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Quantification of uric acid, xanthine and hypoxanthine in human serum by HPLC for pharmacodynamic studies

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Abstract

A simple HPLC method was developed and validated for the determination of uric acid (UA), xanthine (X) and hypoxanthine (HX) concentrations in human serum to support pharmacodynamic (PD) studies of a novel xanthine oxidase inhibitor during its clinical development. Serum proteins were removed by ultrafiltration. The hydrophilic analytes and the I.S. were eluted by 100% aqueous phosphate buffer mobile phase. The hydrophobic matrix components (late peaks) were eluted with a step gradient of a higher organic mobile phase. Validation on linearity, sensitivity, precision, accuracy, stability, and robustness of the method for PD biomarkers (UA, X, and HX) was carried out in a similar manner to that for pharmacokinetic (PK) data where applicable. Issues of selectivity for endogenous biomarker analytes and individual concentration variations were addressed during method validation. Standards were prepared in analyte-free phosphate buffer. Quality control samples were prepared in control serum from individuals not dosed with the xanthine oxidase inhibitor. The method was simple and robust with good accuracy and precision for the measurement of serum UA, X, and HX concentrations.

Keywords: Uric acid; Xanthine oxidase inhibitor; HPLC; Pharmacodynamic biomarkers; Human serum

1. Introduction

Xanthine oxidase (EC 1.1.3.22) and the NAD^+-dependent xanthine dehydrogenase (EC 1.1.1.204) are inter-convertible forms of xanthine oxidoreductase in mammalian cells [1–4]. Xanthine dehydrogenase can be irreversibly converted to xanthine oxidase by proteolysis and cysteine-oxidation. Xanthine oxidase catalyzes the oxidation of hypoxanthine (HX) to xanthine (X) to uric acid (UA) (Fig. 1). The enzyme action is the rate-limiting enzyme for the formation of UA. In humans, xanthine oxidase is present predominantly in the liver and small intestine, although activities have been reported in plasma, the endothelium, bronchial wall, kidney and heart [5]. Xanthine oxidase plays an important role in the purine metabolism, and catabolizes xenobiotics as part of the detoxification process. The oxidative hydroxylation of the substrate occurs at the molybdenum center of the enzyme with subsequent reduction of O_2 to either H_2O_2 or O_2^- at the flavin center [6]. The generation of oxidants O_2^- and H_2O_2 during the enzymatic reaction may contribute to oxidative stress and tissue injury [7–11].

High concentrations of UA in blood (hyperuricemia) cause deposition of urate crystals, which could ultimately result in chronic joint inflammation and renal impairment. In addition, injury to membranes by urate, as a result of hyperuricemia, has been linked to the increase in platelet adhesiveness, inflammation, and smooth muscle cell proliferation [12–14]. Increases in cholesterol, UA and xanthine oxidase activity were observed in atherosclerotic carotid plaques [15]. The study of UA, X, and HX in serum would provide data on the homeostasis of the xanthine oxidase system and the disease condition of gout patients. For a drug development program of an inhibitor of xanthine oxidase, febuxostat, serum concentrations of UA, X, and HX could provide data for PD assessment of the drug effect and patient compliance, and for PK/PD modeling to guide drug development [16,17].

To date, the activity of xanthine oxidase has been determined by HPLC method by measuring the conversion of pterin to isoxanthopterin in human plasma by HPLC [18–21], radiometric

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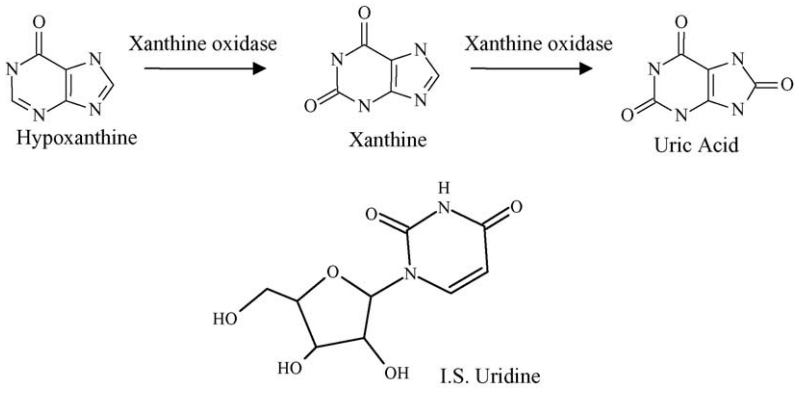

Fig. 1. Conversion of HX to X to UA by xanthine oxidase, and structure of uridine (I.S.).

[22], fluorometric [23,24] and ELISA [25] methods in serum. The serum/plasma activities were reported to be very low. An enzymatic method using pterin as substrate was reported for cell culture [26]. These methods used a relatively long incubation time with an artificial substrate. Two chromatographic methods were reported for UA, X, and HX in serum and two methods for urine [27–30]. These methods for xanthine oxidase activity or UA/X/HX measurements are subject to problems of low sensitivity, poor selectivity, and/or inefficient sample throughput. The determination of UA has been one of the tests in the clinical chemistry laboratory performed for patient diagnosis of gout. The methods usually involve enzyme reactions of uricase and peroxidase to generate H_2O_2 from serum UA, followed by colorimetric or fluorometric measurement of the oxidative product of a substrate [31–33]. A biosensor using immobilized enzymes has been developed recently to provide simple measurements [34]. Even though these assays used in clinical laboratories may provide accurate and precise measurement of UA concentrations, they do not provide homeostasis data on X and HX. Therefore, the development and validation of a multi-analyte assay with established sensitivity, selectivity, precision, and accuracy [36,37], capable of quantifying all three pharmacodynamic measurements (i.e. UA/X/HX) would provide homeostasis information as well as efficacy data and be of significant application during the clinical development of febuxostat.

The method development and validation of a PD biomarker assay can be more challenging and in some respects different from that of a drug analyte assay used in PK studies. Some of the specific considerations during development of an assay method for the measurement of PD biomarkers are: (1) the lack of analyte-free biological matrix for calibrator standard preparations; (2) the need to define analytical ranges based on subject data, as well as the biological variability of individual matrices; and (3) the application of spike recovery tests to demonstrate matrix-selectivity. Currently, there is no guidance for biomarker method validation in the updated Crystal City Conference report [38] or the FDA 2002 Industry Guidance [39]. Suggestions for biomarker method validation were made in the AAPS Biomarker Workshop conference report [40] and several other publications [41–43]. We used the current guidance for drug compound as a framework for method validation with special considerations on the PD biomarkers to demonstrate the method validity and suitability for the study objectives [35,39,40].

2. Materials and methods

2.1. Reagents

UA potassium salt, X, HX and the internal standard (I.S.), uridine, the caffeine metabolites 1-methyl X, 1,7-dimethyl X and 1-methyl UA were purchased from Sigma Chemical Co. (St. Louis, MO). The inorganic salts, acids and NaOH were purchased from Mallinckrodt (Paris, KY). Water was HPLC Grade from Fisher Scientific (Fair Lawn, NJ) or purified by NANOpure®, Barnstead system (Dubuque, IA). HPLC Grade acetonitrile (ACN) was purchased from Fisher Scientific (St. Louis, MO). Centrifree® YM-30 Filters were purchased from Amicon (Bedford, MA). Control serum was purchased from Biochemed (Winchester, VA) or Monobind Inc. (Costa Mesa, CA). Charcoal stripped serum was purchased from Biochemed.

2.2. Instruments

The HPLC system was comprised of a Waters (Milford, MA) model WISP 717+ autosampler, a Beckman 118 (Fullerton, CA) pump, a Waters 510 pump, a Rheodyne (Rohnert Park, California) 10-port column switch valve, and a Beckman 166 UV26 detector operated at 260 nm. The injection volume was 50 μl. The analytical column was a Shiseido (Tokyo, Japan) Capcell Pak C_{18}, UG120, 4.6 mm × 250 mm, 5 μm maintained at constant temperature by a Jones Chromatography (Lakewood, CO) heater/chiller set at 35 °C. A precolumn by Keystone (Bellefonte, PA), BDS Hypersil C_{18}, 10 mm × 2.0 mm was used. The configuration of the HPLC system setup is shown in Fig. 2. The column switcher was at position 1 when the sample was injected onto the column and eluted by a 47 mM KH_2PO_4 solution for ∼7.5 min using a Beckman 118 pump at a flow rate of 1.0 ml/min. After the UA peak was eluted, the column switcher was changed to position 2 eluting with the second mobile phase of ACN:47 mM KH_2PO_4 (50:50, v/v) at the same flow rate for ∼7 min. The pump's event output was connected to both the UV detector and the column switcher. The event times were programmed for switching position after the elution of UA peak

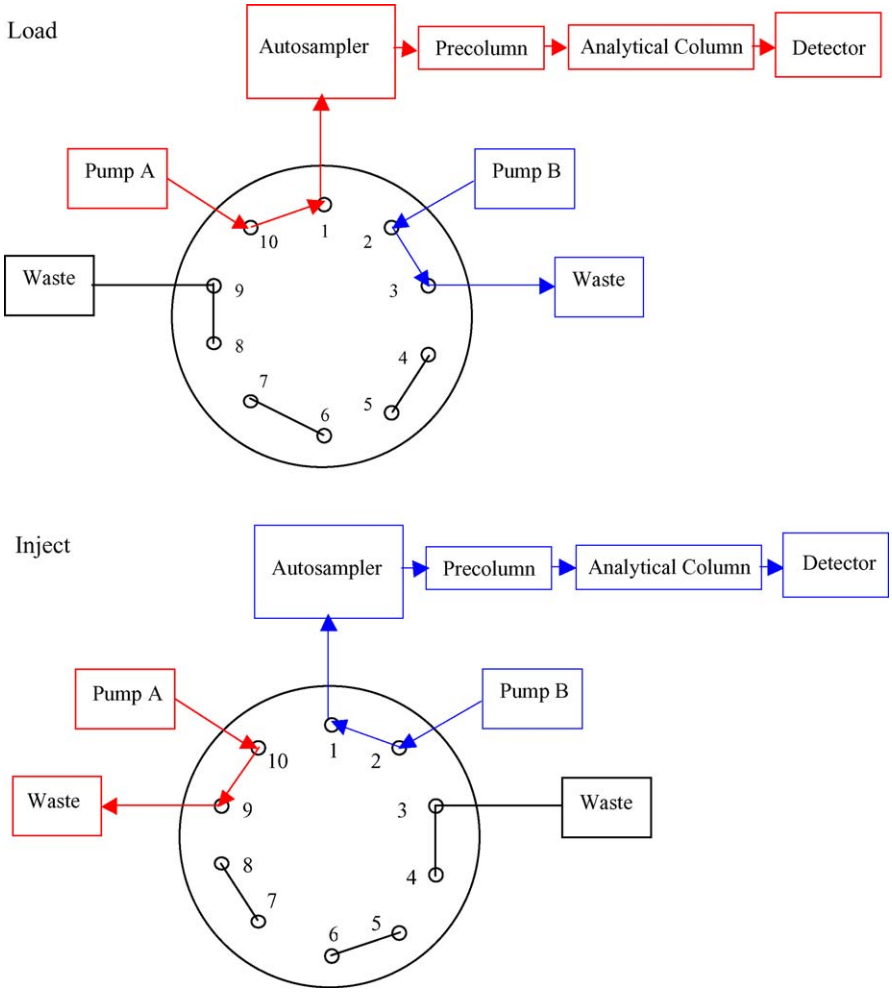

Fig. 2. HPLC system setup. Position 1 was for sample injection from the autosampler and elution using 100% phosphate solution from pump A. At approximately 7 min (after the UA peak), the column switch valve was changed to position 2 to 50% ACN in phosphate solution from pump B. The exact switch time and program events were determined by the system suitability samples prior to each analytical batch or day.

and the stopping of data acquisition before the high organic flow reached the detector. The stop time was ~19 min, with 2 min re-equilibration to the first mobile phase. The needle wash was ACN:H_2O (50:50, v/v). Several different lots of columns were used along the long history of the drug development program. The chromatographic retention times might vary slightly, but without change in the elution order or total separation of the compounds of interest. The second mobile phase of 50% ACN in phosphate solution was used to elute late interference peaks and maintain bonded phase stability. Prior to the injection of a standard curve, the retention time of the UA peak was determined at the beginning of each analytical batch (or day) to set the switch time. The preliminary test also included determination of late-eluting interference components of clinical samples. The test consisted of the following injections: system suitability solution, a clinical sample, and another system suitability. The chromatograms were evaluated for the effect of the clinical sample on the next system suitability sample. The late-eluting components were system and batch dependent; and the high organic elution time could be adjusted for the removal of the late-eluting components for that batch.

2.3. Solution preparation

A system suitability solution was prepared in non-matrix solution at concentrations of UA, X, HX, and I.S. (uridine) at 4.0, 4.0, 198, and 41 μM, respectively, in 47 mM KH_2PO_4. It was used for checking the suitability of the system before each batch. The primary standard stock solutions of X and HX at 1000 μM were prepared in 0.1 N NaOH after weight correction for purity. The standard stock solutions of UA at 5000 μM were prepared in phosphate buffer. Working standards were prepared by adding the appropriate volumes of UA/X/HX standard solution into volumetric flasks and diluting with 47 mM KH_2PO_4. Because the analytes were endogenous compounds, variable concentrations were found in control serum from individuals not dosed with the xanthine oxidase inhibitor. Standards were prepared in analyte-free phosphate solution solutions at eight different concentrations from 10/0.2/0.2 to 1000/20/20 μM of UA/X/HX and were stored at −20 °C for up to 44 weeks. Fifty microliters of working standard was spiked into 100 μl of water to yield the final standard concentration.

Table 1
Design of UA, X, and HX standards and QC

	Serum (μM)	
	Standard Range	QC concentrations
UA	10–1000	29.6, 150, 750
X	0.2–20	0.57, 3, 15
HX	0.2–20	0.57, 3, 15

QC samples were prepared by spiking known amounts of analytes into a pool of sera that had the lowest observed analytes concentrations. The low QC values were determined by the validation mean, while the mid and high QCs were nominal values.

A separate weighing of each analyte from that of the standard stock was used for quality control (QC) primary stock solution preparation. QCs were diluted with pre-screened human serum from individuals that had the lowest concentrations of the analyte. QCs at low, middle and high concentrations were prepared; their concentrations were ~30/0.6/0.6, 150/3/3, and 750/15/15 μM of UA/X/HX, respectively. Because the endogenous analytes contributed substantially to the low QC concentrations, only approximate concentrations of the low QCs could be estimated initially. Therefore, their real values were estimated to be the mean of the predictions obtained for the low QC during the prestudy validation step. For the middle and high QCs, the contribution of the endogenous analytes was insignificant, and nominal values of the spiked concentrations were used. The QCs were distributed in 0.25 ml aliquots in Sarstedt tubes and stored with the clinical samples in a freezer at −20 °C. The standards and QC concentrations of the analytes are listed in Table 1.

2.4. Assay procedures

All solutions, reagents, standards, QCs and samples were brought to room temperature before being assayed. An aliquot of 100 μl of study samples, QC or working standard was introduced into a 13 mm × 100 mm tube after 50 μl of working I.S. had been introduced and dried down in the tube. After 350 μl of 47 mM KH_2PO_4 was added, the tubes were mixed by vortexing approximately 1 min. The samples were transferred to Amicon Centrifree® filters and centrifuged for 30 min in a fixed angle centrifuge. Serum proteins were retained by the filter while the analytes passed through the filter. The filtrates were transferred to autosampler vials and 50 μl was injected onto the HPLC.

2.5. Analytical data treatment

Chromatograms were integrated using a VG® Multichrom data system for VAX®/VMS. Raw data was subsequently transferred into the VAX®/VMS Oracle® database. A weighted [(1/x) where x = analyte concentration] linear regression was used to determine slopes, intercepts and correlation coefficients. The resulting parameters were used to calculate concentrations:

$$\text{Concentration} = \frac{\text{Ratio} - (y - \text{intercept})}{\text{Slope}}$$

where "Ratio" is the ratio of the compound peak area to the internal standard peak area.

The extraction recovery was calculated from the peak area counts of the control sample with analyte added to the sample (prior to the ultrafiltration) versus that to the filtrate (after the ultrafiltration).

3. Results and discussion

3.1. Method development

The extraction recovery was 95.8% (CV ≤ 4.4%) for UA over a concentration range of 10–1000 μM, 98.3% (CV ≤ 7.8%) for X and 101.0% (CV ≤ 8.0%) for HX over a concentration range of 0.2–20.0 μM. The recovery of the I.S. was 99.6% (CV ≤ 4.5%). The high recovery values indicated that none of the analytes were significantly bound to serum proteins, or adsorbed to the ultrafiltration filter or test tube walls. Protein precipitation with an organic solvent and liquid/liquid extractions with various organic solvents were attempted. All of the methods, with the exception of the ultrafiltration, resulted in interference peaks at the retention times of the analytes.

The HPLC method was based on Kock et al. [27] with modifications. The analytical column and precolumn were optimized using current technology for better performance and assurance of consistent quality. Several different analytical columns, mainly C_{18} columns from various manufacturers were tested. The Shiseido Capcell column gave the most reproducible and consistent retention. This column also gave adequate resolution between all of the analytes and the I.S., as shown in Fig. 3. The hydrophilic analytes and I.S. were eluted by the 100% aqueous mobile phase of 47 mM KH_2PO_4. A second mobile phase, 50% ACN in 47 mM KH_2PO_4 was used to elute late interference

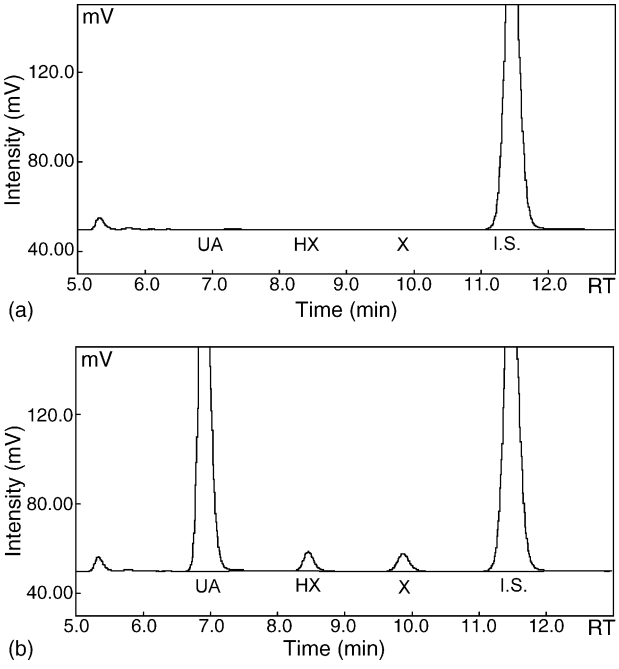

Fig. 3. Representative chromatograms of samples from (a) charcoal-stripped serum with I.S. and (b) charcoal-stripped serum spiked with UA/X/HX at 500/10/10 μM. The retention times were 6.8, 8.4, 9.8, and 11.4 min for UA, HX, X, and I.S., respectively.

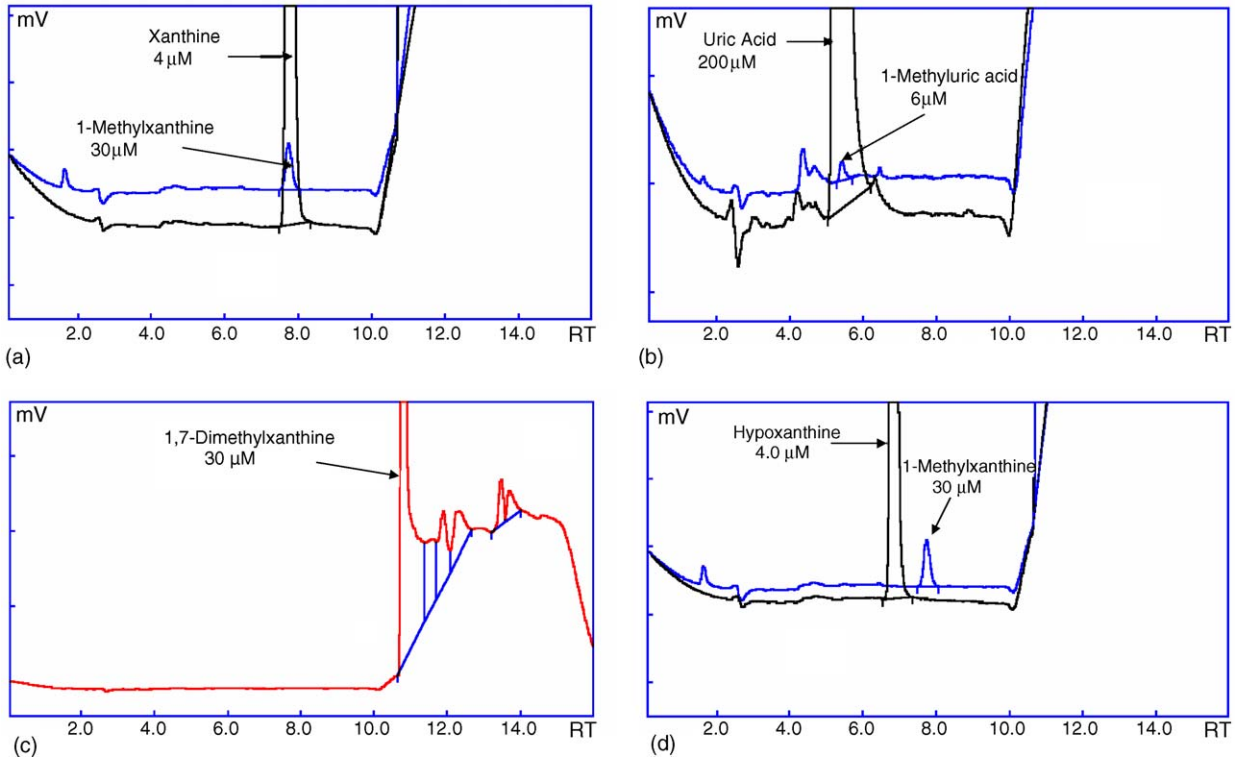

Fig. 4. Chromatographic tests for caffeine metabolite interference on UA, X, and HX. (a) Overlaid chromatograms of 4 μM X (peak area counts 415,496 mV) and 30 μM 1-methyl-X (peak area counts 15,421 mV). (b) Overlaid chromatograms of 4 μM UA (peak area counts >9,000,000 mV) and 60 μM 1-methyl-UA (peak area counts 1633 mV). (c) Chromatogram of 1,7-dimethyl-X. (d) Overlaid chromatograms of 4 μM HX and 30 μM 1-methyl-X.

peaks using a column-switching valve. In addition to cleaning up the column, the higher organic in the second mobile phase also maintained the C_{18} bonded phase in good condition to avoid the bonded phase collapse after continuous elution with a total aqueous mobile phase [44]. Kock's method used 100% phosphate buffer. The guard columns had to be changed every 30th injection; the run time was 20 min. The stability of the analytical column was not discussed. The HPLC method on allantoin, UA, X, and HX in ovine plasma by Czanderna et al. [28] used protein precipitation by $HClO_4$ and gradient elution of 10–95% aqueous phosphate solution mobile phase from C_{18} columns. The retention times of UA/X/HX observed in our results were similar to those observed by Czanderna et al. [28]. However, their method had a longer gradient and re-equilibration time of 60 min.

3.2. Approaches of method development and validation of biomarkers

Since UA, X, and HX are endogenous compounds, two issues needed to be addressed during the planning for method development of this type of endogenous biomarker assays [35,40,42,43]. First, "analyte-free" reference matrices would not be available for standard and quality control sample preparation. Second, the design of the standard curve ranges to encompass the expected analytes concentrations is not as straight forward as that of a drug analyte since the disease state (i.e. gout) itself could have an impact on the concentrations of the biomarker of interest. A survey of literature information for the biomarker was initially used to estimate the potential range of concentration in clinical samples. The reported reference ranges from 171 healthy normal subjects were 151–442 μM, 0.2–5.8 μM, and 1.2–17.9 μM for UA, X, and HX, respectively [27]. Thus, the standard and QC sample concentrations in Table 1 were designed to cover the expected concentration range in clinical study samples. Next, the concentrations of analytes were determined in serum samples from multiple individuals. Serum QC samples were then prepared by spiking known amounts of each analyte into a pool of serum from individuals that had the lowest concentrations of endogenous analytes. Because of the likely contribution of basal analyte at a concentration below the lowest standard, the true concentration of the low QC was estimated to be the mean of the predicted concentrations obtained for the low QC during the prestudy validation part. The nominal spiked concentrations were used for the middle and high QCs. Standards were prepared by 'spiking' into a non-matrix phosphate solution instead of the serum matrix. For each method, the accuracy (mean bias) and inter-assay coefficient of variation (CV) for each QC were assessed with the a priori 15% acceptance limits. The in-study run acceptance criteria (4–6–15 rule) was the same as that used to support bioanalytical assays of the investigational drug, i.e. 67% of QC results had to be within 15% of the target values [36,38,39].

3.3. Selectivity tests

For bioanalysis of drug compounds, the standards are to be prepared in an analyte-free biological matrix representative of

the study samples [36,38,39]. However, all of the compounds of interest in this PD marker study are endogenous and we were unable to find human sera depleted of the analytes. Therefore, the calibration standards were prepared in phosphate solution. Spike-recovery tests were carried out to test if the quantification in serum would be precise and accurate against standard calibrators in phosphate solution and that there was insignificant lot-to-lot variability. Multiple serum lots were spiked with UA/X/HX at 500/10/10 μM, respectively, for the test. Because of the structural similarity of X and HX to caffeine (1,3,7-trimethyl X), three major metabolites of caffeine from hydroxylation, oxidative demethylation by cytochrome P-450 1A2 and xanthine oxidase metabolism [45–47] were tested for possible chromatographic interference on the HPLC system. The results showed that 1-methyl-X at 30 μM (approximately 30-fold the expected serum concentration) contributed as a small interference peak at the retention time of X as depicted in the overlaid chromatograms of X and 1-methyl-X in Fig. 4a. The UV response in mV peak area counts of 30 μM 1-methyl-X was ∼1/30 of that of 4 μM X. The overlaid chromatograms in Fig. 4b show a small interference to UA from 1-methyl-UA at 60 μM (approximately 60-fold the expected serum concentration). The signal intensity of the 200 μM UA was greater than 5000-fold of that of 60 μM 1-methyl-UA. Fig. 4c shows that 1,7-dimethyl-X was eluted after 10 min by the high organic mobile phase step gradient. No interference was observed at the retention time of HX from the caffeine metabolites tested (Fig. 4d). The data indicated that even though the two caffeine metabolites have the potential to artificially contribute to higher serum X and UA concentrations, any over estimations are not expected to be clinically significant (less than 1 and 0.001% overestimation for X and UA, respectively) or measurable at concentrations observed in caffeine users [48].

Additional selectivity tests were conducted for serum samples from normal individuals. Representative chromatograms of charcoal stripped serum and unaltered serum samples are shown in Figs. 3a and 5a, respectively. Compared to the charcoal stripped serum, there were variable amounts of endogenous analytes among the unaltered sera. Unlike the blank control samples of drug compounds, due to the presence of endogenous amounts of PD biomarkers, it would not be possible to establish assay selectivity based on zero signal response at the analytes' retention times. Therefore, spike recovery test was performed to demonstrate method accuracy as well as the lack of matrix effect and interference from the endogenous analytes. UA/X/HX were spiked into the samples of unaltered and charcoal stripped serum. Eleven lots of unaltered serum and three lots of charcoal-stripped serum were tested. Representative chromatograms of the spike samples were presented in Figs. 3b and 5b for charcoal stripped serum and unaltered serum samples, respectively. The unspiked and spiked samples were analyzed. The spike recovery was calculated by subtraction of the unspiked basal values from the spiked values. The data were presented in Table 2 for UA, Table 3 for X and HX. The accuracy and precision of the spike recovery in all unaltered serum and stripped serum samples were acceptable. The apparent basal values range was 153–448 μM for UA, 0.93–4.63 μM for X, and 0.47–79.4 μM for HX in the 11

Table 2
Spike recovery test of UA in human serum

Serum lot number	Basal (unspiked)	STD500 spiked	STD500-basal
Healthy individual sera, $n = 11$			
449	389	886	497
450	311	807	496
451	439	922	483
452	366	864	498
453	448	951	503
454	259	751	492
377	275	742	467
378	305	812	507
380	263	743	480
432	153	647	494
433	344	886	542
Mean	323	819	496
CV%	20.9	9.17	2.40
RE%			−0.8
Charcoal stripped sera, $n = 3$			
448	0.000	500	500
447	0.000	502	502
431	0.000	496	496
Mean	0.00	499	499
CV%			0.6
RE%			−0.1

Serum from individual lots: 11 from healthy individuals and 3 from commercial lots that were stripped with charcoal in an attempt to remove the analytes. Concentrations in μM for samples of unspiked, spiked with standard 500, and calculated value after subtraction.

Table 3
Spike recovery test of X and HX in human serum

Serum lot number	Basal (unspiked)		STD10 spiked		STD10-basal	
	X	HX	X	HX	X	HX
Healthy individual sera, $n = 11$						
449	1.19	0.47	10.8	10.2	9.61	9.73
450	1.06	0.56	10.8	10.6	9.74	10.0
451	1.15	0.78	11.4	10.8	10.3	10.0
452	1.15	1.64	11.0	11.6	9.85	9.96
453	0.929	0.63	11.0	10.6	10.1	9.97
454	1.11	1.25	11.0	11.3	9.89	10.1
377	4.63	79.4	14.1	92.8	9.47	13.4
378	2.79	15.1	13.2	26.3	10.4	11.2
380	3.33	74.7	12.9	85.1	9.57	10.4
432	3.46	13.0	13.6	23.0	10.1	10.0
433	3.33	1.90	14.6	12.8	11.3	10.9
Mean	2.19	17.22	12.22	27.74	10.03	10.51
CV%	54.5	126	10.89	80.3	3.72	6.84
RE%					0.3	5.1
Charcoal stripped sera, $n = 3$						
448	0.010	0.000	10.2	10.4	10.2	10.4
447	0.073	0.000	10.3	10.5	10.2	10.5
431	0.027	0.000	10.4	10.5	10.4	10.5
Mean	0.037	0.000	10.3	10.5	10.3	10.5
CV%					0.943	0.6
RE%					2.6	4.7

Serum source: 11 from healthy individuals and 3 from commercial charcoal-stripped serum. Concentrations in μM for samples of unspiked, spiked with standard 10, and calculated value after subtraction.

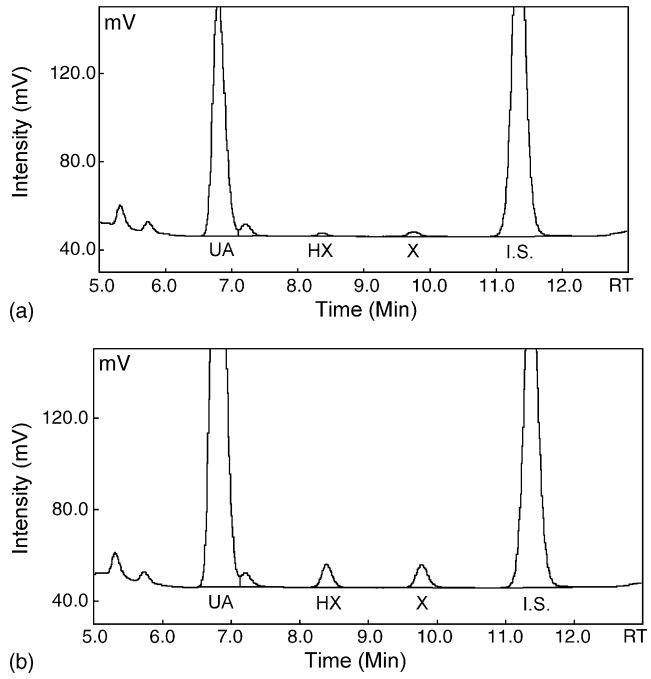

Fig. 5. Representative chromatograms of samples from (a) unaltered serum with I.S. and (b) unaltered serum spiked with UA/X/HX at 500/10/10 μM.

lots of serum. Thus, the standard curve range of 10–1000 μM for UA, 0.2–20 μM for X and HX would appropriately cover the expected concentrations of study samples except for two HX samples.

The spike-recovery precision for all analytes was very tight (1.2–11.8 CV%) despite their highly variable basal levels. The analytes were not detectable or at minimal amount in the charcoal-stripped sera; all spike recoveries were accurate and precise. All lots were quantitated within 15% of the theoretical spiked concentration when regressed against the calibration standards, validating that phosphate solution could be used for the preparation of standard calibrators. The data also showed that accurate and precise quantification would not be affected by matrix lot differences or from individual subjects.

Table 5
Precision and accuracy of interday phosphate solution and urine QC

	29.6 μM	150 μM	750 μM
Uric acid			
Mean	28.7	146	744
CV%	3.5	2.8	3.3
RE%	NA	−2.4	−0.8
	0.57 μM	3.0 μM	15 μM
Xanthine			
Mean	0.564	3.1	15.3
CV%	5.5	2.7	3.2
RE%	NA	3.3	2.2
Hypoxanthine			
Mean	0.567	3.13	15.5
CV%	9.2	4.1	3.6
RE%	NA	4.4	3.6

Data from eight validation batches, six replicates from each batch run. NA, not applicable, due to the endogenous contribution to low QC. The validation mean was used as the target value for in study monitoring.

3.4. Accuracy and precision

Eight pre-study validation runs with six determinations for each QC within a run were conducted to assess the analytical performance. The validation data presented in Table 4 shows good precision and accuracy of standards. CV at the low limit of quantification (LLOQ) was ≤7.1%, and relative error (RE) ≤2.1%. The precision and accuracy of QCs were shown in Table 5. CV was ≤9.2%, and RE ≤4.4%. Note that the endogenous analytes in the low concentration QCs values were determined by the validation mean. The absolute accuracy for the low QC could not be available during prestudy validation. Once the target values were determined by the prestudy validation, the values were used for in-study validation on clinical sample analysis to monitor accuracy, precision and stability. For the mid and high QCs, the

Table 4
Precision and accuracy of analyte standards

Standards	10.0 μM	25.0 μM	50.0 μM	100.0 μM	250.0 μM	500.0 μM	800.0 μM	1000.0 μM
UA								
Mean	10	25.3	50.3	98	249	502	790	1010
CV%	4.6	1.4	2.2	2.9	3.9	2.9	3.5	4.0
RE%	0.4	1.1	0.6	−2.0	−0.3	0.4	−1.2	1.0

Standards	0.2 μM	0.5 μM	1.0 μM	2.0 μM	5.0 μM	10.0 μM	17.5 μM	20.0 μM
X								
Mean	0.196	0.506	1.02	1.96	5.03	10.1	17.2	20.2
CV%	3.6	5.2	2.7	2.7	3.6	2.4	3.3	3.4
RE%	−2.1	1.3	1.8	−2.1	0.6	0.9	−1.9	1.2
HX								
Mean	0.200	0.506	0.994	1.97	5.01	10.2	16.9	20.5
CV%	7.1	2.6	2.6	2.8	3.7	2.7	3.5	4.0
RE%	0.1	1.2	−0.6	−1.4	0.2	1.6	−3.6	2.5

Data from eight validation batches.

Table 6
Analytes stability in human serum and processed samples

	Period	As percent of control		
		UA	X	HX
In human serum				
Benchtop	22 h at RT under white light	99–101	99–106	94–100
Freeze/thaw	Three cycles	97–100	98–99	95–99
Storage	79 weeks at −20 °C	84–104	99–105	96–105
In processed sample				
Reinjection in HPLC	40 h	95–100	98–100	93–99
Refrigeration	65 h	100–101	99–100	91–99

basal concentrations were insignificant for the known amount of analyte added, their nominal values were used for accuracy assessments during prestudy and in-study validation.

3.5. Analyte stability

The stability of the analytes in serum were tested for room temperature exposure on benchtop under white lights, after three cycles of freezing and thawing and after one and a half year of storage at −20 °C. As shown in Table 6, the analytes were stable under all the conditions tested, which were within the environments expected for the study samples. Stability of the analytes in processed samples was also established as shown in Table 6.

3.6. Method robustness

Method robustness was established by performing prestudy and in-study validation tests for instrumentation and sample handling procedures. For prestudy validation, column stability was tested over long analytical runs of up to 112 injections. The retention times were found to be stable throughout the run. Two additional HPLC systems were validated, consisting of an autosampler, pumps, and a detector from either a different manufacturer or a different model from the first system. Five analytical columns of different commercial lot were used in the prestudy validation to test column variability. The defined procedures of the system suitability tests at the beginning of analytical batch or day provided consistent results between days, batched and system set-ups. The robustness of the sample processing during prestudy validation was established by two extraction analysts performing acceptable validation runs using two lots of Amicon Centrifree® filters. For the in-study validation, the HPLC method was applied to more than 7500 serum sample analyses in clinical trials, which utilized eight chemists, more than eight lots of analytical columns and at least four HPLC systems. Assay performance was maintained due to the simplicity of the sample extraction method and the well-defined process of system suitability test prior to sample injections.

4. Application to clinical sample analysis

The method was able to distinctly show differential changes in the concentrations of UA, X, and HX between the pre-dosed and the post-dosed samples following oral administrations of febuxostat in a clinical study. Fig. 6 shows a representative concentration time profile of UA, X, and HX prior to and following once daily oral dosing with 70 mg of febuxostat. Serum samples were taken at 0, 6, 12, and 24 h prior to the first dose for baseline measurements and post-dose on Days 1, 8, and 14. Fig. 6

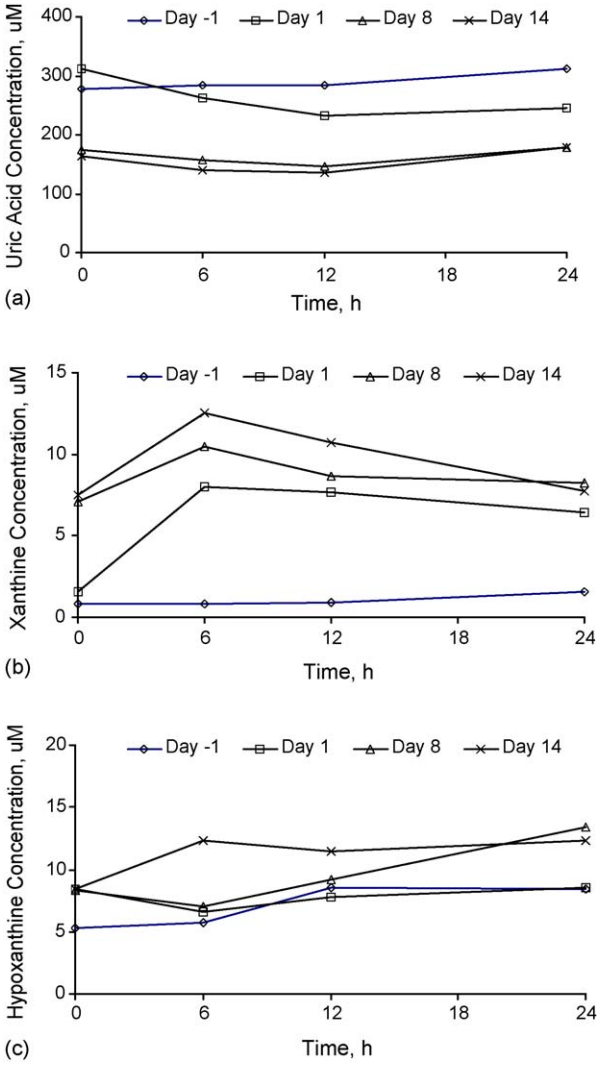

Fig. 6. Representative serum concentration time profiles of UA, X, and HX in a healthy subject on Days −1 (baseline), 1, 8, and 14 following oral dosing with febuxostat 70 mg once daily on Day 1 and Days 3 through 14.

indicates that the concentrations of UA in serum decreased even after a single dose of febuxostat. In conjunction with the decrease in serum UA concentrations there was an increase in serum X concentrations. Interestingly, there appeared to be no substantial increase in serum HX concentrations possibly due to the increase in the renal clearance of HX as well as a decrease in production of HX through HX to inosinate salvage pathway and subsequent feedback inhibition of the amidotransferase [48–50]. The results showed that the bioanalytical method is sensitive with a suitable assay range for selective determinations of UA, X, and HX in serum samples before and after dosing, and capable of evaluating the PD effects of the novel drug, febuxostat. The method is shown to be robust since it was applied to thousands of clinical samples collected from phases I–II study subjects.

5. Conclusions

A simple, robust and reliable HPLC-UV method was developed and validated for the determination of UA, X, and HX in human serum. This method validation was appropriately designed and carried out, addressing issues of biomarkers bioanalysis such as: (1) sample concentration ranges; (2) variation of endogenous concentrations of analytes in the biological matrix; (3) substitution of analyte-free matrix for standard preparation; and (4) potential matrix lots interference. The ultrafiltration method to remove serum proteins was simple and provided cleaner sample preparations than protein precipitation. The HPLC procedure, which encompasses a high organic step gradient, removed late-eluting interfering materials and maintained the bonded phase in good conditions. The run time was relatively short for good sample throughput. We validated a method for the measurement of serum concentrations of UA, X, and HX which provides homeostasis and efficacy data in clinical trials evaluating febuxostat. The method application demonstrated that the method was simple, with good throughput, accuracy, and precision, and robust to meet the demands of bioanalysis of large numbers of clinical samples.

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