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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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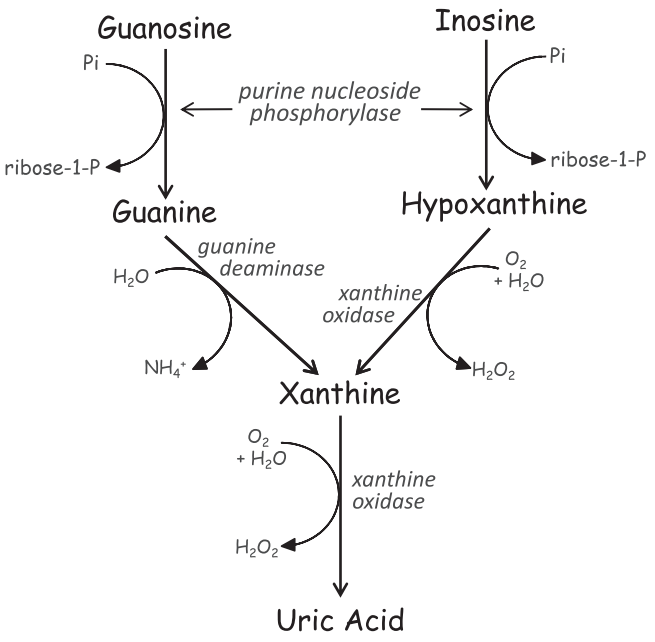

Fig. 1. Schematic presentation of the enzymatic pathways involved in degradation of purine nucleosides.

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 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, xanthine (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).

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2.2. HPLC equipment and conditions

The chromatographic apparatus was an Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) equipped with a degasser (G1379A), a quaternary pump (G1311A), an autosampler (G1313A), a thermostated column compartment (G1316A), a Diode Array Detector (DAD) (G1315B) and a fluorescence detector (G1321A). Instrumental control and data processing were performed using EZChrom Elite software (Agilent Technologies). Chromatographic separation was achieved using a Phenomenex Kinetex Pentafluorophenyl (PFP) (5 μm pore size, 100 Å particle size, 250 × 4.6 mm) analytical column fitted with a Phenomenex Security Guard System containing a PFP (for 4.6 mm ID) pre-column (Phenomenex INC, Castelmaggiore, Bologna, Italy), kept at 35 °C.

The analyte separation was obtained using 0.1% (v/v) formic acid in water (mobile phase A) and methanol (mobile phase B) under the following gradient elution: from 0 to 9 min an isocratic step at 100% A, from 9 to 10 min a linear increase from 0 to 50% of B, from 10 to 14 min an isocratic step at 50% of B, and subsequently a flushing step with an increase to 100% B from 14 to 15 min followed with a return to 0% of B within 3 min. The column was reconditioned at 100% A (initial condition) in order to equilibrate the analytical column before the next run. The flow rate of the mobile phase was set at 1 ml/min while the injection volume was 10 μl. GUO and GUA have been detected using either fluorescence and UV detector while INO, HYPO, XAN and UA with UV detector. The fluorescent compounds were monitored at an excitation wavelength of 260 nm and an emission wavelength of 375 nm whereas the UV based detection was performed at 254 nm for all the analytes except UA at 290 nm. Purine compounds were identified and quantified by comparison with pure external standards.

2.3. Preparation of calibration standards, quality control and calibration curves

Separate stock standard solutions of each purine compound were prepared in ultrapure water at the concentration of 10 mM adding appropriate amounts of NaOH to improve their own solubility. Aliquots of each stock solution were stored at −20 °C.

To establish HPLC retention time of GUO, GUA, INO, HYPO, XAN, UA, a working standard solution of each compound was prepared by diluting each stock solution in the assay buffer (HEPES 50 mM, pH 7.0). Moreover, equal volumes of the different stock solutions were mixed and diluted with the assay buffer to obtain 1 mM final mixture of each standard. Further serial dilutions of this standard mixture were carried out to achieve the appropriate standard concentrations used to construct calibration curves. Quality control (QC) samples were prepared from the standard mixture at three concentration levels named low, middle and high corresponding to 5, 75 and 150 μM respectively, and were used to evaluate intra- and inter-day precision and accuracy. The calibration standards and QC were made freshly and injected at least in duplicate. A calibration curve consisted of a sample containing only the enzymatic assay buffer (HEPES 50 mM, pH 7.0) and a series of standard samples containing the assay buffer with the investigated analytes. A minimum of ten different concentrations of the standard mixture were analyzed in duplicate in each run.

2.4. Method validation

The developed analytical method was validated for linearity, range, limit of detection and quantification (LOD and LOQ respectively), specificity, intra and inter-day precision and accuracy in agreement with ICH Harmonised Tripartite Guideline [29].

Calibration curves were constructed by plotting the peak areas of each analyte versus their concentrations. To determine the linearity of the method, the regression parameters, including correlation coefficient (r), slope and intercept were evaluated. Minimally acceptable r for the calibration curves was at least 0.99. For each analyte, the value of LOD and LOQ were calculated as the lowest concentration which give rise to a signal-to-noise ratio of 3 and 10, respectively.

The specificity is the ability to differentiate and quantify the analyte of interest in presence of other components, which could be present in the sample. Thus, the degree of separation of the different analytes of interest from potentially interfering substances (other than the possible endogenous purine nucleosides and nucleobases found in plasma) was evaluated in the enzymatic assay buffer containing only small aliquots of human plasma without substrate (see below in Section 2.5).

The intra- and inter-day precision and accuracy of the method was calculated by analyzing the QC samples at the three different concentrations reported above. ICH Harmonised Tripartite Guideline [29] defines the intra-day precision (also termed repeatability) as the precision under the same operating conditions over a short interval of time (same day). For inter-day precision (also named intermediate precision), the QC samples were assayed over at least three different days. The precision was expressed as percent coefficient of variation (% CV). The accuracy of an analytical method is defined as the closeness of agreement between the nominal concentration and the concentration found and is expressed as a percentage value.

2.5. Purine nucleoside phosphorylase activity

The activity of PNP present in the human plasma was evaluated measuring the conversion of the substrate, GUO, into its product GUA. In the enzymatic reaction, Pi, formed from a mixture of K_2HPO_4 plus KH_2PO_4 to obtain pH 7.0, was used as co-substrate. The reaction mixture, composed of 50 mM HEPES pH 7.0 plus 50 mM Pi, was incubated at 37 °C for 2 min before the initiation of the enzyme reaction. Various volume of plasma, as source of PNP, were added to the reaction mixture and the enzyme activity was started by the addition of different GUO concentrations. The final reaction volume was 200 μl. The mixture was then incubated by shaking at 37 °C for different incubation times (from 7.5 to 30 min). For kinetic studies, eight different concentrations of substrate were used: 1, 5, 10, 25, 50, 100, 250 and 500 μM. The reaction was stopped by heating the mixture at 70 °C for 5 min and the precipitated proteins were removed by centrifugation. The supernatant was filtrated with 0.2 μm filters (Millipore, Vimodrome, Italy) before injection in the HPLC system. Reaction mixtures, to which only substrate or plasma were added, were run and served as blanks. Each sample was run in duplicate in at least three independent experiments.

2.6. Statistical analysis

Data were expressed as the mean ± SEM and results were analyzed with GraphPad Prism 6 (La Jolla, California, USA) or Microsoft Excel.

3. Results and discussion

3.1. HPLC separation and detection

Since we were interested in evaluating PNP activity in blood and it is known that PNP metabolizes INO and GUO into their respective nucleobases (HYPO and GUA, respectively) that, in turn, can be transformed into XAN and then UA by enzymes present in blood [30,31] (Fig. 1), the aim of this study was to develop an HPLC method able to simultaneously assay purine nucleosides (i.e. INO and GUO), and nucleobases (i.e. HYPO, GUA and XAN), as well as UA.

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Our method showed several features useful to ensure an adequate separation of the different purine compounds, improving the detection sensitivity towards the different analytes. Firstly, since purines are heterocyclic aromatic organic compounds, we chose a Phenomenex Kinetex PFP column to separate purine nucleosides and nucleobases given the high performance of this column for aromatic and halogenated compounds. Thus, using RP-HPLC technique, analytical chromatography was performed applying a gradient elution (described in Section 2; subsection 2.2) and a flow rate of 1 ml/min. As expected, the samples containing only the assay buffer (HEPES 50 mM, pH 7.0), without analytes, always showed a flat baseline without any chromatographic peak when recorded using both UV and fluorescence detectors. Because of their structural similarity, the separation of INO and GUO and mainly of HYPO and GUA is still often a problem with RP-HPLC technique [32]. However, using this new method, the GUO and INO peaks, as well as those for GUA and HYPO were well resolved and completely separated (Fig. 2A). Also UA was separated from the other analytes while the peak of XAN was close to that of HYPO but still adequately separated. Overall, the retention time of the analytes, including the last two ones, was sufficiently distinct, allowing the simultaneous detection of all the investigated compounds. Furthermore, using the UV-DAD detector, all selected purines, except UA, were detected at UV wavelength of 254 nm, whereas, due to its absorption spectrum, UA peak was recorded at 290 nm thus improving the sensitivity of the assay for this compound (Fig. 2A and B).

As fluorimetry is often chosen for its high sensibility and specificity and given that GUA and related derivatives are the most fluorescent compounds among purines [28], we were also interested in developing an HPLC-fluorescence detection assay to improve the sensitivity of the method. Since guanine-based purines are more fluorescent under acidic conditions, a solution of 0.1% (v/v) formic acid in water, with pH 2.6–2.8, was used as mobile phase without damaging the analytical column which is stable at pH from 1.5 to 8.5 under gradient conditions, as reported by the manufacturer (Phenomenex Inc).

Using the spectral characteristics of each analyte, we tested different wavelengths of excitation and emission. In no case, INO and HYPO emitted appreciable fluorescence, while the highest fluorescence detection for GUA and GUO was measured with emission and excitation wavelength set at 375 and 260 nm, respectively (Fig. 2C). This is in agreement with other papers in which similar emission and excitation wavelengths were used to assay antiviral drugs such as penciclovir, acyclovir and ganciclovir, which are GUA nucleoside analogs [27,33]. In particular, using wavelengths of 260 nm (excitation) and 380 nm (emission), Dao et al. [34] selected GMP as internal standard due to its fluorescence spectrum. It is to note that, using our selected emission and excitation wavelengths, XAN and UA were detected only at concentrations higher than those reported by UV detection.

3.2. Method validation

The linearity, regression, LOD, LOQ, precision and accuracy were taken into account to evaluate the validation of the developed method. Then, the results obtained using the UV detector were compared to those found with the fluorescence detector.

3.2.1. Linearity, limits of quantitation and detection

The calibration curves were obtained running, in duplicate, different concentrations of working standard solutions containing all analytes of interest over several days. The linearity of the analytical method was evaluated by calculating r, y-intercept and slope of the regression line for each analyte (Table 1).

The correlation coefficients were always higher than 0.998 using either UV or fluorescence detector. These findings indicated a good

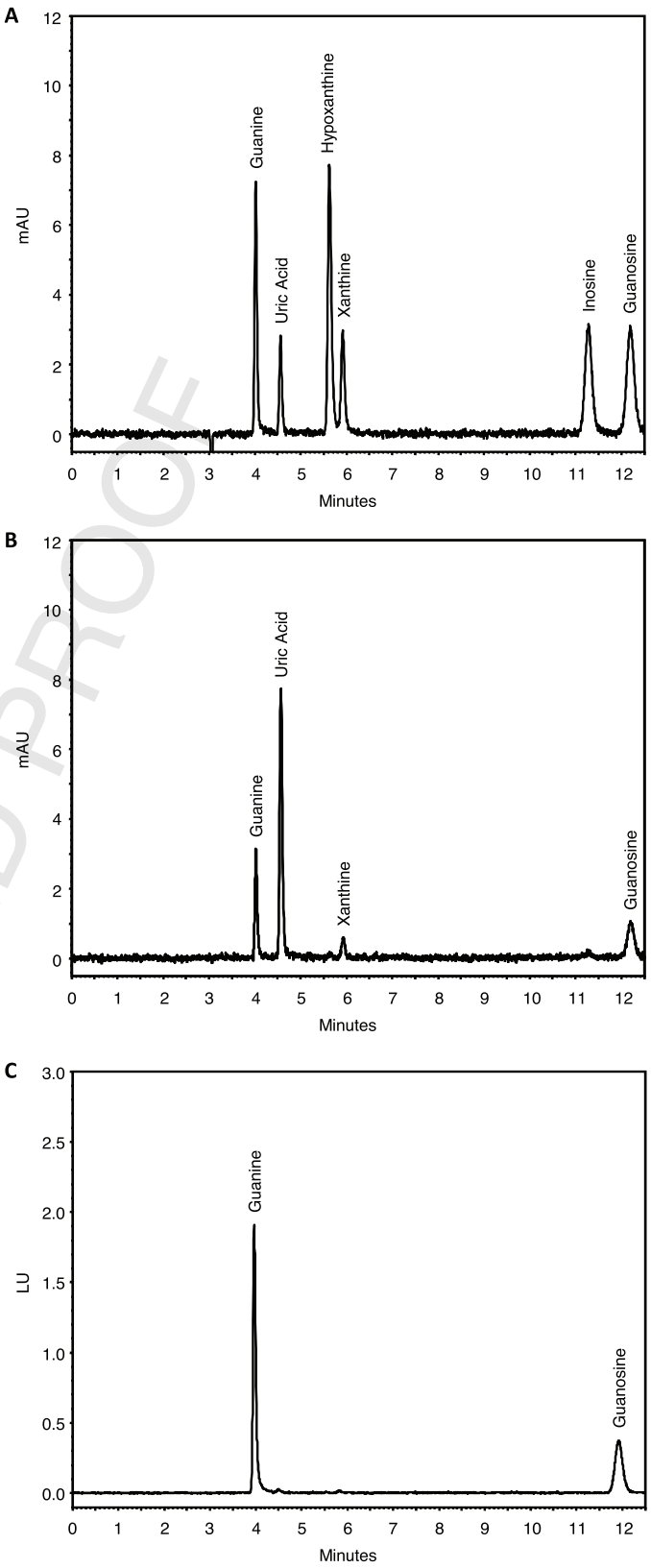

Fig. 2. Representative chromatograms of a standard mixture of six purine compounds separated and analyzed by HPLC coupled to UV detector set at (A) 254 nm and (B) 290 nm or (C) coupled to fluorescence detector (λ exc 260 nm/em 375 nm). The concentration of each analyte was 5 μM. Separation was achieved using 0.1% formic acid in water and methanol under gradient elution. Flow rate of the mobile phase was 1 ml/min.

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Table 1
Q4 Regression equation, correlation coefficient and limit of detection (LOD) and quantification (LOQ) for the investigated purine compounds by HPLC coupled with fluorescence or UV detector.

Analyte	Detector	Regression equation	Correlation coefficient	LOD μM (μg/ml)	LOQ μM (μg/ml)
Guanine	Fluorescence	$y = 559196\,x + 880122$	0.9986	0.06 (0.009)	0.21 (0.032)
Guanosine	Fluorescence	$y = 256520\,x + 806709$	0.9997	0.29 (0.082)	0.96 (0.272)
Guanine	UV at 254 nm	$y = 21820\,x + 75855$	0.9995	0.68 (0.103)	2.27 (0.343)
Hypoxanthine	UV at 254 nm	$y = 18993\,x + 66419$	0.9998	0.77 (0.105)	2.58 (0.351)
Xanthine	UV at 254 nm	$y = 13638\,x + 39558$	0.9999	1.06 (0.161)	3.53 (0.537)
Inosine	UV at 254 nm	$y = 21444\,x + 70999$	0.9998	1.12 (0.300)	3.73 (1.000)
Guanosine	UV at 254 nm	$y = 24663\,x + 67753$	0.9999	1.03 (0.292)	3.43 (0.972)
Uric acid	UV at 290 nm	$y = 16646\,x + 99501$	0.9988	0.55 (0.092)	1.83 (0.308)

correlation between concentrations and peak areas for each compound within the interval concentrations ranging from LOQ value to 500 μM for all investigated analytes except GUA, whose linearity range was from LOQ value to 250 μM with the fluorescence detector. Using the UV detector, LOD values ranged from 0.55 μM for UA up to 1.12 μM for INO while LOQ values were from 1.83 μM for UA to 3.73 μM for INO (Table 1). As expected, using the fluorescence detector, the LOD and LOQ values for GUA and GUO were lower than those found with UV detection. In particular, the LOD and LOQ values for GUA were 10-fold lower than those reported using UV-coupled HPLC (Table 1). These data clearly demonstrated that fluorescence detection significantly increased the sensitivity of the method.

3.2.2. Precision and accuracy

Precision and accuracy of the method were carried out using QC samples containing low, medium, and high concentrations (5, 75 and 150 μM, respectively) of the standard mixture. Six to nine determinations per concentration were made. The intra- and inter-day precision was evaluated for the retention time and peak area. As shown in Table 2, for retention time, the intra- and inter-day precision ranged from + 0.09 up to + 1.27% and from + 0.26 to + 1.15%, respectively. The intra- and inter-day precision for peak area ranged from + 0.40 up to + 3.85% and from + 2.16 to + 6.67%, respectively. The accuracy of the method varied from 98.40% up to 107.86% (Table 2). All results were much lower than the ± 15% limit indicated by ICH Harmonised Tripartite Guideline [29], indicating that the developed method had a very good accuracy and a very high precision regarding both retention time and peak area for each investigated analyte.

3.2.3. Specificity

It is well known that plasma contains many substances that might interfere with the assay preventing a successful analysis as well as endogenous purines that must be taken into account in evaluating plasma PNP activity. Thus, even if we used only small amounts of plasma as source of PNP, the selectivity of the HPLC method was tested by injecting 10 μl of blank samples consisting of 200 μl of the assay buffer supplemented with plasma from a pool of healthy donors (from 1 to 8 μl) but without addition of enzyme substrate. When the fluorescence detector was used, no interference was present at the retention times corresponding to those of GUA and GUO, and the baseline was extremely clean as illustrated in Fig. 3A which, shows a comparison of the representative chromatogram of a blank sample containing 4 μl of human plasma in the reaction mixture (trace 1) and that of a standard mixture of analytes in the absence of plasma (trace 2). On the contrary, using UV detector, the same blank sample showed a peak of UA at 254 nm which was even more evident at 290 nm (trace 1 of Fig. 3B and C). The measured UA concentration was in agreement with that physiologically present in human plasma. Furthermore, the baseline of the blank sample was quite unstable (trace 1 of Fig. 3B and C), but

Table 2
Intra- and inter-day precisions were determined for retention time (t_R) and peak area (AUC) and expressed as a coefficient of variation (CV%). Accuracy was expressed as a percentage value between nominal versus measured concentration (%).

Analyte	Detector	QC level	Intra-day precision (CV%)		Inter-day precision (CV%)		Accuracy (%)
			for t_R	for AUC	for t_R	for AUC	
Guanine	Fluorescence	Low	0.99	2.60	0.92	4.00	105.69
		Middle	0.72	3.26	1.06	4.52	98.40
		High	0.78	2.45	1.15	4.87	100.36
Guanosine	Fluorescence	Low	1.27	2.05	1.05	5.35	105.04
		Middle	0.82	3.85	1.03	5.86	98.64
		High	0.60	3.77	1.11	5.82	100.32
Guanine	UV at 254 nm	Low	0.06	2.28	0.48	6.67	103.61
		Middle	0.10	1.83	0.50	2.67	98.88
		High	0.09	1.59	0.49	2.60	100.27
Hypoxanthine	UV at 254 nm	Low	0.11	3.12	0.45	4.49	107.86
		Middle	0.18	1.80	0.46	2.83	98.41
		High	0.16	1.64	0.43	2.91	100.38
Xanthine	UV at 254 nm	Low	0.13	3.18	0.33	5.10	103.49
		Middle	0.19	2.48	0.34	3.05	98.65
		High	0.15	1.96	0.30	2.59	100.33
Inosine	UV at 254 nm	Low	0.15	3.33	0.59	3.94	107.31
		Middle	0.34	1.14	0.60	2.78	98.46
		High	0.27	1.17	0.57	3.11	100.37
Guanosine	UV at 254 nm	Low	0.19	2.31	0.81	3.09	107.06
		Middle	0.37	0.40	0.80	3.30	98.43
		High	0.29	0.74	0.77	3.28	100.37
Uric acid	UV at 290 nm	Low	0.07	2.22	0.26	2.72	106.21
		Middle	0.16	2.83	0.30	3.03	98.75
		High	0.12	1.32	0.26	2.16	100.29

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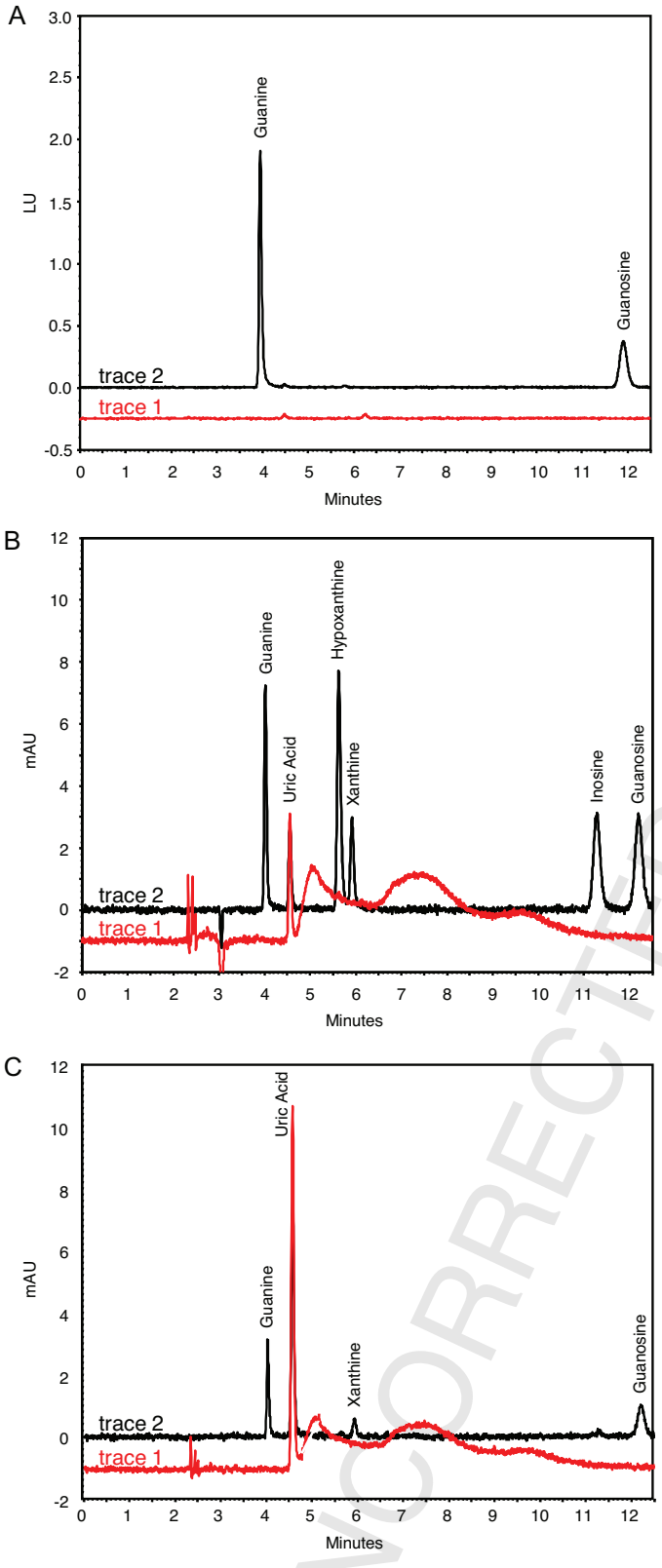

Fig. 3. Representative overlaid HPLC chromatograms for comparison of (trace 1) the buffer assay containing 4 μl of human plasma without substrate and (trace 2) a mixture of the six investigated purines (5 μM) by (A) fluorescence detector (λ exc 260 nm/em 375 nm) or UV detector set at (B) 254 nm and (C) 290 nm.

apart from UA peak before mentioned, no other interfering substance was found at the retention time of the other investigated purine compounds, as shown in Fig. 3B and C by the overlaid of the blank sample (trace 1) with the standard mixture (trace 2).

3.3. Application of the HPLC/fluorescence detection assay to study PNP kinetics

Having thus validated the method, we found that the fluorescence detection had a better sensitivity and specificity than the UV one. Thus, GUO was used as substrate for the PNP and the amount of formed product (i.e. GUA) was assayed by the fluorescence HPLC method to evaluate the enzyme activity. It is noteworthy that, regardless of the type of detector used, a further advantage of this method in evaluating PNP activity is represented by the order of appearance of GUO and GUA peaks and their excellent separation. Indeed, the enzyme product peak (GUA) appears a long time prior to that of the substrate (GUO), thus avoiding possible interference due to the high concentrations of substrate used. Furthermore, we never observed any carryover effect even at the highest doses of substrate used. When only the enzyme substrate (GUO) or only plasma sample was added to the reaction mixture, no GUA formation was observed even after the longest incubation period considered in this study (i.e. 30 min). Similarly, no enzymatic reaction occurred in the reaction mixture containing both plasma sample and GUO and assessed at time zero min. Furthermore, when plasma samples were first heated at 70 °C for 5 min and then added to the reaction mixture containing GUO and incubated at 37 °C, no metabolism of the added substrate was observed, indicating that the heat treatment is effective in inactivating the enzyme and thus in terminating the reaction (data not shown).

Since it has been reported that both GUA deaminase and XAN oxidase are present in plasma [30,31], the GUA formed in the reaction mixture could be further deaminated into XAN and then oxidized into UA by the consecutive action of the two enzymes mentioned above (Fig. 1). So, it was important to evaluate by HPLC if, after the incubation period, a peak of XAN appeared in the assay buffer or if the UA peak increased. Regardless of the incubation period and the volume of plasma used, XAN levels were never detectable in the reaction mixture as well as no increase of UA concentrations was observed. Furthermore, the disappearance of the substrate (i.e. GUO) was always of the same order of magnitude of the increase of the reaction product (i.e. GUA). These data indicated that, in our experimental conditions, plasma PNP activity measured through the formation of GUA was not underestimated by further metabolism of the neo-formed GUA.

To achieve the appropriate assay conditions for testing plasma PNP activity, we first evaluate the optimal incubation time and plasma volume to use. Under our experimental conditions, the formation of GUA induced by plasma PNP increased linearly along with increasing volumes of plasma (ranging from 1 to 8 μl) or increasing reaction times (from 7.5 to 30 min) (Fig. 4A and B). Thus, to evaluate the kinetics of the enzyme reaction we chose an incubation period of 15 min and a plasma volume of 4 μl. The kinetic curve has been obtained using increasing concentrations of substrate (GUO) in presence of fixed co-substrate concentration (Pi 50 mM) and measuring the initial velocity. Within the range of GUO concentrations used (from 1 to 500 μM) to reach the saturation, the curve was best fitted by non-linear regression analysis to the Michaelis–Menten hyperbolic function (Fig. 5). Km and Vmax values were estimated to be 69.24 ± 16.96 μM and 36.85 ± 2.94 nmol/min/ml plasma, respectively. The Km value obtained in this work was roughly in the same range of those reported in literature by other authors (from about 35–65 μM) who used the same substrate, GUO, but different methods and enzyme sources [35–37].

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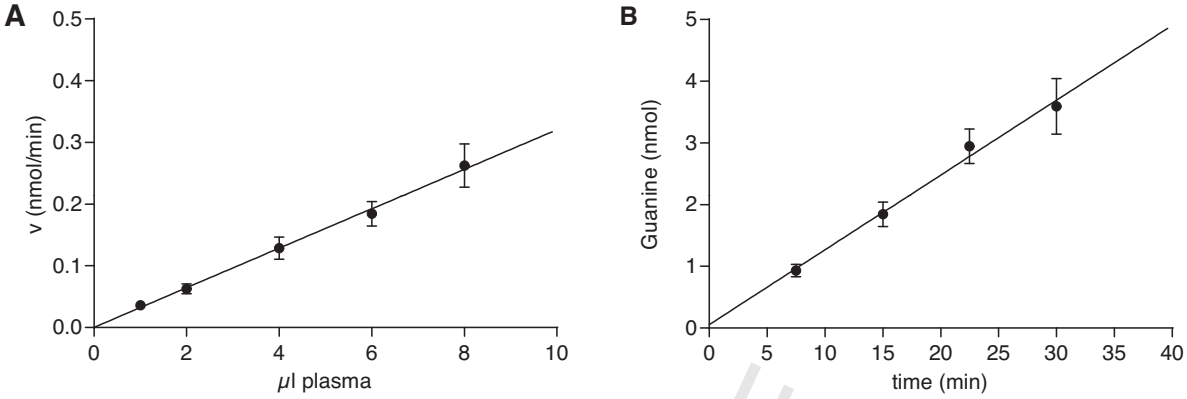

Fig. 4. Dependence of PNP activity on plasma volume and reaction time. The amount of GUA produced in the reaction mixture was evaluated (A) using different volumes of plasma incubated with 500 μM GUO plus 50 mM Pi for 15 min at 37 °C or (B) after different periods of incubation at 37 °C using 500 μM GUO plus 50 mM Pi and 4 μl of plasma as enzyme source. Data points represent the mean ± S.E.M. from at least three different experiments, each run in duplicate.

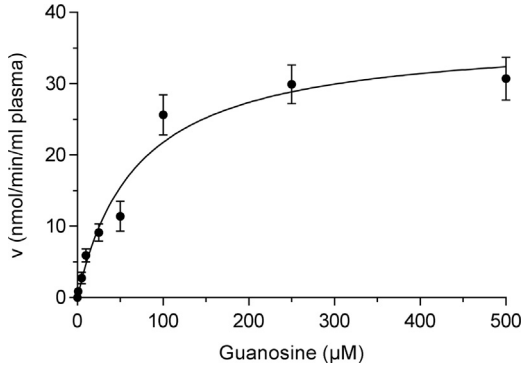

Fig. 5. Michaelis–Menten plot of the initial velocities of PNP reaction versus GUO concentrations. Reactions were conducted at 37 °C for 15 min, and Pi concentration was set to 50 mM. Data points represent the mean ± S.E.M. from at least three different experiments, each run in duplicate.

4. Conclusions

The aim of this study was to develop a simple, fast and sensitive HPLC method to determine PNP activity in biological samples, in particular human plasma since it is emerging that an abnormal PNP activity in blood could be considered as a possible indicator of various pathological conditions.

The HPLC assay developed in this study allowed the simultaneous quantification of purine nucleosides and nucleobases that, in the PNP reaction, represent the natural enzyme substrates (INO and GUO), products (HYPO and GUA), and the subsequent metabolic products (XAN and UA). The method proposed herein has been validated and exhibited an excellent selectivity, precision, accuracy and a short run time since the chromatographic separation of all selected purines was achieved within 13 min. Compared with other HPLC assays used so far, the main advantage of this method was the use of a fluorescence detector that improved the sensibility and selectivity of the analysis. This was possible since GUO and its nucleobase GUA are both native fluorescent molecules and their fluorescence is sufficiently intense to allow their simultaneous assay without the need for derivatization procedures which are not always fast, simple and able to guarantee a good reproducibility and stable reaction products. Even using a complex matrix such as plasma and unlike the UV detector, the fluorescence detection allowed us to obtain an excellent baseline stability. To the best of our knowledge, this is the first time that the naturally fluorescence of these compounds is exploited to set up an HPLC method.

Thus, our data indicate that this method is suitable and reliable for the evaluation of PNP activity in human plasma. Due to its high sensitivity and selectivity, it might be useful not only to evaluate an increased activity of the enzyme as an indicator of pathological conditions such as cancers, but also to detect a reduced activity occurring i.e. in patients with PNP deficiency. Furthermore, since PNP inhibitors represent a new promising class of drugs that could be used as selective immunosuppressive agents [38,39] or for the treatment of hyperuricemia of gout [40], our assay, using the native plasma PNP and its natural substrate, might be useful for selectively screening new PNP inhibitors as novel drugs. This method could be also adapted for monitoring the PNP activity in other biological fluids, such as the gingival crevicular fluid. Indeed, high PNP activity has been detected in that fluid, close to sites affected by periodontal disease. Since this activity was reduced after periodontal treatment [41], this indicates that PNP could potentially serve as a marker of disease status.

Overall, this new method proved to be simple, straightforward and did not involve any complicated equipment or expensive reagents, which makes it particularly suitable for routine laboratory uses.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

[1] A. Bzowska, E. Kulikowska, D. Shugar, Purine nucleoside phosphorylases: properties, functions, and clinical aspects, Pharmacol. Ther. 88 (2000) 349–425.
[2] B. Wielgus-Kutrowska, A. Modrak-Wojcik, A. Dyzma, K. Breer, M. Zolkiewski, A. Bzowska, Purine nucleoside phosphorylase activity decline is linked to the decay of the trimeric form of the enzyme, Arch. Biochem. Biophys. 549 (2014) 40–48.
[3] P.O. de Giuseppe, N.H. Martins, A.N. Meza, C.R. dos Santos, H.D. Pereira, M.T. Murakami, Insights into phosphate cooperativity and influence of substrate modifications on Binding and Catalysis of hexameric purine nucleoside phosphorylases, PloS One 7 (2012) e44282, http://dx.doi.org/10.1371/journal.pone.0044282.
[4] P. Dessanti, Y. Zhang, S. Allegrini, M.G. Tozzi, F. Sgarrella, S.E. Ealick, Structural basis of the substrate specificity of *Bacillus cereus* adenosine phosphorylase, Acta Crystallogr. D Biol. Crystallogr. 68 (2012) 239–248.

[5] E.M. Bennett, C. Li, P.W. Allan, W.B. Parker, S.E. Ealick, Structural basis for substrate specificity of *Escherichia coli* purine nucleoside phosphorylase, J. Biol. Chem. 278 (2003) 47110–47118.
[6] J. Tebbe, A. Bzowska, B. Wielgus-Kutrowska, W. Schröder, Z. Kazimierczuk, D. Shugar, W. Saenger, G. Koellner, Crystal structure of the purine nucleoside phosphorylase (PNP) from *Cellulomonas* sp. and its implication for the mechanism of trimeric PNPs, J. Mol. Biol. 294 (1999) 1239–1255.
[7] M. Camici, V. Micheli, P.L. Ipata, M.G. Tozzi, Pediatric neurological syndromes and inborn errors of purine metabolism, Neurochem. Int. 56 (2010) 367–378.
[8] S. Balasubramaniam, J.A. Duley, J. Christodoulou, Inborn errors of purine metabolism: clinical update and therapies, J. Inherit. Metab. Dis. 37 (2014) 669–686.
[9] N. Nohata, T. Hanazawa, N. Kikkawa, D. Sakurai, K. Sasaki, T. Chiyomaru, K. Kawakami, H. Yoshino, H. Enokida, M. Nakagawa, Y. Okamoto, N. Seki, Identification of novel molecular targets regulated by tumor suppressive miR-1/miR-133a in maxillary sinus squamous cell carcinoma, Int. J. Oncol. 39 (2011) 1099–1107.
[10] S. Kojima, T. Chiyomaru, K. Kawakami, H. Yoshino, H. Enokida, N. Nohata, M. Fuse, T. Ichikawa, Y. Naya, M. Nakagawa, N. Seki, Tumour suppressors miR-1 and miR-133a target the oncogenic function of purine nucleoside phosphorylase (PNP) in prostate cancer, Br. J. Cancer 106 (2012) 405–413.
[11] T. Yamasaki, H. Yoshino, H. Enokida, H. Hidaka, T. Chiyomaru, N. Nohata, T. Kinoshita, M. Fuse, N. Seki, M. Nakagawa, Novel molecular targets regulated by tumor suppressors microRNA-1 and microRNA-133a in bladder cancer, Int. J. Oncol. 40 (2012) 1821–1830.
[12] O. Sanfilippo, M. Camici, M.G. Tozzi, M. Turriani, A. Faranda, P.L. Ipata, R. Silvestrini, Relationship between the levels of purine salvage pathway enzymes and clinical/biological aggressiveness of human colon carcinoma, Cancer Biochem. Biophys. 14 (1994) 57–66.
[13] E.L.L. Roberts, R.P. Newton, A.T. Axford, Plasma purine nucleoside phosphorylase in cancer patients, Clin. Chim. Acta 344 (2004) 109–114.
[14] S.K. Vareed, V.B. Bhat, C. Thompson, V.T. Vasu, D. Fermin, H. Choi, C.J. Creighton, S. Gayatri, L. Lan, N. Putluri, G.S. Thangjam, P. Kaur, M. Shabahang, J.G. Giri, A.I. Nesvizhskii, A.A. Asea, A.G. Cashikar, A. Rao, J. McLoughlin, A. Sreekumar, Metabolites of purine nucleoside phosphorylase (NP) in serum have the potential to delineate pancreatic adenocarcinoma, PLoS One 6 (2011) e17177, http://dx.doi.org/10.1371/journal.pone.0017177.
[15] J. Ozer, M. Ratner, M. Shaw, W. Bailey, S. Schomaker, The current state of serum biomarkers of hepatotoxicity, Toxicology 245 (2008) 194–205.
[16] J.D. Stoeckler, R.E. Parks, Purine nucleoside phosphorylase. Measurement of activity and use of inhibitors, in: D.M. Paton (Ed.), Methods Used in Adenosine Research, Plenum Press, New York, 1985, pp. 147–162.
[17] C.H. Chang, L.L. Bennett Jr, R.W. Brockman, A new isotopic assay for purine nucleoside phosphorylase, Anal. Biochem. 183 (1989) 279–282.
[18] A. Modrak-Wojcik, A. Kirilenko, D. Shugar, B. Kierdaszuk, Role of ionization of the phosphate cosubstrate on phosphorolysis by purine nucleoside phosphorylase (PNP) of bacterial (*E. coli*) and mammalian (human) origin, Eur. Biophys. J. 37 (2008) 153–164.
[19] P.A. Ropp, T.W. Traut, Allosteric regulation of purine nucleoside phosphorylase, Arch. Biochem. Biophys. 288 (1991) 614–620.
[20] T. Yamamoto, Y. Moriwaki, S. Takahashi, Y. Nasako, J. Yamakita, K. Hiroishi, K. Higashino, Determination of plasma purine nucleoside phosphorylase activity by high-performance liquid chromatography, Anal. Biochem. 227 (1995) 135–139.
[21] J. Iqbal, C.E. Müller, High-sensitivity capillary electrophoresis method for monitoring purine nucleoside phophorylase and adenosine deaminase reactions by a reversed electrode polarity switching mode, J. Chromatogr. A 1218 (2011) 4764–4771.
[22] R.I. López-Cruz, M.B. Pérez-Milicua, D.E. Crocker, R. Gaxiola-Robles, J.A. Bernal-Vertiz, A. de la Rosa, J.P. Vázquez-Medina, T. Zenteno-Savín, Purine nucleoside phosphorylase and xanthine oxidase activities in erythrocytes and plasma from marine, semiaquatic and terrestrial mammals, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 171 (2014) 31–35.
[23] W. Struck, M. Waszczuk-Jankowska, R. Kaliszan, M.J. Markuszewski, The state-of-the-art determination of urinary nucleosides using chromatographic techniques hyphenated with advanced bioinformatic methods, Anal. Bioanal. Chem. 401 (2011) 2039–2050.
[24] D.P. Bhatt, X. Chen, J.D. Geiger, T.A. Rosenberger, A sensitive HPLC-based method to quantify adenine nucleotides in primary astrocyte cell cultures, J. Chromatogr. B 889–890 (2012) 110–115.
[25] N.K. Kochetkov, V.N. Shibaev, A.A. Kost, New reaction of adenine and cytosine derivatives, potentially useful for nucleic acids modification, Tetrahedron Lett. 22 (1971) 1993–1996.
[26] A.A. Kost, M.V. Ivanov, Etheno derivatives of adenine and cytosine, Chem. Heterocycl. Compd. 16 (1980) 209–221.
[27] A. Maes, B. Garré, N. Desmet, K. van der Meulen, H. Nauwynck, P. De Backer, S. Croubels, Determination of acyclovir in horse plasma and body fluids by high-performance liquid chromatography combined with fluorescence detection and heated electrospray ionization tandem mass spectrometry, Biomed. Chromatogr. 23 (2009) 132–140.
[28] S. Udenfriend, P. Zaltzman, Fluorescence characteristics of purines, pyrimidines, and their derivatives: measurement of guanine in nucleic acid hydrolyzates, Anal. Biochem. 3 (1962) 49–59.
[29] International Conference on Harmonisation (ICH), Validation of Analytical Procedures: Text and Methodology, ICH Harmonised Tripartite Guideline, ICH Topic Q2 (R1), 2005.
[30] E.L.L. Roberts, R.P. Newton, Estimation of guanine deaminase using guanosine as a "prosubstrate", Anal. Biochem. 324 (2004) 250–257.
[31] R. Aranda, E. Doménech, A.D. Rus, J.T. Real, J. Sastre, J. Viña, F.V. Pallardó, Age-related increase in xanthine oxidase activity in human plasma and rat tissues, Free Radic. Res. 41 (2007) 1195–1200.
[32] H. Fan, S.P. Li, J.J. Xiang, C.M. Lai, F.Q. Yang, J.L. Gao, Y.T. Wang, Qualitative and quantitative determination of nucleosides, bases and their analogues in natural and cultured *Cordyceps* by pressurized liquid extraction and high performance liquid chromatography?electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS), Anal. Chim. Acta 567 (2006) 218–228.
[33] F. Schenkel, S. Rudaz, Y. Daali, M.K. Oestreicher, J.L. Veuthey, P. Dayer, Development and validation of a new reversed-phase ion pairing liquid chromatographic method with fluorescence detection for penciclovir analysis in plasma and aqueous humor, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 826 (2005) 1–7.
[34] Y.J. Dao, Z. Jiao, M.K. Zhong, Simultaneous determination of aciclovir, ganciclovir, and penciclovir in human plasma by high-performance liquid chromatography with fluorescence detection, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 867 (2008) 270–276.
[35] P.E. Daddona, W.P. Wiesmanni, W. Milhouse, J.W. Chernll, L.B. Townsendn, M.S. Hershfieldn, H.K. Webster, Expression of human malaria parasite purine nucleoside phosphorylase in host enzyme-deficient erythrocyte culture. Enzyme characterization and identification of novel inhibitors, J. Biol. Chem. 261 (1986) 11667–11673.
[36] M. Ghanem, L. Li, C. Wing, V.L. Schramm, Altered thermodynamics from remote mutations altering human toward bovine purine nucleoside phosphorylase, Biochemistry 47 (2008) 2559–2564.
[37] M. Ghanem, S. Saen-oon, N. Zhadin, C. Wing, S.M. Cahill, S.D. Schwartz, R. Callender, V.L. Schramm, Tryptophan-free human PNP reveals catalytic site interactions, Biochemistry 47 (2008) 3202–3215.
[38] O.A. O'Connor, Novel agents in development for peripheral T-cell lymphoma, Semin. Hematol. 47 (2010) S11–S14.
[39] T.S. Chan, Y.L. Kwong, E. Tse, Novel therapeutic agents for T-cell lymphomas, Discov. Med. 16 (2013) 27–35.
[40] C. Diaz-Torné, N. Perez-Herrero, F. Perez-Ruiz, New medications in development for the treatment of hyperuricemia of gout, Curr. Opin. Rheumatol. 27 (2015) 164–169.
[41] E.L. Batista Jr, C. Deves, L. Ayub, R.G. da Silva, L.C. Filho, L.A. Basso, D.S. Santos, Purine nucleoside phosphorylase activity and expression are upregulated in sites affected by periodontal disease, J. Periodontal. Res. 45 (2010) 664–671.

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