid any interference from diseases and to assess the magnitude of the plasma PNP activity in healthy subjects. This new technique should enable an easy determination of PNP activity in plasma that, in turn, could be used as an indicator of several pathological conditions.

2. Materials and methods

2.1. Materials

Chemical standards of GUO, GUA, INO, HYPO, XAN and UA were all of analytical grade and purchased from Sigma–Aldrich (Milan, Italy). HPLC-grade (>99.8%) methanol, water with 0.1% formic acid (LC–MS CHROMASOLV), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), sodium hydroxide (NaOH), dipotassium phosphate (K_2HPO_4) and monopotassium phosphate (KH_2PO_4) were from Sigma–Aldrich. Ultrapure water was obtained by means of a MilliQ apparatus by Millipore (Molsheim, France) and was used in the preparation of the samples and buffer solutions. Human plasma was purchased from Biopredic International (Rennes, France).