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Optimization and validation of a UPLC-MS/MS assay for simultaneous quantification of 2,8-dihydroxyadenine, adenine, allopurinol, oxypurinol and febuxostat in human plasma

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ABSTRACT

Adenine phosphoribosyltransferase (APRT) deficiency is a rare , hereditary disorder characterized by renal excretion of 2,8-dihydroxyadenine (DHA), leading to kidney stone formation and chronic kidney disease (CKD). Treatment with a xanthine oxidoreductase inhibitor, allopurinol or febuxostat, reduces urinary DHA excretion and slows the progression of CKD. The method currently used for therapeutic monitoring of APRT deficiency lacks specificity and thus, a more reliable measurement technique is needed. In this study, an ultra-performance liquid chromatography-tandem mass spectrometry method for simultaneous quantification of DHA, adenine, allopurinol, oxypurinol and febuxostat in human plasma was optimized and validated. Plasma samples were prepared with protein precipitation using acetonitrile followed by evaporation. The chemometric approach design of experiments was implemented to optimize gradient steepness, amount of organic solvent, flow rate, column temperature, cone voltage, desolvation temperature and desolvation flow rate. Experimental screening was conducted using fractional factorial design with addition of complementary experiments at the axial points for optimization of peak area, peak resolution and peak width. The assay was validated according to the US Food and Drug Administration guidelines for bioanalytical method validation over the concentration range of 50 to 5000 ng/mL for DHA, allopurinol and febuxostat, 100 to 5000 ng/mL for adenine and 50 to 12,000 ng/mL for oxypurinol, with $r^2 \geq 0.99$. The analytical assay achieved acceptable performance of accuracy (−10.8 to 8.3 %) and precision (CV < 15 %). DHA, adenine, allopurinol, oxypurinol and febuxostat were stable in plasma samples after five freeze–thaw cycles at −80 °C and after storage at −80 °C for 12 months. The assay was evaluated for quantification of the five analytes in clinical plasma samples from six APRT deficiency patients and proved to be both efficient and accurate. The proposed assay will be valuable for guiding pharmacotherapy and thereby contribute to improved and more personalized care for patients with APRT deficiency.

1. Introduction

Adenine phosphoribosyltransferase (APRT) deficiency is a rare autosomal recessive disorder of adenine metabolism characterized by production and urinary excretion of the poorly soluble 2,8-dihydroxyadenine (DHA). DHA precipitates in the urine and forms crystal aggregates that accumulate in the kidney parenchyma [1,2]. The clinical manifestations vary considerably among patients, ranging from asymptomatic state to recurrent kidney stones and progressive chronic kidney disease (CKD) [1–3]. The diagnosis is based on demonstrating absence of APRT enzyme activity in red blood cells or biallelic pathogenic variants in the APRT gene [4]. Furthermore, an ultra-performance

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liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assay for absolute quantification of urinary DHA has been developed by our group for diagnosis of APRT deficiency [5] and has been successfully used in patient care [5,6].

Treatment with a xanthine oxidoreductase (XOR) inhibitor, allopurinol (oxypurinol is the active metabolite) or febuxostat, has been shown to reduce the urinary excretion of DHA, prevent further stone formation and even improve kidney function [1,3,6]. The dosing of XOR inhibitor therapy in patients with APRT deficiency has been largely empiric, and urine microscopy has generally been used for therapeutic monitoring, where the absence of urinary DHA crystals is considered indicative of adequate treatment. However, urine microscopy has several limitations that render it unsatisfactory for diagnosis and pharmacotherapy monitoring in the clinic. The aforementioned urinary UPLC-MS/MS assay has shown promise as a diagnostic test in APRT deficiency and has the potential to improve monitoring of pharmacotherapy with the exception of patients with advanced CKD who frequently have minimal urinary DHA excretion. A method for accurate measurement of DHA concentration in plasma is therefore needed. Furthermore, a reliable method to simultaneously measure the plasma concentration of DHA, adenine, allopurinol, oxypurinol and febuxostat for therapeutic monitoring of patients with APRT deficiency would be highly valuable.

Several liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for quantification of either allopurinol and oxypurinol [7–10] or febuxostat [11–18] in plasma samples from patients with gout have been reported. Quantification of DHA in serum samples from APRT deficiency patients has been reported using high performance liquid chromatography (HPLC) with electrochemical detection [19], and in plasma samples from healthy individuals following administration of adenine using HPLC [20]. However, no method currently exists for the simultaneous determination of DHA, adenine, allopurinol, oxypurinol and febuxostat. Accurate quantification of this constellation of analytes will lead to more precise dosing of pharmacotherapy in patients with APRT deficiency.

In the present study, the design of experiments (DoE) methodology was applied for the development and optimization of a UPLC-MS/MS assay for simultaneous quantification of DHA, adenine, allopurinol, oxypurinol and febuxostat in human plasma. The aim was to develop a robust assay for absolute quantification of these biomarkers and pharmacological agents for diagnosis and pharmacotherapy monitoring in patients with APRT deficiency. The method was validated according to the US Food and Drug Administration (FDA) guidelines for validation of bioanalytical methods [21,22], through evaluation of selectivity, sensitivity, concentration curve, accuracy, precision, recovery, matrix effect, carryover, dilution integrity and stability.

2. Experimental

2.1. Chemicals and reagents

2,8-Dihydroxyadenine (DHA; 95 % pure) was synthesized at the Department of Chemistry, University of Iceland, using a previously reported protocol [23,24]. Adenine (99.6 % pure), adenosine (100.0 % pure), allopurinol (99.0 % pure), hypoxanthine (99.6 % pure), xanthine (100.0 % pure), inosine (99.2 % pure), 2-deoxyadenosine (99.9 % pure), 2-deoxyinosine (99.8 % pure), ammonium acetate (LC-MS grade), ammonium hydroxide (NH_4OH, reagent grade), acetic acid (LC-MS grade), formic acid (≥98 %) and dimethyl sulfoxide (DMSO, Chromasolv grade) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Febuxostat (99.4 % pure) and oxypurinol (99.0 % pure) were purchased from Santa Cruz (TX, USA). Acetonitrile (Chromasolv, LC-MS Ultra) was purchased from Honeywell (Charlotte, North Carolina, USA). Deionized water was produced by Millipore Q-POD ultrapure water system (Merck Millipore, Burlington, MA, USA).

2,8-Dihydroxyadenine-2-^{13}C-1,3-$^{15}N_2$ (DHA-2-^{13}C-1,3-$^{15}N_2$) was synthesized at the Department of Chemistry, University of Iceland, following a previously reported protocol [23,24], for use as an internal standard for DHA. Other reagents used as internal standards in the experiments were: allopurinol-^{13}C ($^{15}N_2$) (98.0 % pure) and oxypurinol-^{13}C ($^{15}N_2$) (96.6 % pure) purchased from Toronto Research Chemicals (Toronto, ON, Canada), febuxostat-d7 (99.8 % pure) purchased from Santa Cruz (Dallas, TX, USA) and adenine $^{15}N_5$ (98.0 % pure) purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and applied as internal standards for allopurinol, oxypurinol, febuxostat and adenine, respectively.

2.2. Preparation of stock solutions

Stock solutions for DHA and adenine were prepared as 100 μg/mL solutions in 100 mM NH_4OH and placed in a sonicator bath for 20 min. Stock solutions for allopurinol and febuxostat were prepared as 1 mg/mL solutions in DMSO. Stock solution for oxypurinol was prepared as 1 mg/mL solution in 100 mM NH_4OH.

Stock solutions were prepared for 2-deoxyadenosine, 2-deoxyinosine, adenosine, hypoxanthine, inosine and xanthine separately as 500 μg/mL solutions and placed in a sonicator bath for 20 min. These solutions were solely used in the DoE screening, because these analytes can interfere with the analysis of adenine, allopurinol and oxypurinol.

Stock solutions for 2,8-dihydroxyadenine-2-^{13}C-1,3-$^{15}N_2$ (DHA-2-^{13}C-1,3-$^{15}N_2$) and adenine$^{15}N_5$ were prepared as 100 mM NH_4OH solutions at a concentration of 100 μg/mL and placed in a sonicator bath for 20 min. Oxypurinol^{13}C, $^{15}N_2$ was prepared in 100 mM NH_4OH at a concentration of 1 mg/mL, febuxostat-d7 and allopurinol^{13}C, $^{15}N_2$ were prepared in DMSO at a concentration of 1 mg/mL.

2.3. Preparation of calibration standards and quality control samples

A mixed working standard solution was prepared by dilution of the DHA, adenine, allopurinol, oxypurinol and febuxostat stock solutions in 10 mM NH_4OH. The mixed working standard solution was then diluted with 10 mM NH_4OH to prepare a concentration series of 250, 500, 1000, 2000, 5000, 7500, 15,000, 25,000, 40,000 and 60,000 ng/mL. Calibration curve standards were prepared by spiking human plasma samples from healthy controls with the working standard solution to obtain concentrations of 50, 100, 200, 400, 1000, 1500, 3000, 5000, 8000 and 12,000 ng/mL. The quality control (QC) working solution samples were prepared in the same manner at lower limit of quantification (LLOQ), as low QC (LQC), medium–low QC (MLQC) and medium–high QC (MHQC). An additional QC sample for oxypurinol was prepared to cover the concentration range of 5000–12,000 ng/mL, denoting high QC (HQC). The QC working solutions were prepared at a concentration of 250, 500, 750, 3000, 10,000 and 35,000 ng/mL. Human plasma samples from healthy controls were spiked with the QC working solutions to obtain a concentration of 50, 100, 150, 600, 2000 and 7000 ng/mL for LLOQ, LQC, MLQC, MHQC and HQC, respectively.

2.4. Sample preparation

Protein precipitation was performed using a 96-well Ostro Protein Precipitation & Phospholipid Removal Plate (Ostro plate) (Waters, Milford, MA, USA). A 100 μL plasma sample and 20 μL of internal standard working solution were pipetted into the plate, and 400 μL of acetonitrile containing 1 % formic acid subsequently added using a Tecan Freedom Evo pipetting robot (Tecan, Männedorf, Switzerland), followed by mixing of samples three times. The Ostro plate was then placed on a positive pressure manifold. The collected samples were evaporated to dryness under a gentle stream of nitrogen (Ultravap, Porvair Sciences, North Wales, UK) and reconstituted in 100 μL mobile phase A. The sample preparation procedure was carried out for the standards, QC and clinical samples prior to analysis with the UPLC-MS/MS assay.

2.5. UPLC-MS/MS instrumentation and analysis conditions

UPLC-MS/MS analyses were performed on a ACQUITY™ UPLC™ coupled to a Xevo™ TQ-XS Tandem Quadrupole Mass Spectrometer system equipped with electrospray ionization (ESI) probe (Waters Corporation, Milford, MAs, USA). Nitrogen was used as desolvation gas and cone gas and argon as collision gas. Source temperature was set at 150 °C and cone voltage at 20 V for all analytes. ACQUITY UPLC HSS T3 column (1.8 μm, 100 x 2.1 mm) was used for chromatographic separation (Waters Corporation, Milford, MA, USA) and injection volume was 2 μL. Mobile phase A consisted of 2 mM ammonium acetate in MQ water at pH 5.7 and mobile phase B consisted of 2 mM ammonium acetate in acetonitrile with 5 % mobile phase A (95:5 v/v). Electrospray ionization was used in positive and negative ionization mode. Quantification was performed in multiple reaction monitoring (MRM). MRM transitions and collisions energies for each analyte were determined by infusing neat standards into the ion source at 20 μL/min. Masslynx 4.2 and Targetlynx XS softwares (Waters Corporation, Milford, MA, USA) were used for data acquisition and data processing. Gradient steepness, amount of organic solvent, flow rate, column temperature, cone voltage, desolvation temperature and desolvation flow rate were optimized using DoE as described in section 2.6 below.

2.6. Design of experiments

Selection of experimental factors and their ranges for the DoE screening was based on the DoE design from the previously published UPLC-MS/MS urinary assay [5], as well as literature review and preliminary experiments. The experimental screening by fractional factorial (FF) design of resolution V + with an interaction model was created in Modde 13 (Sartorius Stedim Data Analytics, Umeå, Sweden). Before running the experimental design, a test run was conducted with all experimental factors at either high, low or middle value to investigate if they would produce a response and thus a valid model. The following factors were optimized with the FF design: gradient steepness (Gra), amount of organic solvent (%B), flow rate (Flo), column temperature (Temp), capillary voltage (Cap), desolvation temperature (DesT) and desolvation flow rate (DesF). The factor levels are shown in Table 2. The settings of all other UPLC and MS/MS parameters were as described in section 2.7. The investigated responses were peak area, retention time, peak width and resolution between xanthine (peak 1)/oxypurinol (peak 2) and allopurinol (peak 1)/inosine (peak 2). Resolution was calculated using the following equation:

$$Rs = 1.18^{*}\frac{t_{R2} - t_{R1}}{W_{0.5h1} + W_{0.5h2}}$$

t_R = retention time

$W_{0.5}$ = peak width at half height.

Following the FF design, complemented axial points for significant experimental factors were added to the design, using the same factors and factor range. The experimental factors were optimized and related to the UPLC-MS/MS responses using partial least squares (PLS) regression. The working solution used for the DoE screening was an EDTA plasma sample from a healthy control subject spiked with DHA, adenine, allopurinol, oxypurinol, febuxostat, 2-deoxyadenosine, 2-deoxyinosine, adenosine, hypoxanthine, inosine and xanthine at 1000 ng/mL. A list of all responses included in the DoE screening is provided in Supplementary Table S2.

2.7. Method validation

The UPLC-MS/MS assay was validated according to the US FDA guidelines for bioanalytical method validation for selectivity, calibration curve, sensitivity, accuracy and precision, recovery, matrix effect, carryover, dilution integrity and stability [21,22].

2.8. Sensitivity, selectivity and calibration curve

The limit of detection (LOD) was defined as the lowest concentration that gave a signal-to-noise (S/N) ratio of minimum 3:1. Sensitivity was determined using the LLOQ and calculated as the lowest point in the calibration curve with acceptance criteria of within ± 20 % and < 20 % for accuracy and precision, respectively.

Selectivity was evaluated by comparing non-spiked blank plasma samples and blank plasma samples spiked with the analytes at the LLOQ from six healthy individuals, to verify the absence of interfering substances at the retention time of all tested analytes and internal standards. For acceptable selectivity, the mean peak response obtained for the non-spiked plasma samples at the expected retention time of the analytes had to be less than 20 % of the response of the analytes in the LLOQ samples and less than 5 % of the response of the IS.

Calibration curves were constructed by plotting the peak area ratio (ratio of peak area of analyte and peak area of internal standard) at eight (seven for adenine and 10 for oxypurinol) concentration levels on three consecutive days for assessment of calibration range. The correlation of the calibration curve was estimated through the coefficient of determination (r2) and a linear 1/x weighted regression analysis. Acceptance criteria were r2 ≥ 0.99 and a relative error ± 15 % from the nominal value for each concentration standard, except ± 20 % for the LLOQ. A minimum of 75 % of the calibrator standards had to meet the above criteria in each validation run.

2.9. Accuracy and precision

Intra-assay accuracy and precision were assessed by repeated analyses ($n = 6$) at LLOQ, LQC, MLQC, MHQC and HQC on the same day (intra-day). Inter-day accuracy and precision were determined by conducting repeated analyses ($n = 6$) of each QC sample at each concentration level on three consecutive days. The precision was calculated as the coefficient of variation (%CV) and the accuracy as the %bias using the following equation:

$$Accuracy = \frac{\text{Amount of analyte determined} - \text{Amount of analyte spiked}}{\text{Amount of analyte spiked}} x100$$

Acceptance criteria were %bias within ± 15 % (±20 % for LLOQ) of the nominal concentration and %CV below 15 % (20 % for LLOQ). A minimum of two-thirds of all the QC samples and at least 50 % at each concentration level had to fall within the acceptance criteria.

2.10. Matrix effect and recovery

The matrix effect was determined at two concentration levels (LQC and MHQC) in plasma samples from six healthy individuals. The matrix effect was determined by comparing the analyte peak area to internal standard peak area ratio in post-extracted plasma to the analyte peak area to internal standard peak area ratio in neat solution (mobile phase A). Matrix effect was calculated using the following equation:

$$Matrix\ effect = \frac{\text{Peak area analyte}_{post-extracted} / \text{Peak area internal standard}_{post-extracted}}{\text{Peak area analyte}_{neat-solution} / \text{Peak area internal standard}_{neat-solution}} x100$$

Acceptance criteria for matrix effect were %bias within ± 15 % of the nominal concentration and %CV below 15 %.

The recovery of the analytes was determined at two concentration levels (LQC and MHQC) in plasma samples from six healthy individuals. The recovery was calculated by comparing the analyte peak area to internal standard peak area ratio in extracted plasma samples to the analyte peak area to internal standard peak area ratio in post-extracted plasma. The recovery of the analytes from the spiked sample had to be consistent and reproducible.

2.11. Dilution integrity and carryover

For dilution integrity, pooled plasma samples from healthy controls ($n = 6$) were prepared with analyte concentration above the highest calibration standard (1.5 times the concentration for DHA and adenine and 2 times the concentration for adenine, oxypurinol and febuxostat), and diluted fivefold and tenfold with blank plasma from healthy controls. Acceptance criteria for dilution integrity were %bias within ± 15 % of the nominal concentration and %CV below 15 %. Assessment of carryover was made by analyzing blank samples from healthy controls injected following the highest calibration standard. Acceptance criteria for carryover was a response in the blank sample of less than 20 % of the LLOQ response.

2.12. Stability

The stability of DHA, adenine, allopurinol, oxypurinol and febuxostat in human plasma samples from healthy controls was assessed by analyzing QC samples ($n = 6$) at two concentration levels (LQC and MHQC) under the following conditions: after storage at room temperature for 4 h, after five freeze–thaw cycles (thawed at room temperature and frozen at − 80 °C for at least 12 h) and following storage at −80 °C for 12 months. Post-processing stability was evaluated after storage in the autosampler for 48 h. Stability of stock solutions was determined in neat solution at 1000 ng/mL after five freeze–thaw cycles (thawed at room temperature and frozen at − 20 °C for at least 12 h) and after storage at − 20 °C for 12 months. Samples were considered stable if % bias was within ± 15 % of the nominal concentration and %CV below 15 %.

2.13. Evaluation of the clinical applicability of the UPLC-MS/MS assay

The applicability of the assay was evaluated by analyzing EDTA plasma samples from six APRT deficiency patients, both untreated (n = 6) and on treatment with allopurinol 400 mg/day (n = 6) or febuxostat 80 mg/day (n = 6). This part of the study was approved by the National Bioethics Committee of Iceland (NBC 09–072) and the Icelandic Data Protection Authority. Informed consent was obtained from all participants. Venous blood was drawn from patients and healthy individuals and the blood samples were centrifuged at 3000 rpm for 10 min at 4 °C. Plasma was extracted, aliquoted and stored at − 80 °C. Plasma samples were processed using the previously described sample preparation procedure in section 2.4.

3. Results and discussion

3.1. Method development

Our group has previously developed and optimized a UPLC-MS/MS assay for quantification of urine DHA and adenine using DoE [5]. The urine study provides good understanding of which UPLC and MS/MS factors and factor levels affect the sensitivity of DHA measurement and the resolution of the purines. The same analytical column and organic solvent in the mobile phases were used in the current study. The pH of the mobile phase was changed from 6.7 to 5.7 since more analytes were included in the plasma assay, such as the XOR inhibitors febuxostat and allopurinol and its major metabolite, oxypurinol. A new DoE screening was conducted, where the selection of the experimental factors was largely based on the urinary assay findings.

Table 1
MRM transitions and collision energies of each analyte.

Analyte	Molecular mass, g/mol	Precursor ion, *m/z*	Product ion, *m/z*	ESI mode	CE, eV
DHA	167.1	168.1	125.0	ESI +	18
Adenine	135.1	136.1	119.2	ESI +	17
Allopurinol	136.1	137.0	110.0	ESI +	18
Oxypurinol	152.1	151.0	41.9	ESI –	18
Febuxostat	316.3	317.2	261.0	ESI +	9
DHA ^{13}C, $^{15}N_2$	170.1	171.1	126.1	ESI +	18
Adenine $^{15}N_5$	140.1	141.0	123.0	ESI +	20
Allopurinol ^{13}C, $^{15}N_2$	139.1	140.1	112.0	ESI +	18
Oxypurinol ^{13}C, $^{15}N_2$	155.1	154.0	41.9	ESI –	15
Febuxostat-d7	323.4	324.2	262.1	ESI +	11

ESI, electrospray ionization; CE, collision energy; DHA, 2,8-dihydroxyadenine.

Table 2
Experimental factors and settings for the design of experiments optimization study.

Variable parameters	Abbreviation	Experimental domain		
		(–)	(0)	(+)
Gradient steepness (min)	Gra	3.5	4.5	5.5
Amount of organic solvent (%)	%B	5	10	15
Flow rate (mL/min)	Flo	0.3	0.4	0.5
Column temperature (°C)	Temp	25	30	35
Capillary voltage (kV)	Cap	0.3	0.65	1
Desolvation temperature (°C)	DesT	500	550	600
Desolvation flow rate (L/hr)	DesF	800	1000	1200

Obtaining a chromatographic resolution was important as spontaneous fragmentation (in-source fragmentation) of adenosine (mass to charge ratio (*m/z*) 268.1 > 136.1) and 2-deoxyadenosine (*m/z* 252.1 > 136.1) into the same ion fragment as adenine (*m/z* 136.1 > 119.2) and of inosine (*m/z* 269.1 > 137.0) and 2-deoxyinosine (*m/z* 253.1 > 137.1) into the same ion fragment as allopurinol (*m/z* 137.0 > 110.0) may occur. Furthermore, hypoxanthine (*m/z* 137.0) and xanthine (*m/z* 151.0) have the same *m/z* as allopurinol (*m/z* 137.0) and oxypurinol (*m/z* 151.0), respectively, and need to be separated chromatographically as they cannot be separated by their *m/z* ratio. [7]. Therefore, 2-deoxyadenosine, 2-deoxyinosine, adenosine, inosine, hypoxanthine and xanthine were included in the working solution for the DoE study to ensure chromatographic resolution. The most critical resolution required for the assay was between allopurinol and the in-source fragmentation of inosine, and between oxypurinol and xanthine. Hence, the goal of the DoE screening was to obtain separation between allopurinol and inosine and between oxypurinol and xanthine, without increasing the peak width of adenine and oxypurinol, as well as to optimize the sensitivity for DHA, oxypurinol and febuxostat.

Prior to the DoE screening, the ion modes and MRM transitions were determined for each analyte, and all internal standards and mass spectrometry factors for the DoE screening were selected upon infusion of the analytical standards into the ion source. Both ESI + and ESI – modes were tested for all analytes and the ion mode that provided the highest signal intensity for the precursor ion of each analyte was selected. ESI + mode was selected for DHA, adenine, allopurinol and febuxostat and ESI – mode for oxypurinol.

Collision energies were set for each analyte individually, while cone voltage was not included in the DoE screening since changing the voltage setting across a large range did not affect the intensity of any of the analytes, and thus was set at 20 V (data not shown). Capillary

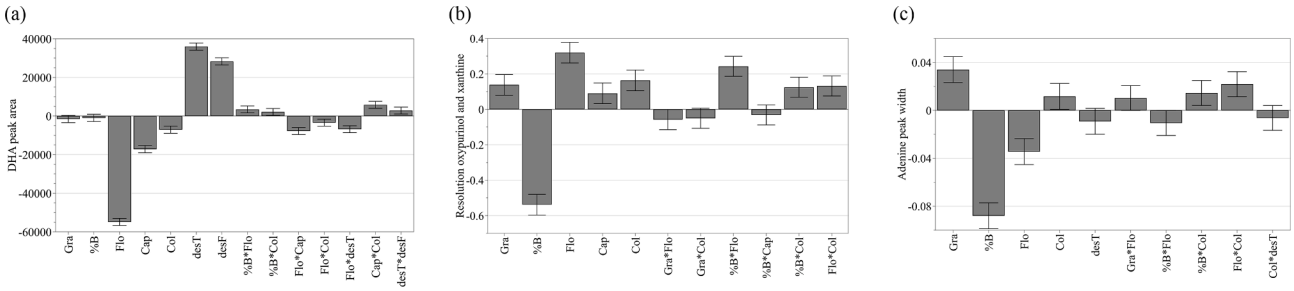

Fig. 1. Coefficient plots from the complementary design for the peak area of 2,8-dihydroxyadenine (DHA) (Area1) (a), resolution between oxypurinol and xanthine (Rs2) (b), and peak width of adenine (Width1) (c). The x-axis displays experimental factors and factor interactions that have a significant effect on the responses, and the y-axis displays the responses. Error bars represent the 95 % confidence interval. Gra, gradient steepness (min); %B, amount of organic solvent (%); Flo, flow rate (mL/min); Cap, capillary voltage (kV); Col, column temperature (°C); desT, desolvation temperature (°C); desF, desolvation flow rate (L/h).

voltage, however, did affect the intensity of the analytes (data not shown) and was therefore included in the DoE screening. MRM transitions and collision energies for each analyte and all internal standards are shown in Table 1, and MRM transitions and collision energy for 2-deoxyadenosine, 2-deoxyinosine, adenosine, inosine, hypoxanthine and xanthine can be found in Supplementary Table S3.

3.2. Method optimization by design of experiments

Experimental screening was conducted using aFF design that included a total of 13 responses: peak area of DHA (Area1), oxypurinol (Area2) and febuxostat (Area3), resolution between inosine and allopurinol (Rs1) and between xanthine and oxypurinol (Rs2), peak width (half height) for adenine (Width 1) and oxypurinol (Width2). Furthermore, the retention times of DHA, allopurinol, oxypurinol, febuxostat, inosine and xanthine were included in the model. The complete list of responses and their respective R2 and Q2 is shown in Supplementary Table S2.

According to PLS regression analysis of the responses, the fraction of variance (R2) was > 87 % for all responses with an acceptable predictive ability of the model, Q2 > 80 % (See Supplementary Table S2). The regression coefficient plots shown in Fig. 1 revealed the effect of the experimental factors on the responses. The results show that the desolvation temperature and desolvation flow rate had a significant positive effect on Area1, meaning that increasing these factors would increase the peak area of DHA. The flow rate, capillary voltage and column temperature had a significant negative effect on the DHA peak area, meaning that reducing these factors would increase the peak area of DHA. Significant interaction effect was observed between flow rate and capillary voltage, flow rate and column temperature, flow rate and desolvation temperature, and between capillary voltage and column temperature (Fig. 1**a**). For Rs2, gradient steepness, flow rate, column temperature and capillary voltage had a positive effect, while %organic solvent had a negative effect. Interaction effect was observed between % organic solvent and flow rate, %organic solvent and column temperature and flow rate and column temperature (Fig. 1**b**). For Width1, the gradient steepness and column temperature had a positive effect and % organic solvent and flow rate had a negative effect. Interaction effect was observed between %organic solvent and column temperature and between flow rate and column temperature (Fig. 1**c**).

For Rs1, gradient steepness, flow rate and column temperature had a positive effect and %organic solvent had a negative effect. Interaction effect was observed between the flow rate and column temperature (Supplementary Fig.S1). For Width2, gradient steepness, flow rate and column temperature had a positive effect and %organic solvent had a negative effect. Interaction effect was noted between %organic solvent and flow rate and between flow rate and column temperature (Supplementary Fig. S1**b**). For Area2, column temperature, desolvation temperature and desolvation flow rate had a positive effect, and %organic solvent and capillary voltage had a negative effect. Interaction effect was observed between %organic solvent and flow rate, %organic solvent and column temperature and between flow rate and capillary voltage (Supplementary Fig. S1**c**). For Area3, capillary voltage, desolvation temperature and desolvation flow rate had a positive effect whereas flow rate and column temperature had a negative effect. Interaction effect was seen between flow rate and capillary voltage, flow rate and desolvation temperature and between flow rate and desolvation flow rate (Supplementary Fig. S1**d**). The presence of interaction effects between UPLC and MS/MS factors highlights the importance of optimizing these factors simultaneously.

The coefficient plots from the FF design revealed that all seven main experimental factors were significant for at least one of the responses

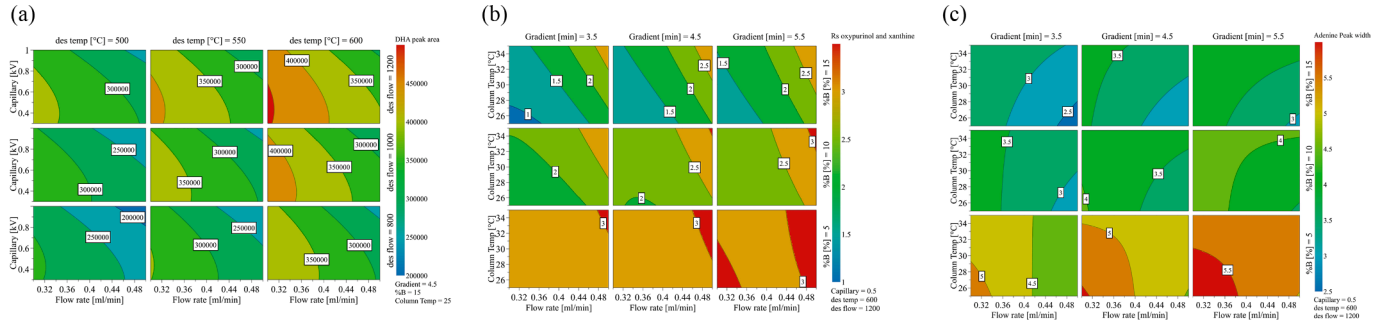

Fig. 2. Counter plots from the complementary design for peak area of 2,8-dihydroxyadenine (DHA) (Area1) according to flow rate, capillary voltage, desolvation temperature and desolvation flow rate (a), resolution between oxypurinol and xanthine (Rs2) according to flow rate, column temperature, gradient steepness and %B (b), and peak width of adenine (Width1) according to flow rate, column temperature, gradient steepness and %B (c). Gradient, gradient steepness (min); %B, amount of organic solvent (%); Capillary, capillary voltage (kV); Column temp, column temperature (°C); des temp, desolvation temperature (°C); des flow, desolvation flow rate (L/h). The color scale explains the value of each color in the plot with red indicating the highest values and green the lowest values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

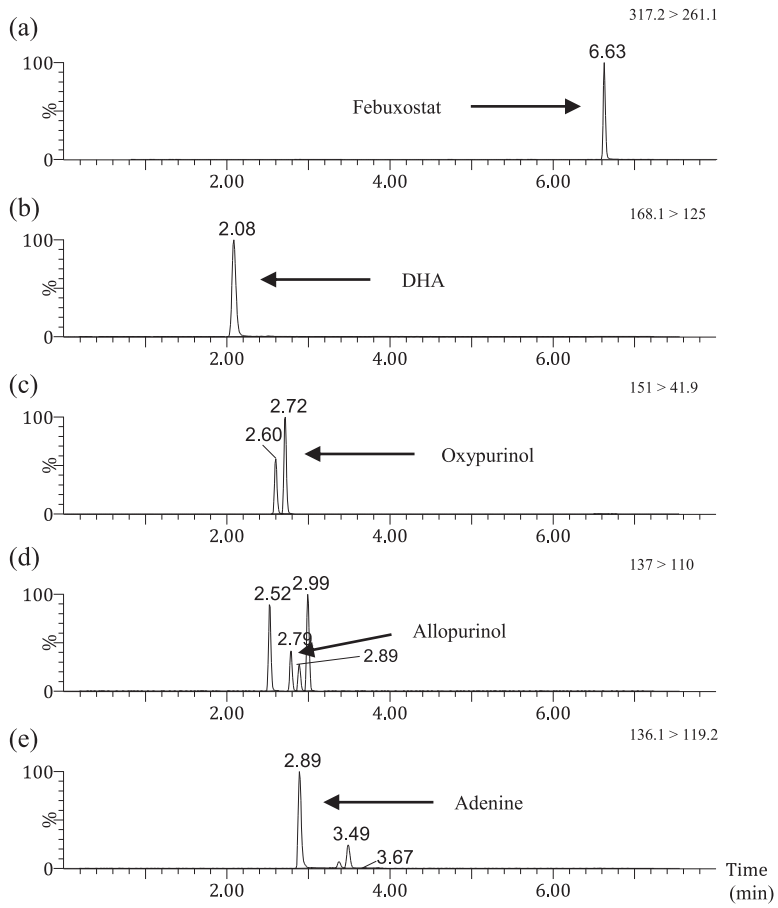

Fig. 3. MRM chromatograms of febuxostat (a), 2,8-dihydroxyadenine (DHA) (b), oxypurinol (c), allopurinol (d) and adenine (e) after design of experiments optimization of the UPLC-MS/MS method.

selected for the model. It was therefore not possible to exclude any of the experimental factors in the optimization design. Furthermore, the normal residual plot showed a curved pattern for several of the responses (Supplementary Fig. S2), which can be an indication of non-modeled quadratic relations. Thus, following the FF design, additional complementary experiments were included at the axial points to investigate potential curvature in the model. The optimized conditions for each response can be visualized in the counter plots from the complementary design in Fig. 2 and Supplementary Fig. S3.

The coefficient plots from the complementary design revealed that several quadratic effects were significant, as seen in Supplementary Fig. S4. The quadratic term was significant for capillary voltage for Area1 and Area3, for %organic solvent for Width1 and Rs1, and for flow rate for Rs2.

The complementary design revealed that the optimal setting of five factors required compromise: gradient steepness, flow rate, amount of organic solvent, capillary voltage and column temperature. The optimal setting of the desolvation temperature and desolvation flow rate did not require compromise, and the highest setting was selected for both factors due to their significant positive effect on Area1, Area2 and Area3.

For gradient steepness, increasing the length of the gradient would increase Rs1 and Rs2, but at the same time increase Width1 which results in wider peak shape. Hence, an intermediate gradient steepness (4.5 min) was selected for the optimized method. Reducing the flow rate would increase Area1 and Area3 while decreasing Area2. For greater resolution and reduced peak width, an increased flow rate is more favorable. Consequently, an intermediate flow rate (0.4 mL/min) was chosen for the optimized method. The lowest setting of the amount of organic solvent (5 %B) would increase Area2, Rs1 and Rs2 but at the same time increase Width1 and Width2, resulting in wider peak shape.

Table 3
Validation results (I). Calibration curve, sensitivity, selectivity and carryover.

Analyte	Concentration range (ng/mL)	Regression selection	Correlation coefficient (r^2)	LLOQ	Sensitivity		Selectivity		Carryover
DHA	50 – 5000	Linear, 1/X	0.999	50 ng/mL	%CV	3.8	Analyte	0.50 %	0.38 %
					%Bias	−4.1	IS	0.02 %	
Adenine	100 – 5000	Linear, 1/X	0.998	100 ng/mL	%CV	2.2	Analyte	4.20 %	8.40 %
					%Bias	−4.5	IS	0.00 %	
Allopurinol	50 – 5000	Linear, 1/X	0.999	50 ng/mL	%CV	4.8	Analyte	0.00 %	0.07 %
					%Bias	−5.1	IS	0.01 %	
Oxypurinol	50 – 12,000	Linear, 1/X	0.996	50 ng/mL	%CV	5.8	Analyte	0.01 %	0.13 %
					%Bias	−8.8	IS	0.01 %	
Febuxostat	50 – 5000	Linear, 1/X	0.999	50 ng/mL	%CV	3.1	Analyte	3.40 %	3.90 %
					%Bias	0.1	IS	0.08 %	

LLOQ, lower limit of quantification; CV, coefficient of variation; DHA, 2,8-dihydroxyadenine; IS, internal standard.

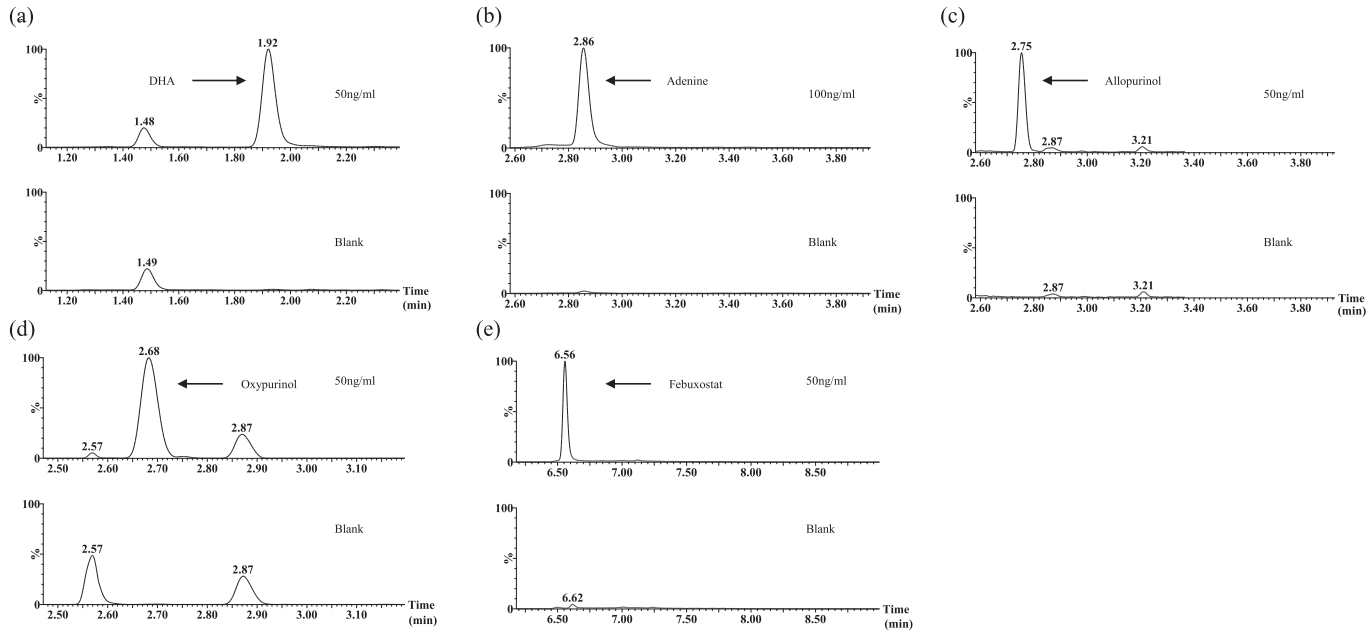

Fig. 4. MRM chromatograms of 2,8-dihydroxyadenine (DHA) (a), adenine (b), allopurinol (c), oxypurinol (d) and febuxostat (e) at the lower limit of quantification (upper panel) and in blank plasma matrix (lower panel).

Table 4
Validation results (II). Intra-assay and inter-assay accuracy and precision.

Analyte	Intra-assay								Inter-assay							
	Accuracy (%Bias)				Precision (%CV)				Accuracy (%Bias)				Precision (%CV)			
	LLOQ	LQC	MLQC	MHQC	LLOQ	LQC	MLQC	MHQC	LLOQ	LQC	MLQC	MHQC	LLOQ	LQC	MLQC	MHQC
DHA	−4.13	−0.09	−2.30	−1.10	3.79	3.87	2.63	1.56	−3.70	2.50	3.20	3.61	6.10	3.24	5.33	4.74
Adenine	−4.5	3.05	−2.29	−0.60	2.15	4.34	4.24	1.63	−8.96	0.60	0.93	2.75	7.32	3.81	4.80	3.79
Allopurinol	−5.13	−2.46	−8.32	0.96	4.82	4.57	1.49	3.91	−0.04	−2.73	−4.13	2.40	6.90	3.76	4.93	6.61
Oxypurinol	−8.80	−6.99	−6.61	−2.68	5.80	4.20	2.20	1.10	−4.20	−2.10	0.30	−5.10	6.40	5.40	5.00	5.50
Febuxostat	0.10	−1.73	−5.88	3.64	3.10	4.07	1.79	1.02	0.97	0.94	−0.63	5.03	5.70	5.90	5.90	4.90

LLOQ, lower limit of quantification; LQC, low quality control; MLQC, medium–low quality control; MHQC, medium–high quality control; CV, coefficient of variation; DHA, 2,8-dihydroxyadenine.

Since Width1 was highly sensitive to the amount of organic solvent, the highest setting (15 %B) was selected for the optimized method. Reducing the capillary voltage settings would increase Area1 and Area2 while decreasing Area3. It was considered more favorable to reduce the setting of capillary voltage, but a slight compromise was still made, and thus, the final optimized setting was closer to the lower limit of the factor range at 0.5 kV. For column temperature, selecting the highest setting would increase Area2, Rs1, Rs2, Width1 and Width2 but decrease Area1 and Area3. Since column temperature had the strongest effect on Width1, it was decided to select the lowest setting at 25 °C.

The best balance with regard to peak shape, resolution and sensitivity was found by selecting the following conditions: gradient steepness of 4.5 min, amount of organic solvent of 15 %B, flow rate of 0.4 mL/min, column temperature of 25 °C, cone voltage of 0.5 kV, desolvation temperature of 600 °C and desolvation flow rate of 1200 L/hr.

Following the DoE optimization, the resolution between oxypurinol and xanthine and between allopurinol and inosine was greater than 1.5 (see Supplementary Fig. S5). Fig. 3 shows the MRM chromatograms of the five analytes under the optimized conditions, with chromatographic separation between oxypurinol and xanthine and between allopurinol and inosine. Outline of the final optimized UPLC-MS/MS conditions can be found in Supplementary Table S1.

3.3. Method validation

3.3.1. Sensitivity, selectivity and calibration curve

Analysis of blank plasma samples from six healthy individuals showed no significant interfering peaks at the retention times of the five analytes, with the peak areas < 5 % of the corresponding LLOQ level (Table 3). The peak area at the retention times of the internal standards in the blank sample were < 0.1 % of the mean internal standards peak area (Table 3). MRM chromatograms of blank plasma samples are shown in Fig. 4 for each of the five analytes, demonstrating the absence of significant interfering endogenous components. The LOD and LLOQ were 20 ng/mL and 50 ng/mL, respectively. The LLOQ for adenine was 100 ng/mL. The intra- and inter-day accuracy (%bias) and precision (% CV) at the LLOQ were within the acceptance criteria of %bias ± 20 % and %CV > 20 % (Table 3).

The calibration range in plasma was 50–5000 ng/mL for DHA, allopurinol and febuxostat, 100–5000 ng/mL for adenine and 50–12,000 ng/mL for oxypurinol. The calibration curves were linear over the standard ranges, showing reproducibility between runs and R2 values > 0.99 (see Table 3). Typical calibration equations and regression parameters for all analytes are shown in Supplementary Fig. S6a-e.

3.3.2. Accuracy and precision

The intra- and inter-day accuracy (%bias) and precision (%CV) in plasma were determined by running six replicates at three concentration

Table 5
Validation results (III). Recovery and matrix effect.

Analyte	Concentration (ng/mL)	Mean (%)	
		Matrix effect	Recovery
DHA	150	98.2	107.0
	2000	100.6	105.3
Adenine	150	102.1	110.2
	2000	104.9	110.7
Allopurinol	150	102.7	107.8
	2000	101.0	110.9
Oxypurinol	150	103.1	115.7
	2000	100.2	111.7
Febuxostat	150	100.9	110.0
	2000	99.6	111.6

DHA, 2,8-dihydroxyadenine.

levels (LQC, MLQC and MHQC) for all five analytes (Table 4). The %CV ranged between 1.0 % and 5.8 % and between 3.1 % and 6.9 % for intra-assay and inter-assay precision, respectively. The %bias ranged between −8.8 % and 3.6 % and between −8.9 % and 5.0 % for intra-assay and inter-assay accuracy, respectively. For HQC, the intra-assay accuracy and precision were −10.8 % and 1.07 %, respectively, and the inter-assay accuracy and precision were −5.07 % and 5.46 %, respectively (data not shown) These results are within the acceptance criteria for accuracy (within ± 15 %) and precision (>15 %), demonstrating the reliability and reproducibility of the assay.

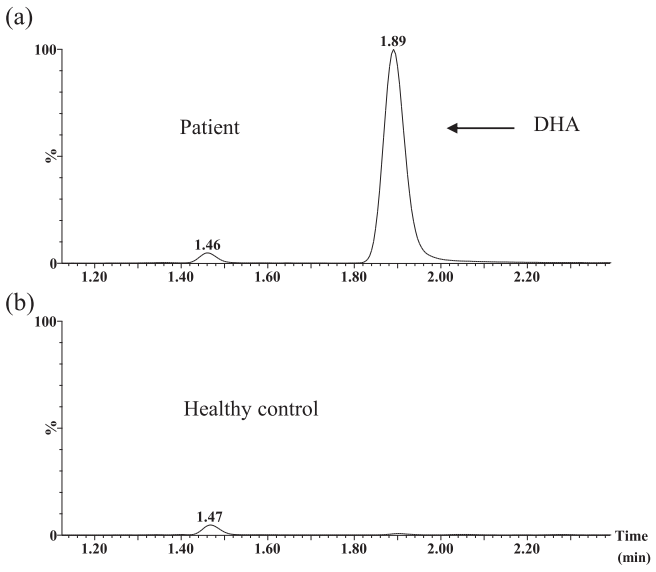

Fig. 5. MRM chromatograms of 2,8-dihydroxyadenine (DHA) in plasma samples from an untreated patient with adenine phosphoribosyltransferase deficiency (a) and from a healthy control (b).

Table 6
Validation results (IV). Determination of stability under different storage conditions in stock solution (i), plasma (ii) and working solution (iii).

Stability in stock solution (i)				
Analyte	Conentration (ng/mL)	%Bias		
		Room temperature (4 h)	Freeze-thaw	12 months (-20 C)
DHA	1000	−3.0 %	1.3 %	13.0 %
Adenine	1000	−0.7 %	13.2 %	−6.0 %
Allopurinol	1000	6.1 %	0.5 %	1.1 %
Oxypurinol	1000	−0.7 %	7.4 %	9.2 %
Febuxostat	1000	−1.8 %	−2.7 %	−4.3 %
Stability in plasma (ii).				
Analyte	Conentration (ng/mL)	%Bias		
		Room temperature (4 h)	Freeze-thaw (-80 C)	12 months (-80 C)
DHA	150	2.0 %	−1.0 %	3.2 %
	2000	0.8 %	2.2 %	7.8 %
Adenine	150	2.4 %	11.6 %	8.4 %
	2000	−2.0 %	13.4 %	12.0 %
Allopurinol	150	0.8 %	1.6 %	3.8 %
	2000	−0.2 %	8.9 %	6.4 %
Oxypurinol	150	−4.0 %	−5.9 %	−10.9 %
	2000	0.8 %	−1.9 %	−1.0 %
Febuxostat	150	−1.8 %	−5.6 %	1.8 %
	2000	−0.1 %	−0.3 %	3.3 %
Stability in working solution (iii).				
Analyte		Conentration (ng/mL)		%Bias Room temperature (4 h)
DHA		150		−5.0 %
		2000		−1.0 %
Adenine		150		−3.7 %
		2000		−3.9 %
Allopurinol		150		−6.4 %
		2000		−3.8 %
Oxypurinol		150		−1.0 %
		2000		−6.0 %
Febuxostat		150		−5.6 %
		2000		−1.6 %

DHA, 2,8-dihydroxyadenine

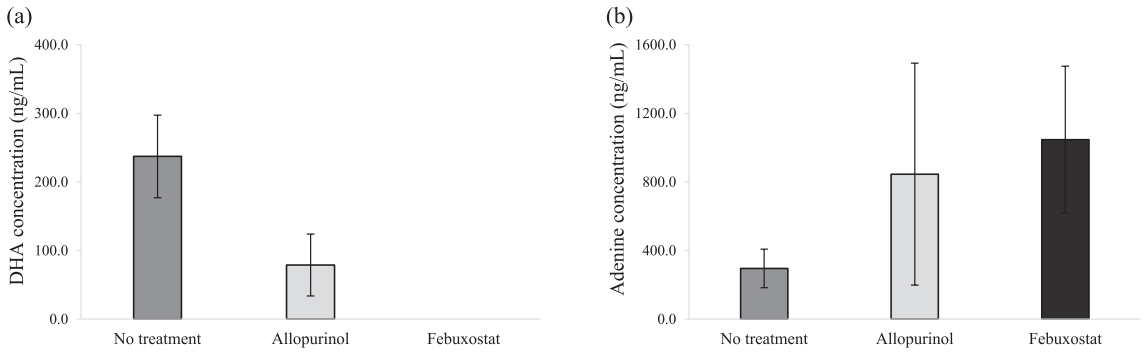

Fig. 6. Average plasma concentration of 2,8-dihydroxyadenine (DHA) (a) and adenine (b) in adenine phosphoribosyltransferase (APRT) deficiency patients, untreated and treated with either allopurinol or febuxostat. The data represents mean values and SD of samples from six APRT deficiency patients enrolled in a clinical study.

3.3.3. Matrix effect and recovery

The matrix effect and recovery were evaluated by comparing the peak response of the post-extracted plasma samples and neat solution at high and low concentrations (LQC and MHQC) for each analyte. The matrix effect in plasma samples was between 98.2 and 104.9 % and the recovery between 105.3 and 115.7 % for all analytes (Table 5). Both the matrix effect and recoveries were similar at the high and low concentrations for each analyte, as shown in Table 5, indicating that the sample preparation procedure and analysis are reproducible across the concentration range. The %CV for post-extracted samples varied between 1.1 and 3.0 % at the high and low concentration for all analytes (data not shown), implying that the source of the plasma matrix does not affect the quantification of the analytes.

3.3.4. Dilution integrity and carryover

Dilution integrity was evaluated by preparing samples with concentrations exceeding the concentration of the highest calibration standard and diluting them fivefold or tenfold with blank plasma. The % bias for the fivefold and tenfold dilutions ranged between 102 and 108 % for all five analytes and was thus within the acceptance criteria of ± 15 % of the nominal concentration (Supplementary Table S4). The %CV for the fivefold and tenfold dilutions was between 3.7 and 7.5 % and therefore within the acceptance criteria of < 15 % (Supplementary Table S4). These results indicate that diluting the samples with concentrations above the highest calibration standard does not impact the accuracy and precision of the measurement.

After injection of the highest calibration standard, the carryover in the blank plasma sample was below 20 % of the LLOQ for all five analytes (Table 3).

3.3.5. Stability

Assessment of the stability of the five analytes in plasma and in working solution was carried out by analyzing LQC and MHQC samples (n = 6), and stability of the stock solutions of each analyte was evaluated in neat solution. The analytes were found to be stable in plasma after storage for 4 h at room temperature, at −80 °C for 12 months and after 5 freeze–thaw cycles at −80 °C. The analytes were also stable in working solution after 4 h at room temperature. Stock solutions of the analytes were found to be stable for 4 h at room temperature, at −20 °C for 12 months and after 5 freeze- thaw cycles at −20 °C. The results of the stability study are presented in detail in Table 6. The current method is therefore suitable for long-term storage and repeated freeze–thaw cycles.

3.3.6. Analysis of clinical samples

The applicability of the assay was demonstrated by analyzing plasma samples collected from six patients with APRT deficiency and 13 controls, using the optimized method. Samples from the APRT deficiency patients were collected both off and on treatment with either allopurinol or febuxostat. The MRM chromatogram of DHA in a plasma sample from an untreated patient revealed a peak corresponding to DHA at the appropriate retention time (Fig. 5**a**). DHA was not detected in a plasma sample from a healthy control (Fig. 5**b**), and the same results were observed for the other healthy control subjects (data not shown). The average concentration of DHA in plasma samples from six untreated APRT deficiency patients was 237.1 ng/mL (Fig. 6**a**). These results are in line with a previous report demonstrating serum DHA concentration ranging from 230 to 750 ng/mL in APRT deficiency patients, as well as undetectable DHA in serum samples from heterozygotes and healthy controls [19]. However, the method for measurement of serum DHA required a high sample volume (500 μL), and no internal standard was used in the analysis. The average plasma DHA concentration in the current study was 78.8 ng/mL in the patients treated with allopurinol (400 mg/day) and below the limit of quantification (BLQ) when febuxostat (80 mg/day) was used to treat the disorder (Fig. 6**a**). The average plasma adenine concentration was 295.5 ng/mL in the untreated patients, and 845.0 and 1046.4 ng/mL in those on treatment with allopurinol and febuxostat, respectively (Fig. 6**b**). Hence, the inhibitory effect of febuxostat, in the daily dose of 80 mg, on XOR seems to be greater than that of allopurinol 400 mg daily, reflected by a much lower plasma DHA concentration and higher plasma adenine concentration on febuxostat compared to allopurinol therapy. However, analysis of more plasma samples from patients with APRT deficiency is needed to confirm these observations.

While a handful of studies targeting the quantitation of either allopurinol and oxypurinol or febuxostat in urine or plasma have been reported [7–18], no published method exist for simultaneous quantification of the two drugs in a single assay. Furthermore, to our knowledge no methods exists for the simultaneous quantification of DHA, adenine, allopurinol, oxypurinol and febuxostat in plasma. Information on the concentration of DHA, adenine and the XOR inhibitors in plasma markedly enhances the monitoring of pharmacotherapy in APRT deficiency patients. More accurate monitoring would be expected to improve the dosing of XOR inhibitor therapy in these patients. Furthermore, the assay can be used for diagnostic purposes. Together with the urinary UPLC-MS/MS assay, the novel plasma assay will be valuable in the care of patients with APRT deficiency. Future work will focus on the correlation between plasma DHA concentration and urinary DHA excretion. Implementation of sample collection by microsampling devices is also a future perspective, as it would enable easier sample collection and shipment from remote locations [25].

4. Conclusion

The present work describes the development, optimization and validation of a UPLC-MS/MS assay for absolute quantification of DHA, adenine, allopurinol, oxypurinol and febuxostat in human plasma. A simple sample preparation procedure that includes protein precipitation provided consistent recovery across the concentration range for all analytes. The optimum conditions for the analytical method were found by using DoE and by exploring the relationship between the responses and the experimental factors, although a compromise had to be made regarding sensitivity, resolution and peak width. The assay was successfully validated according to the US FDA guidelines for bioanalytical method validation with respect to selectivity, sensitivity, concentration curve, accuracy, precision, recovery, matrix effect, dilution integrity, carryover and stability. The analysis of plasma samples from patients and healthy controls demonstrated the efficiency and accuracy of the assay as a tool for diagnosis of APRT deficiency and monitoring of pharmacotherapy. The proposed assay will be valuable for guiding XOR inhibitor treatment and thereby contributes to improved and more personalized care of patients with APRT deficiency.

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CRediT authorship contribution statement

Unnur A. Thorsteinsdottir: Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Conceptualization. **Hrafnhildur L. Runolfsdottir:** Writing – review & editing. **Finnur F. Eiriksson:** Writing – review & editing. **Inger M. Sch. Agustsdottir:** Data curation. **Vidar O. Edvardsson:** Writing – review & editing, Project administration, Funding acquisition. **Runolfur Palsson:** Writing – review & editing, Supervision, Funding acquisition. **Margret Thorsteinsdottir:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2024.124041.

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