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Simultaneous Determination of Uric Acid, Xanthine and Hypoxanthine in Human Plasma and Serum by HPLC–UV: Uric Acid Metabolism Tracking

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Abstract A simple reversed phase HPLC method with UV detection in isocratic conditions was developed and validated for the simultaneous determination of hypoxanthine, xanthine and uric acid levels in human plasma and serum. One analysis run takes 6.5 min including a short organic mobile phase gradient for column regeneration. Concentrations of uric acid, xanthine and hypoxanthine in plasma and serum samples were highly comparable. However, hypoxanthine levels were increased in serum compared to plasma samples due to a prolonged time between serum and blood elements separation. The method was validated for linearity, precision, accuracy, sensitivity and robustness in a similar manner to that for pharmacokinetic data and it is appropriate for physiological and pathophysiological levels of all analytes. The stability of stock standard solutions was verified using spectrophotometric analysis in different conditions. The method is simple and robust with a good precision for the measurement of hypoxanthine, xanthine and uric acid in human plasma and serum.

Keywords HPLC · Uric acid · Xanthine · Hypoxanthine · Plasma · Serum

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Introduction

Determination of uric acid (UA) is highly relevant nowadays, as elevated levels are markers of pathological mechanisms involved in a plethora of diseases. UA is generated by enzymatic conversion of hypoxanthine (HX) and xanthine (X) catalyzed by xanthine oxidoreductase (XOR) as a terminal and rate limiting step of purine degradation in the higher primates [1]. The mammalian enzyme exists predominantly in a form of NAD^+ dependent dehydrogenase (EC 1.17.1.4) which does not form reactive oxygen species, however, the enzyme can be converted to a dioxygen dependent xanthine oxidase (EC 1.17.3.2) by several mechanisms including reversible sulfhydryl oxidation or irreversible limited proteolysis. The dioxygen dependent form can produce reactive oxygen species (superoxide anions and hydrogen peroxide) [2] and, therefore, it is responsible for cytotoxicity due to the formation of hydroxyl radicals [3]. On the other hand, UA is considered as an important antioxidant in vivo with a significant antioxidant capacity. In spite of being a terminal waste product of purine metabolism, UA is extensively reabsorbed (approximately 90%) in the kidneys, and thus maintained in relatively high levels in blood [4]. The protection against oxidative stress provided by UA has been proposed as one of the reasons for increased life span in humans [5].

An increase in XOR activity has been reported in several pathological conditions including rheumatic and auto-immune diseases, pneumonia, schizophrenia, several viral diseases and after certain transplantations and surgeries [3]. Hyperuricemia is associated not only with gout, urate nephrolithiasis and tumor lysis syndrome but also with atherosclerosis and vascular diseases associated with metabolic syndrome (high levels of UA are commonly clustered with visceral obesity, hypertension, dyslipidemia, insulin

resistance and type 2 diabetes) [6], and its advanced consequences such as stroke, infarction [3] and kidney dysfunction [7]. UA metabolism disturbances can be treated by available and efficient inhibitors of XOR. The understanding of the role of terminal metabolites of purine metabolism and monitoring of XOR activity as the most pharmacologically targetable source of free radicals provides the need for extensive exploration and it requires fast and simple method for analyzing HX, X and UA levels which is suitable for determination in both human plasma and serum.

The determination of serum UA in clinical laboratories usually involves enzyme reactions with uricase and peroxidase followed by colorimetric or fluorimetric measurement of a reaction product [8]. One of the drawbacks of this method is that plasma samples containing EDTA (neither citrate nor oxalate) cannot be used because uricase requires divalent ions for preserving optimal conditions. Furthermore, the uricase method does not provide data on HX and X levels reflecting XOR activity. Finally, since X is a strong competitive inhibitor of uricase and its increased levels can result in false positive elevated uricemia [9], indeed, hyperxanthinemia has also been associated with hyperuricemia [10]. Moreover, if the direct peroxidase method is used bilirubin [11], hemoglobin, ascorbic acid, glutathione and hyperlipidemia interfere [12].

Simultaneous determination of HX, X and UA using reversed phase HPLC (C18) under various conditions has been described by several authors [13–21], however, the previously reported methods were time-consuming (15 min or more per sample) with long analytical columns (250 mm or longer) and more complicated conditions of separation and furthermore, none of them compared levels of HX, X and UA in plasma and serum samples. Recently, the use of mass spectrometry for UA detection in plasma samples was described by Zhong et al. [22] and Stentoft et al. [23], however, these methods require both rather complex procedure and equipment. Moreover, coefficients of variation are higher than they should be (Zhong et al. <10%, Stentoft et al. ≤25%). The aim of this study was to develop fast and simple reversed phase HPLC method for the simultaneous determination of HX, X and UA and to validate its use for both human plasma and serum.

Materials and Methods

Reagents

All standards were purchased from Sigma Aldrich (St. Louis, MO, USA), all other chemicals were purchased prom Fluka (Buchs, Switzerland) and were of analytical grade. HPLC-grade acetonitrile was purchased from Scharlau Chemie S. A. (Barcelona, Spain). Water was purified using Milli-Q system (Merck Millipore, Darmstadt, Germany). The lyophilized two-level control PreciControl ClinChem based on human serum was manufactured by Roche Diagnostics (Mannheim, Germany). The plasma and serum samples were obtained from 15 healthy volunteers (median age 28, interquartile range (IQR) [25–35], 67% of women) and frozen at −70 °C for one month.

Equipment

HPLC was performed on a Shimadzu LC-10 Series (Shimadzu, Kyoto, Japan) equipped with Kinetex EVO C18 core–shell column (150 × 4.6 mm id, particle size 5 μm) protected by SecurityGuard ULTRA Cartridges UHPLC C18 (Phenomenex, Torrance, CA, USA). Two other columns Luna 3u C8(2) 100A and Kinetex 2.6u C18 100A were tested (both Phenomenex, Torrance, CA, USA). The guard column was replaced after 200 injections to avoid significant reduction in separation properties of column. UV-detection was performed with a photodiode array detector SPD-M10A (Shimadzu, Kyoto, Japan). The calibration and data processing was based on the peak heights for HX and X and on the peak areas for UA carried out with LabSolutions software (Shimadzu, Kyoto, Japan). The flow-rate was 1 mL min^{-1} corresponding to a back pressure 10.5 MPa. Stability of standard solutions was verified using a Spectronic Helios Gamma spectrophotometer (Thermo Electron Corporation, Beverly, MA, USA).

Method

Because of limited solubility of UA, the stability of standard solutions was checked spectrophotometrically using their maximum absorption wavelengths. Absorption maxima of HX, X and UA in a solution of 100 mmol L^{-1} potassium dihydrogen phosphate buffer (pH 1.5) were determined at 248 nm, 263 nm, and 282 nm, respectively. Stock standard solutions (1 mmol L^{-1}) were prepared (i) in solutions of sodium, potassium and ammonium hydroxide with different concentration (1 mol L^{-1}, 100 mmol L^{-1} and 10 mmol L^{-1}), (ii) in a solution of 1.5 mmol L^{-1} sodium hydroxide, (iii) in solutions of 1.5 mmol L^{-1} sodium hydroxide supplemented with magnesium ions (15 μmol L^{-1} to 1 mmol L^{-1}) or disodium salt of EDTA (100 μmol L^{-1} to 5 mmol L^{-1}) for all standards and (iv) in a solution of 15 mmol L^{-1} sodium hydroxide supplemented with 150 μmol L^{-1} magnesium ions for the stock solution of X only. Stock solutions of all analytes under conditions (i) to (iv) were diluted to a working concentration of 50 μmol L^{-1} with 100 mmol L^{-1} phosphate buffer pH 1.5 and the absorbance was measured. The conditions providing the best stability of analytes were

Table 1 Previously used conditions for the preparation of standard stock solutions

Analyte (mM)		Solvent used for stock solutions*	Frozen	Reference
HX, X	1	100 mM NaOH	Yes	[13]
UA	5	47 mM phosphate buffer	Yes	[13]
HX, X	0.5	100 mM NaOH	No	[14]
UA	0.5	13 mM NH_3	No	[14]
HX, X, UA	1	Water with addition of 1000 mM NaOH	Yes	[15]
HX	0.5	Water	No	[19]
X, UA	0.5	Alkalinized hot water pH > 10	No	[19]
HX, X	6	20 mM NaOH	Yes	[20]
UA	6	2.6 mM Li_2CO_3	Yes	[20]
HX, X	1	Water alkalinized with Li_2CO_3	No	[21]
UA	1	Saturated Li_2CO_3	No	[21]
HX, X, UA	Unknown	2 mM NH_3	No	[22]
HX, X	0.5 and 5	Water	Yes	[23]
UA	0.5 and 2	Water	Yes	[23]

* The concentrations of ions are stated as described in original papers

chosen as follows: HX and UA standards were dissolved in 1.5 mmol L^{-1} sodium hydroxide solutions containing 150 μmol L^{-1} magnesium chloride and X was dissolved in 15 mmol L^{-1} sodium hydroxide solution containing 150 μmol L^{-1} magnesium chloride to form 1 mmol L^{-1} stock solutions. Stock solutions were kept at room temperature in dark while stirring under argon atmosphere for 5 days maximally. Working standard solutions were freshly prepared by diluting the stock solutions with 1.5 mmol L^{-1} sodium hydroxide containing 150 μmol L^{-1} magnesium chloride each day.

The EDTA plasma was separated by centrifugation (1000g, 10 min, 4 °C). Serum samples were separated after 30 min of hemocoagulation at room temperature using centrifugation (2000g, 10 min, 4 °C). All samples were stored until analysis at −70 °C. Proteins in plasma/serum samples were precipitated with 7.2% perchloric acid (1:1, v/v), vortexed for 30 s, incubated 10 min on ice, vortexed again for 30 s, incubated 5 min on ice and finally centrifuged (12,800g, 6 min, 4 °C). Blank and working standard solutions were mixed (1:1, v/v) with a solution of 7.2% perchloric acid containing 0.25 mol L^{-1} sodium hydroxide to reach the same pH as plasma/serum samples after protein precipitation (pH 2.5). Optimal injection volume of the sample onto column was determined as 20 μL.

The separation of HX, X and UA was performed at 30 °C under isocratic conditions, using a mobile phase consisting of 100 mmol L^{-1} potassium phosphate buffer (pH 1.5). The column was regenerated using acetonitrile gradient after each run. Conditions of separation and following column regeneration were set as: 1.5 min 100% of mobile phase, followed by a step to 70% of mobile phase and 30% of acetonitrile for 2 min to regenerate the column, followed by a step to 100% of mobile phase for 3 min to equilibrate the initial conditions. The complete analysis time was 6.5 min only. The flow-rate was set to 1 mL min^{-1}.

Photodiode array detector with semi-microcell was set to the wavelengths range 245–295 nm. HX, X were quantified at their absorption maxima (248 and 263 nm, respectively), but UA was quantified near to its absorption maximum (292 nm), because the peak for X was not apparent at this wavelength. All analytes were quantified using external standards.

Results and Discussion

Standard Stock Solutions

UA is virtually insoluble in water and common organic solvents. Its solubility increases with increasing pH and the solubility of monosodium urate the most common form of UA under physiological conditions increases with ionic strength [24]. Thus, it is recommended by the manufacturer (Sigma Aldrich–Product Information U0881) to dissolve UA in 1 mol L^{-1} sodium hydroxide. However, the stability of UA standard stock solution was very low (more than 15% decline each day) under these conditions. Also the stability of frozen standard stock solutions was unsatisfactory.

Standard solutions of analytes of interest were diluted under different conditions by other authors as summarized in Table 1. Several results from articles concerned with simultaneous determination of HX, X and UA are in correlation with our data. Czauderna et al. [16] showed that approximately 1 mmol L^{-1} UA is decomposed even by
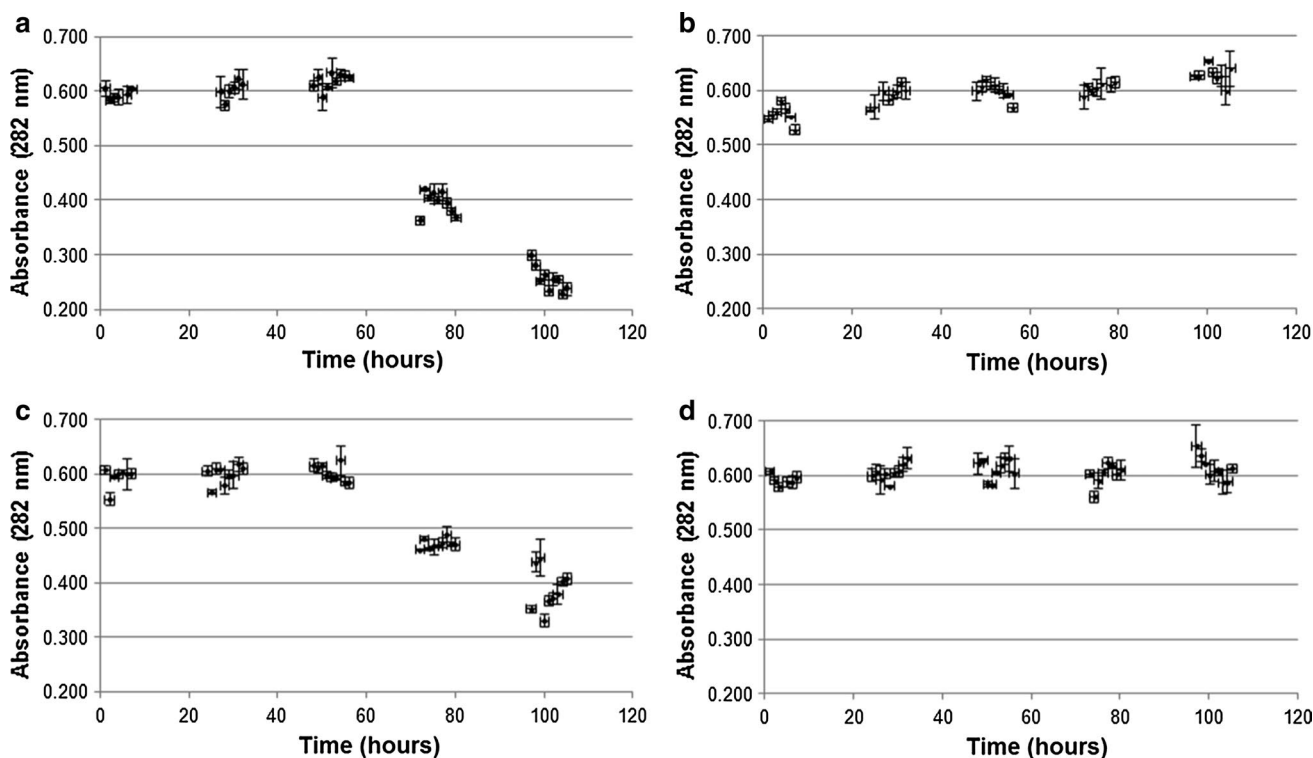
Fig. 1 Stability of uric acid. Absorbance at the absorbtion maximum of 50 µmol L^{-1} uric acid during time; **a** in 1.5 mmol L^{-1} sodium hydroxide; **b** in 1.5 mmol L^{-1} sodium hydroxide containing 15 µmol L^{-1} EDTA; **c** in 1.5 mmol L^{-1} sodium hydroxide containing 15 µmol L^{-1} magnesium ions; **d** in 1.5 mmol L^{-1} sodium hydroxide containing 150 µmol L^{-1} magnesium ions. The measurements were provided every hour in triplicates (8 h each day). Data are provided as means and standard deviations

addition of 6 mmol L^{-1} sodium hydroxide to allantoin in 85 °C after 1 h, even in room temperature UA was not stable in alkaline pH for more than one day. Also Valik and Jones [21] mentioned that UA is decomposed in the presence of sodium and potassium hydroxide.

According to our data standards of UA were highly unstable in concentrated alkaline solutions (more than 10 mmol L^{-1} of sodium, potassium or ammonium hydroxide). The best stability was achieved in 1.5 mmol L^{-1} sodium hydroxide. Different concentrations of magnesium ions (15 µmol L^{-1} to 1 mmol L^{-1}) and disodium salt of EDTA (100 µmol L^{-1} to 5 mmol L^{-1}) were used to stabilize the stock solutions. Addition of EDTA slowed down the dissolving of UA up to 120 h, magnesium ions made this process faster and a good stability was achieved (Fig. 1) when using 150 µmol L^{-1} concentration. Magnesium ions were proposed to form magnesium hydrogen urate octahydrate more soluble than sodium hydrogen urate and UA itself [25], so the presence of divalent ions could probably enhance UA solubility according to our data. Similar results were obtained for HX and X (data not shown),

however, X required more alkaline conditions to dissolve completely (Fig. 2)—15 mmol L^{-1} sodium hydroxide containing 150 µmol L^{-1} magnesium ions.

Method Development

The separation of analytes was tested at various conditions. Mobile phase containing 100 mmol L^{-1} acetate buffer (pH 4.8) was used, however, the peak for HX was split under these conditions. The optimal separation conditions were achieved in 100 mmol L^{-1} potassium phosphate buffer with pH 1.5. One C8 (Luna 3u C8(2) 100A) and two C18 columns (Kinetex 2.6u C18 100A and Kinetex 5u EVO C18 100A) were examined. The Kinetex 5u EVO C18 100A column with core–shell particles gave the most reproducible results. The Kinetex core–shell technology delivers high efficiencies because of its defined structure—homogeneous porous shells grown on a solid silica core. Previously used columns for simultaneous determination of HX, X and UA contained C18 in their stationary phase, however, they did not reach as fast analysis as we did. The Kinetex 5u EVO C18 100A column with core shell particles is compatible with 100%
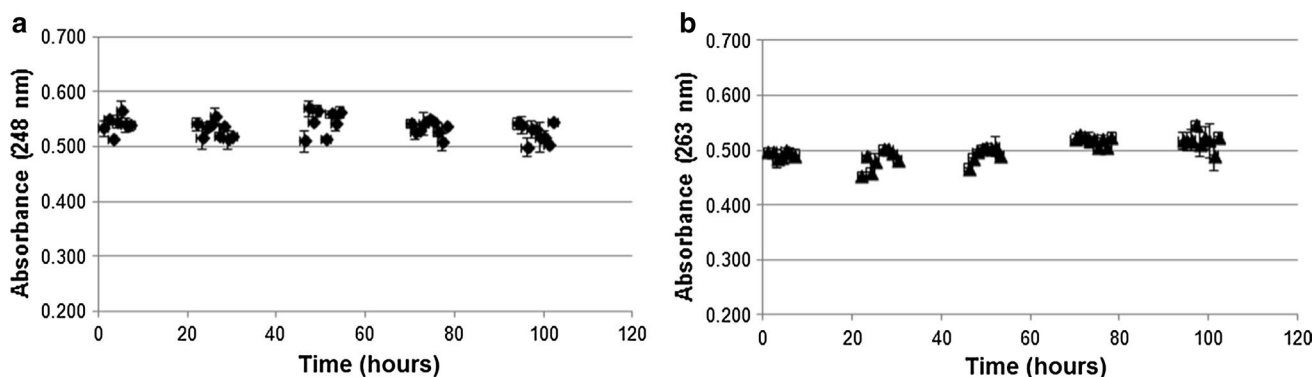
Fig. 2 Stability of hypoxanthine and xanthine. Absorbance at the absorption maxima of 50 μmol L^{-1} hypoxanthine and xanthine during time; **a** hypoxanthine in 1.5 mmol L^{-1} sodium hydroxide containing 150 μmol L^{-1} magnesium ions; **b** xanthine in 15 mmol L^{-1}

sodium hydroxide containing 150 μmol L^{-1} magnesium ions. The measurements were provided every hour in triplicates (8 h each day). Data are provided as means and standard deviations

Fig. 3 Chromatograms of human plasma sample. The separation conditions were set as follows: the mobile phase–100 mmol L^{-1} potassium phosphate buffer pH 1.5, the Kinetex 5u EVO C18 100A column (Phenomenex, Torrance, CA, USA), the flow-rate 1 mL min^{-1} at 30 °C, injection volume 20 μL of deproteinized plasma, photodiode array detector. Concentrations were as follows: 22.3 μmol L^{-1} HX, 7.52 μmol L^{-1} X and 320 μmol L^{-1} UA
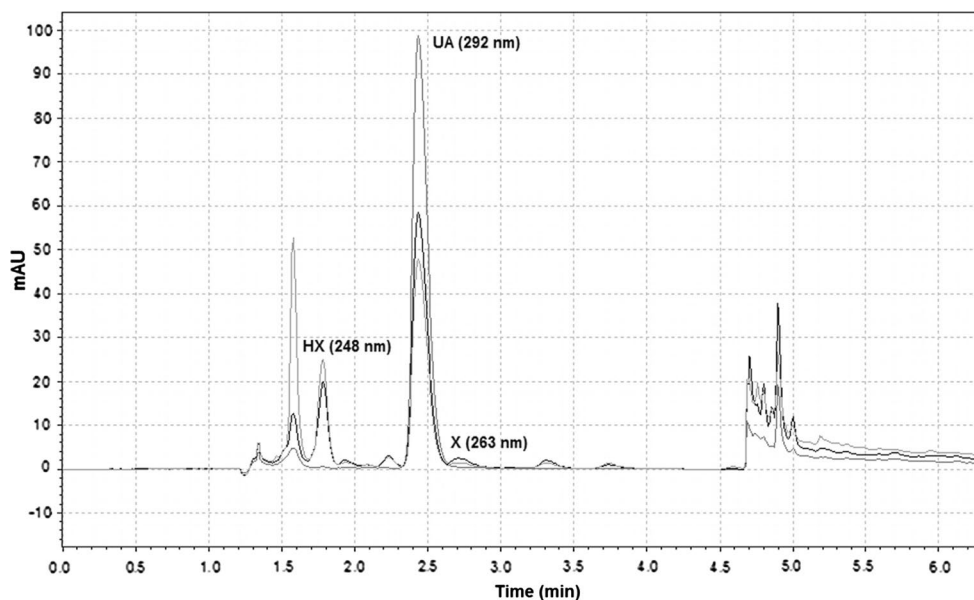
aqueous mobile phase and the core–shell technology results in less peak broadening compared to fully porous particles. This results in better chromatographic resolution. The appropriate sensitivity was achieved with the use of 100 mmol L^{-1} potassium phosphate buffer. Furthermore, this column was highly stable in acidic conditions for more than 600 injections of real samples and more than 2000 injections in total. Low pH (1.5) and the use of concentrated buffer enabled fast elution of all analytes preserving good separation and resolution. The best separation was reached at the flow-rate of 1 mL min^{-1} and temperature of 30 °C.

The efficiency of deproteinization of samples with acetone (1:4, v/v) and acetonitrile (1:1, v/v) was examined. Nevertheless, proteins were detectable in higher amount when using acetone and no peaks for HX and X were

apparent when using acetonitrile precipitation. Protein precipitation with perchloric acid according to Brunnekreeft et al. [26] gave optimal results.

Assay Validation

All analytes of interest are baseline-separated from the neighboring peaks when detected in its absorption maxima (Fig. 3). Over a 4-months period the mean retention times (in minutes) were 1.77 ± 0.03 for HX, 2.63 ± 0.06 for X and 2.35 ± 0.05 for UA (data are expressed as mean ± standard deviation (SD). All validation parameters for UA are peak area based and all validation parameters for X and HX are peak height based because concentration of HX and X is very low in real samples and automated

Table 2 Intra- and inter-day precisions

Analyte	Concentration (μmol L^{-1})	Presicion RSD (%)	
		Intra-day	Inter-day
HX	1	1.27	2.03
	10	0.88	1.24
	20	1.13	1.8
	9.48*	1.44*	3.44*
	12.2**	2.19**	3.96**
X	1	2.55	4.43
	5	1.08	3.14
	10	2.06	3.11
	4.31*	3.12*	3.72*
	4.77**	4.80**	4.06**
UA	1	2.18	4.83
	250	0.42	1.02
	500	0.44	0.99
	211*	0.95*	3.13*
	261**	1.93**	3.07**

* Plasma sample

** Serum sample

Table 3 Recoveries

Description	Recovery (%)		
	HX	X	UA
Above LOQ*	101	105	103
50%**	99.1	102	102
100%**	96.2	101	103
200%**	97.6	102	102
Plasma sample	102	95.6	103
Serum sample	103	99.4	97.1

* Concentrations were established as: 1 μmol L^{-1} HX, 1 μmol L^{-1} X, 1 μmol L^{-1} UA

** Percent of expected concentrations in a mixture of standards (10 μmol L^{-1} HX, 5 μmol L^{-1} X and 250 μmol L^{-1} UA)

integration works more precisely when using peak height. There was no difference between validation parameters for HX and X based on their area and height (data not shown).

Eight-point (triplicated) calibration graphs with standard solutions (1–100 μmol L^{-1} range for HX and X and 1–1000 μmol L^{-1} for UA) showed good linearity: $y = 843.41x - 894.94$ ($R^2 = 0.9931$), $y = 448.31x - 1084.6$ ($R^2 = 0.9911$) and $y = 3104.2x - 50451$ ($R^2 = 0.9919$) for HX, X and UA, respectively. Accuracy of the method was verified on a serum based two-level certified control sample with following concentrations of UA provided by a manufacturer: 293 ± 15 μmol L^{-1} and 568 ± 29 μmol L^{-1} (mean $\pm$ SD). A triplicate analysis revealed following levels of UA: 297 ± 6 μmol L^{-1} and 543 ± 7 μmol L^{-1} (mean $\pm$ SD). No statistically significant differences were found between them (both P = NS, two sample t test). Method was validated in terms of the intra- and inter-day precision (Table 2) established by analyzing replicates of standard solutions and replicates of pooled plasma and serum samples as relative standard deviation (RSD), the values did not exceed 5%.

Recoveries were determined in real plasma/serum samples and in samples of known concentration at 4 different levels with spiking of 3 different concentrations and measured in triplicates (Table 3). The mean recoveries were 99.8% for HX, 100.8% for X and 101.7% for UA; the values did not show more than 5% variation from 100% showing that none of the analytes was bound to plasma or serum proteins and a proper accuracy. Limit of detection (LOD) and limit of quantification (LOQ) were measured in standard solutions based on signal to noise ratio at 3 and 10, respectively, and were as follows: 18.8 and 62.8 nmol L^{-1} for HX, 121 and 403 nmol L^{-1} for X, 68.8 and 229 nmol L^{-1} for UA, respectively.

HX, X and UA levels (median and IQR) were determined in plasma and serum samples from the same donors ($n = 15$), no statistically significant differences were found between plasma and serum levels of analytes (Table 4).

Wung and Howell [15] found out that erythrocytes and platelets can continue to release HX and X after the blood was drawn. In our study, the contact of serum with blood elements was longer by 15 min than of plasma, therefore, we have found lower levels of HX in plasma than in serum (86%) in accordance to previously reported data [15]. Valik and Jones [21] mentioned that blood has to be proceeded as soon as possible (within 15 min) after venipuncture if HX should be determined which supports also our data as shown in Fig. 4. Thus, the pre-analytical phase is very important for the correct determination and assessment of HX. On the other hand, X and UA showed good stability for several hours (Fig. 4).

The analytes of interest are structurally very similar to caffein and its physiologically occuring metabolites. However, major metabolites of caffeine occur in low levels in human serum, so the possible interferences on the HPLC system were not found [13]. In our study we examined the effect of 50 μmol L^{-1} solutions of caffeine (1,3,7-trimethylxanthine), paraxanthine (1,7-dimethylxanthine), 1-methylxanthine, 7-methylxanthine, 1-methyluric acid and 1,7-dimethyluric acid. All methylated derivatives of X and UA were eluted under gradient conditions used for column regeneration (average retention times between 4.72 and 4.86 min of analysis) and they were thus not interfering with HX, X and UA on the system.

Table 4 Comparison of plasma and serum samples

Analyte	Plasma	Serum	Comparison	
	Median [IQR] (μmol L^{-1})	Median [IQR] (μmol L^{-1})	P (Mann–Whitney)	Plasma/serum* (%)
HX	9.49 [7.42–13.1]	11.2 [9.92–13.8]	0.16	86
X	4.17 [4.04–4.28]	4.30 [4.23–4.44]	0.11	95
UA	197 [187–232]	214 [196–242]	0.27	94

* Average of plasma/serum ratios, $n = 15$
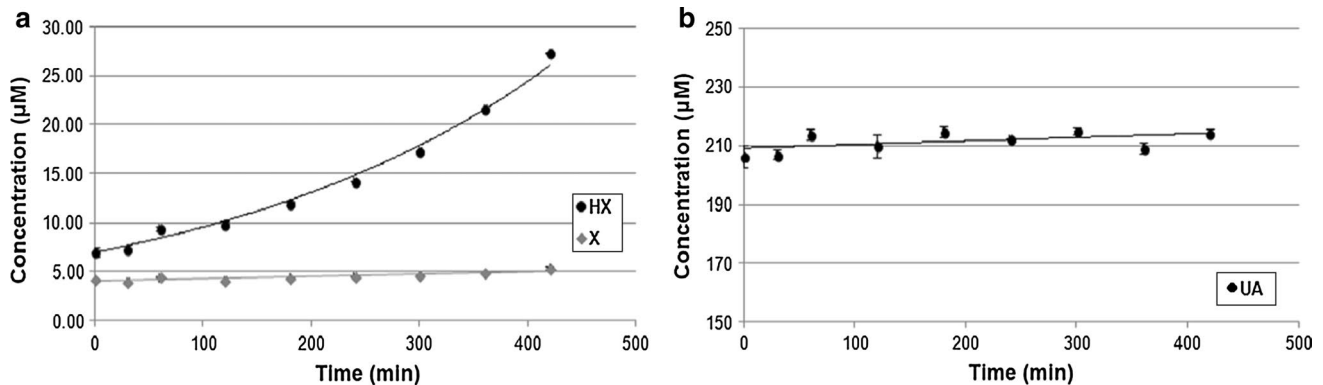
Fig. 4 Hypoxanthine, xanthine and uric acid concentration in EDTA blood. The influence of time of proceeding after EDTA blood collection on concentration of analytes of interest.; **a** for hypoxanthine and xanthine; **b** for uric acid. Concentrations of all analytes were determined with HPLC, all analyses were measured in triplicates. Data are expressed as means and standard deviations

Conclusions

The role of terminal metabolites of purine metabolism is explored extensively nowadays. UA metabolism disturbances are associated to many diseases and pathophysiological states and simple method appropriate for both human plasma and serum is required in clinical research. In brief, optimization of the chromatographic procedure showed that the best separation between the plasma and serum constituents HX, X and UA could be obtained with a 100 mmol L^{-1} potassium dihydrogen phosphate buffer with a pH 1.5. For this solvent one chromatographic run takes only 6.5 min and a sufficient separation is established for all analytes of interest. All analytes can be quantified from the same chromatographic run using photodiode array detector. The recoveries were in range 96–105%, intra- and inter-day precision, limits of detection, limits of quantification and linearity were adequate to a physiologically observable levels of all analytes. No interferences were found.

Compliance with ethical standards

References

1. Morgan EJ, Stewart CP, Hopkins FG (1922) Proc R Soc Lond B Biol Sci 94:109–131
2. Harris CM, Massey V (1997) J Biol Chem 272:28335–28341
3. Battelli MG, Bolognesi A, Polito L (2014) Biochim Biophys Acta 1842:1502–1517. doi:10.1016/j.bbadis.2014.05.022
4. Bobulescu IA, Moe OW (2012) Adv Chronic Kidney Dis 19:358–371. doi:10.1053/j.ackd.2012.07.009
5. Ames BN, Cathcart R, Schwiers E, Hochstein P (1981) Proc Natl Acad Sci USA 78:6858–6862
6. Hayden MR, Tyagi SC (2004) Nutr Metab (Lond) 1:10. doi:10.1186/1743-7075-1-10
7. Lytvyn Y, Perkins BA, Cherney DZ (2015) Can J Diabetes 39:239–246. doi:10.1016/j.jcjd.2014.10.013

8. Zhao Y, Yang X, Lu W, Liao H, Liao F (2009) Microchim Acta 164:1–6. doi:10.1007/s00604-008-0044-z

9. Tiffany TO, Jansen JM, Burtis CA, Overton JB, Scott CD (1972) Clin Chem 18:829–840

10. Yang X, Yuan Y, Zhan CG, Liao F (2012) Drug Dev Res 73:66–72. doi:10.1002/ddr.20493

11. Aoki Y, Ihara H, Nakamura H, Aoki T, Yoshida M (1992) Clin Chem 38:1350–1352

12. Kroll MH, Elin RJ (1994) Clin Chem 40:1996–2005

13. Cooper N, Khosravan R, Erdmann C, Fiene J, Lee JW (2006) J Chromatogr B Analyt Technol Biomed Life Sci 837:1–10. doi:10.1016/j.jchromb.2006.02.060

14. Kock R, Delvoux B, Greiling H (1993) Eur J Clin Chem Clin Biochem 31:303–310

15. Wung WE, Howell SB (1980) Clin Chem 26:1704–1708

16. Czauderna M, Kowalczyk J (1997) J Chromatogr B Biomed Sci Appl 704:89–98

17. McBurney A, Gibson T (1980) Clin Chim Acta 102:19–28

18. Knudson EJ, Lau YC, Veening H, Dayton DA (1978) Clin Chem 24:686–691

19. Balcells J, Guada JA, Peiró JM, Parker DS (1992) J Chromatogr 575:153–157

20. Kojima T, Nishina T, Kitamura M, Kamatani N, Nishioka K (1986) Clin Chem 32:287–290

21. Valik D, Jones JD (1997) Mayo Clin Proc 72:719–725

22. Zhong H, Liang Q, Xia J, Hu P, Wang Y, Tong X, Luo G (2011) Chromatographia 73:149–155. doi:10.1007/s10337-010-1833-1

23. Stentoft C, Vestergaard M, Løvendahl P, Kristensen NB, Moorby JM, Jensen SK (2014) J Chromatogr A 1356:197–210. doi:10.1016/j.chroma.2014.06.065

24. Wilcox WR, Khalaf A, Weinberger A, Kippen I, Klinenberg JR (1972) Med Biol Eng 10:522–531

25. Babić-Ivančić V, Jendrić M, Sostarić N, Opacak-Bernardi T, Zorić ST, Dutour Sikirić M (2010) Coll Antropol 34(Suppl 1):259–266

26. Brunnekreeft JW, Eidhof H, Gerrits J (1989) J Chromatogr 491:89–96