ANALYTICAL SCIENCES SEPTEMBER 2015, VOL. 31

2015 © The Japan Society for Analytical Chemistry

Analysis of Intra- and Extracellular Levels of Purine Bases, Nucleosides, and Nucleotides in HepG2 Cells by High-performance Liquid Chromatography

Tomoko FUKUUCHI,† Noriko YAMAOKA, and Kiyoko KANEKO

The Laboratory of Biomedical and Analytical Sciences, Faculty of Pharma Sciences, Teikyo University, 2-11-1 Kaga, Itabashi, Tokyo 173–8605, Japan

To evaluate cellular uptake and purine transport, we developed a high-performance liquid chromatography method for intra- and extracellular purine quantification. Our aim was to develop an effective method for simultaneously quantifying the substrate and metabolites with high sensitivity. C18 columns from different manufacturers were tested for simultaneous quantification of 22 different purine bases, nucleosides, and nucleotides. We used a YMC-Triart C18 column. The analysis conditions, including extraction solutions for the cells and cell culture medium, were optimized to achieve good quantification. Linearity, accuracy, determination limits, and recovery were assessed and showed good performance. The developed HPLC method was successfully applied to the qualitative analysis of 22 different intra- and extracellular purines, demonstrating that it is useful for studying the overall pattern of purine metabolism. This method could also be useful for evaluating metabolic dynamics of purines under a variety of stimulatory conditions of culture cells.

Keywords Purines, HPLC, HepG2 cells, nucleosides, nucleotides, uric acid

(Received April 30, 2015; Accepted July 2, 2015; Published September 10, 2015)

Introduction

The end product of purine catabolism in man is uric acid. Uric acid (UA) is primarily formed in the liver and excreted by the kidney into the urine. Hyperuricemia can result from increased production, decreased excretion, or a combination of both mechanisms. UA production is influenced by dietary intake of purines and the rates of *de novo* biosynthesis of purines from nonpurine precursors, nucleic acid turnover, and *salvage* by phosphoribosyltransferase activities. Several studies confirm the direct relationship between the consumption of a purine-rich diet and incident hyperuricemia and gout that found that higher meat and fish consumption was associated with increased serum UA levels.[1] Foods containing more adenine evidently have a greater effect on serum UA levels than food containing mainly guanine. Other studies demonstrate that oral hypoxanthine (HX), adenosine monophosphate (AMP), guanosine monophosphate (GMP), inosine monophosphate (IMP), and adenine (A) produce greater hyperuricemic effects, whereas xanthine (X) and guanine (G) do not affect serum UA levels.[2] Furthermore, alcohol consumption increases the level of serum UA.[3-7] In addition to the increase of ATP production by alcohol, the frequency of prevalence of gout is rising through the synergistic effect of purines present in beer.[8,9] Most diets that affect serum UA levels affect either the synthesis or excretory pathways of UA. Thus, the mechanism for serum UA level increase due to other contributory components is of interest to our group.

To date, several methods have been developed for the analysis of purines, including high-performance liquid chromatography (HPLC),[10-15] and LC-MS-based methods.[16] These biochemicals have now been measured in a variety of systems, including cultured cells,[17-19] isolated tissues,[15] food substances,[12-14,20-25] fungi,[26] and biological samples.[11] Previously, we also reported the use of the HPLC method for determining the purine content of food,[14] beer, and beer-like alcoholic beverages,[12] whereas LC-MS was suitable for the simultaneous analysis of 23 species of purine bases, pyrimidines, nucleosides, and nucleotides.[24] However, similar to most methods described, the simultaneous determination of purine bases (A, G, HX, X, and UA), nucleosides [(adenosine (Ado), guanosine (Guo), inosine (Ino), xanthosine (Xao), deoxyadenosine (dAdo), deoxyguanosine (dGuo), and deoxyinosine (dIno)], and nucleotides [Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), AMP, guanosine 5′-triphosphate (GTP), guanosine 5′-diphosphate (GDP), GMP, IMP, and xanthosine 5′-monophosphate (XMP), nicotinamide adenine dinucleotide (NAD^+)] has yet to be reported. A method for the determination of the full profiles of nucleotides and their metabolites (nucleosides and purine bases) in various samples would be valuable for physiological and pharmacological studies.

To evaluate cellular uptake and transport of purines in HepG2 cells, we developed an HPLC method for the quantification of intra- and extracellular purines. The determination of these compounds is challenging because of a large difference in the concentrations of purine bases, nucleosides, and nucleotides, the presence of numerous endogenous interferences in complex matrices, and the high polarity of nucleotides due to the

† To whom correspondence should be addressed.
E-mail: fukuuchi@pharm.teikyo-u.ac.jp

phosphate moiety. Moreover, in the matrices of cell extracts containing enzymes involved in purine metabolism, it is important to optimize a pre-treatment method for blocking the metabolism of each purine immediately by enzyme inactivation. Therefore, we also investigated the optimal pre-treatment method for the determination of purines in cell extracts. Because the purines derived from food are generally absorbed after degrading to nucleosides in the human intestine, we sought to simultaneously quantify purine concentrations in the samples obtained by collecting the cells and cell-cultured medium, which were collected 2 h after the addition of nucleosides to the HepG2 cells at different concentrations. Our method described here was validated and successfully applied to quantify intra- and extracellular purine concentrations in the above samples, demonstrating that it is useful for studying the overall pattern of purine metabolism.

Experimental

Chemicals

All chemicals were of the highest commercial quality available. Five purine bases (A, G, HX, X, and UA), six nucleosides (Ado, dAdo, Guo, dGuo, Ino, and dIno), five nucleotides (ADP, AMP, GDP, GMP, and XMP), nicotinamide adenine dinucleotide (NAD^+), ammonium dihydrogen phosphate, and phosphoric acid, methanol (HPLC grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). ATP, cyclic-adenosine 5′-monophosphate (cAMP), GTP, IMP, and acetonitrile (ACN) (HPLC grade) were purchased from Sigma-Aldrich (Tokyo, Japan). Methanol (MeOH, HPLC grade) was purchased from Kanto Chemical Co., Inc (Tokyo, Japan). Xao was purchased from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were analytical grade and used without further purification. Milli-Q (Milford, MA) water was used throughout the study.

HPLC condition

HPLC analysis was performed on an LC-20A HPLC system (Shimadzu, Kyoto, Japan). The quantitative HPLC separations were performed at 35°C on a YMC-Triart C18 column (25 × 4.6 mm i.d., 3 µm, YMC, Japan) with photodiode array detection (PDA) at 260 nm. The mobile phase comprised 80 mmol/L H_3PO_4/$NH_4H_2PO_4$ ratios of 100:9 (v/v) (A) and 30% MeOH in A (B) and with a flow rate of 0.6 mL/min. The elution began with 1% B for 15 min followed by programmed elution as follows: linear gradient to 25% B from 15 to 30 min; isocratic at 25% B from 30 to 40 min; linear gradient to 50% B from 40 to 50 min; then re-equilibrated back to 1% and re-equilibrated for 20 min. The total run time was 70 min.

Preparation of standard solutions

The stock solution was composed of a mixture of 0.1 mM purines dissolved in water. Some of the compounds with low solubility, such as UA and G, were dissolved using a small amount of 1 M potassium hydroxide and then filled up. The stock solution was diluted with Milli-Q water to working solutions ranging from 0.1 to 100 µM. All samples were freshly prepared.

Cell culture and sample preparation

HepG2 cells are used as an *in vitro* model system for human hepatic cells. HepG2 cell lines that were obtained from RIKEN BRC were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin, 1% (v/v) in a 5% CO_2 at 37°C. Cells (1.0×10^5 cells/well) were seeded in 24-well plates and cultured to confluence over a period of approximately 24 h. These were then used for the experiment after the cell culture medium was removed and replaced with Hank's balanced salt solution (HBSS, pH 7.4). For the nucleoside addition experiment, the cells were pre-incubated for 20 min with HBSS and each tested purine was added and incubated for 2 h more. After this time, the cultured medium was filtered immediately through a centrifuge filter (30K Omega Nanosep, Pall Corp.). The medium was removed completely and then washed twice with phosphate-buffered saline (PBS) and collected by addition of extraction solvents. The extraction solvents evaluated were 0.5 M perchloric acid (PCA), 0.1 M formic acid, and 0.1 M formic acid in MeOH, isopropanol (IPA):water (7:3), ACN:water (7:3), ethanol (EtOH):water (7:3), and MeOH:water (7:3). Acid solvents were neutralized with 1 M KOH and the precipitate was removed by centrifugation and then evaporated. In the case of organic solvent extraction, the extract was centrifuged at 15000*g* for 10 min at 4°C to remove insoluble materials and the supernatant was evaporated. All dried samples were dissolved in 100 µL mobile phase A and filtered through a 0.45-µm filter before HPLC analysis.

Method validation

Calibration standards for each analyte were constructed over the range of 1 pmol – 10 nmol ($n = 6$ at each level). Limits of detection (LOD) were found by injection of 10 µL standard mixture at decreasing concentrations until signal-to-noise ratio (*S*/*N*) decreased to below 3:1 for an individual analyte. Intra-day precision was evaluated by the extraction of standards at concentrations of 250 pmol ($n = 6$) on the same day. To assess the inter-day precision, replicate spiked samples ($n = 6$) were analyzed on six different days. The precision was calculated from the relative standard deviation (RSD, %) of the replicate analyses. Method validation for cell extraction was performed using the ACN:water mixture (7:3). The measured amount of endogenous analyte was subtracted from the total observed analyte concentration in the spiked samples, which was subsequently divided by the spike concentration to yield the analyte recovery (%).

Results and Discussion

Various methods have been reported of the quantification of nucleotides using ion-pairing chromatography,[4,10,24] we aimed to construct a more simple method of using a C18 column. C18 columns from different manufacturers were tested: Wakopak® Ultra C18 (25 × 4.6 mm i.d., 3 µm, Wako Pure Chemical, Japan), InertSustain C18 (25 × 4.6 mm i.d., 3 µm, GL Sciences, Japan), and YMC-Triart C18 (25 × 4.6 mm i.d., 3 µm, YMC, Japan). There was a need to adjust chromatographic conditions to obtain better results for each column. The different mobile phase compositions (generally with increasing pH and/or salt concentration) were tested.

The best results with the Wakopak® Ultra C18 column were attained with a mobile phase A composition of 30 mM ammonium phosphate buffer adjusted to pH 3.9 and mobile phase B composition was 30% MeOH in A at a gradient elution for 15 – 35 min (Fig. 1A). For this column, the separation efficiency for GMP and IMP was found to be insufficient. Even after testing different mobile phase compositions, it was not possible to achieve better peak resolutions. Moreover, because

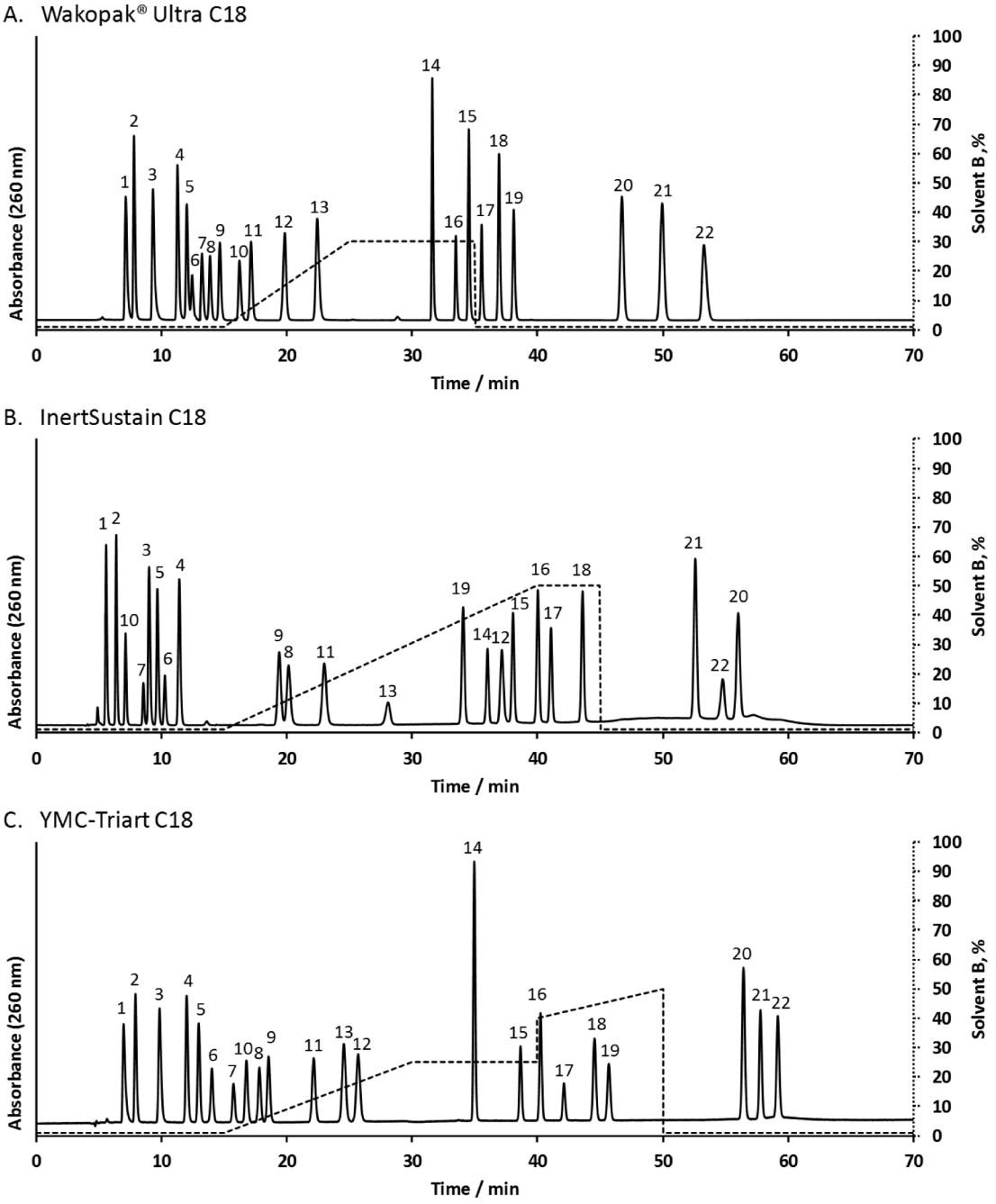

Fig. 1 A comparison of performance of C18 columns for 22 purines in a standard mixture.
Eluting peaks: 1. GTP, 2. GDP, 3. ATP, 4. ADP, 5. GMP, 6. IMP, 7. UA, 8. G, 9. HX, 10. XMP, 11. X, 12. A, 13. AMP, 14. NAD^+, 15. Ino, 16. Guo, 17. dIno, 18. dGuo, 19. Xao, 20. Ado, 21. dAdo, and 22. cAMP.

the ratio of organic solvents was used to separate low and high polarity substances repeatedly, the durability of the columns became a problem. InertSustain C18 shows the best separation ability using a mobile phase A composition of 15 mM ammonium phosphate buffer adjusted to pH 5.8 and mobile phase B was 30% MeOH in A (Fig. 1B). For this column, the separation efficiency for guanine and hypoxanthine was found to be insufficient. The YMC-Triart C18 column was found to be the best separation column using a mobile phase A composition of 80 mM ammonium phosphate buffer adjusted to pH 4.1 and mobile phase B was 30% MeOH in A (Fig. 1C). As a result, good conditions for separating 22 standard samples were determined similarly. The best column for reproducibility was the YMC-Triart C18 columm, which was able to separate purines with high resolution. The total analysis time was 70 min per sample, comprising a column run time of 50 min and a post-run stabilization time of approximately 20 min.

Extractions of HepG2 cells with a variety of acids and organic solvents were conducted to investigate the effects on purine recovery. Acid extractions with PCA and formic acid and formic acid in MeOH as extraction solvents were evaluated. For protein precipitation, PCA was mainly employed when analysis was performed either with HPLC or LC-MS devices.[18,19,27] Highly variable recoveries were observed when PCA was used

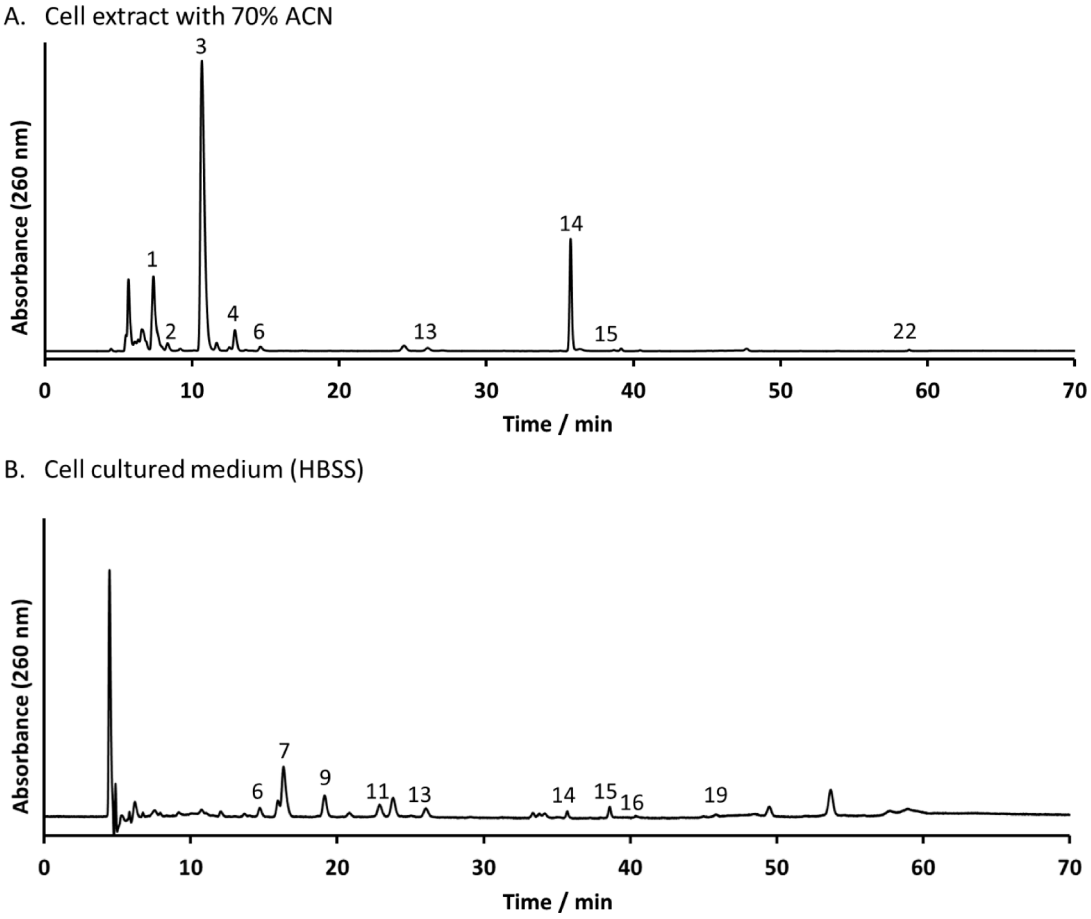

Fig. 2 Chromatograms of 70% ACN extract of (A) HepG2 cells and (B) cell culture medium (HBSS) after incubation for 2 h in the absence of purine-related compounds.
Identities of the relevant peaks: 1. GTP, 2. GDP, 3. ATP, 4. ADP, 6. IMP, 7. UA, 9. HX, 11. X, 13. AMP, 14. NAD^+, 15. Ino, 16. Guo, 19. Xao, 22. cAMP.

as the extraction solvent with the exception of NAD^+. In addition, the peak shape of GTP and GDP highlighted weak retention and became worse (data not shown). Variable negative recoveries were observed, in particular, hydrolyzation of trinucleotides, when formic acid was used as the extraction solvent (data not shown). These results suggested that cellular processes were not being immediately stopped by the addition of acid but were being disrupted in some way leading to erroneous data; hence, this approach was deemed unusable.

Organic solvents as extraction solvents[18,19,27] were widely used and known to have a good ability to precipitate proteins. Organic solvents such as IPA, ACN, MeOH, and EtOH were also evaluated as extraction solvents. In the case of nucleotide extraction, it has been reported that the methods of solely using an organic solvent have a higher recovery rate than those solvents containing water.[17] However, low solubility analytes were included in this study, such as guanine, guanosine, and uric acid; therefore, it was not possible to use an organic solvent alone because these compounds would result in protein precipitation. As a result of considering the ratio of water, it was revealed that the mixture containing 30% water solvent was suitable for the solubility and the deproteinization effect (data not shown). Furthermore, poor extraction recoveries were observed with MeOH:water (70:30), EtOH:water (70:30), and IPA:water (70:30). ACN:water (70:30) extraction yielded the highest recovery and the best reproducibility (data not shown). Compared with the literature reported to date, the existing ratio of endogenous purine seems to be appropriate, although cell types used were different in other studies.[16,19]

The extraction was generally performed at a low temperature; however, there were no effects on the recovery rate using a pre-cooled solvent to –30°C (data not shown). Based on our investigations, the addition of ACN:water (70:30) extraction was found to be the best extraction technique.

The recovery rate of the cell medium samples were also determined by collecting the medium of HepG2 cell cultured for 2 h on a 24-well plate. However, in the assay system using the normal medium containing FBS, we could not monitor purine metabolism changes. This may be because after addition of purine to the medium containing FBS, the tri- and di-phosphate nucleotides were found to be rapidly degraded, regardless of cell presence (data not shown). To monitor the extracellular purine kinetics using cultured cells, we consider it necessary to establish a culture system where cells were conditioned with serum-free medium.

Generally, because HBSS is used for the drug cellular uptake assays in a short period of time, we also studied the recovery rate using HBSS as the cell medium. The recovery rate of spiked cell cultured HBSS was satisfactory and 91.0 – 114.9% (CV 0.2 – 1.2%) at 500 pmol and 94.0 – 109.7% (CV 0.5 – 3.0%) at 50 pmol was obtained, with most contaminants not being found (Table 2). Even if allowed to stand for 3 h at room

temperature after standard addition, large degradation was not observed in HBSS (data not shown). In this study, we decided to perform the cellular uptake experiments for 2 h with HBSS. However, to perform a long duration of study in the future, it is necessary to construct a culture system incorporating a serum-free medium that contains the various trophic factors in place of FBS.

Table 1 Linear regression data, LOD, and LOQ of investigated compounds

Analyte	LOD/pmol	LOQ/pmol	Linearity/r^2
ATP	5.0	20.0	1.0000
ADP	2.5	10.0	1.0000
AMP	3.9	15.6	1.0000
cAMP	3.9	10.0	1.0000
Ado	2.4	7.8	1.0000
A	3.9	15.6	1.0000
GTP	3.9	10.0	1.0000
GDP	2.5	10.0	1.0000
GMP	4.0	15.6	1.0000
Guo	2.5	7.8	1.0000
G	3.9	15.6	1.0000
IMP	5.0	15.6	1.0000
Ino	3.9	15.6	1.0000
HX	3.9	15.6	1.0000
XMP	3.9	15.6	1.0000
Xao	3.9	15.6	1.0000
X	3.9	15.6	1.0000
UA	7.8	15.6	0.9998
dAdo	7.8	15.6	1.0000
dGuo	3.9	15.6	1.0000
dIno	10.0	32.2	1.0000
NAD^+	1.2	3.9	1.0000

The specificity of the method was tested for endogenous interference from samples (as shown in Fig. 2). Calibration curves were obtained using linear regression analysis of eight-point concentration curves (data not shown). The correlation coefficient (r^2) values were good, and linearity was checked for the range of concentrations from 10 nmol down to the LOQ of the respective analytes. The upper limits of quantification were the solubility limits of the purine bases. LOD and LOQ values are shown in Table 1. The overall intra- and inter-day variations (RSD) of the six analytes were 0.1 – 0.6% and 0.3 – 5.4% (Table 2), respectively. A recovery analysis was conducted of the cell extract and cell-cultured HBSS samples independently spiked with two different concentrations of standards containing all analytes. The spiked amount of purines in the recovery studies was 250 and 500 pmol for HepG2 cells and 50 and 500 pmol for cultured medium because they were in closer concentration range with endogenous purines. Actually, we examined, at 50 pmol, a low spiked amount for cell cultured medium (HBSS) because it does not present in the extracellular, except for the added purine compound, whereas nucleotides, such as ATP, GTP, and ADP, contained more than 400 pmol/injection in the cells. As shown in Table 2, the accuracy varied from 84.7 to 114.9% (RSD 0.2 – 6.2%), which is considered a satisfactory level for this method. The identification of the investigated compounds was performed by comparison of their retention time and their PDA spectra with those obtained by injecting standards in the same conditions. Moreover, the reliability of peaks was additionally confirmed by the enzymatic peak-shift assay.[28]

The developed HPLC method was subsequently applied to simultaneous determination of 22 purines. To test the purine uptake in the cells, each purine nucleoside (Ado, Ino, Guo, and Xao) (0, 12.5, 25, 50, 100, and 200 μM) was supplied to HepG2 cells, and the cells were harvested after 2 h. Each data point in

Table 2 Intra- and inter-day precision and recovery for the investigated compounds

Analyte	Precision (RSD, %, n = 6)		Cell extract with ACN:water (7:3)				Cell cultured HBSS with ultrafiltration			
			Recovery (250 pmol, n = 3, %)		Recovery (500 pmol, n = 3, %)		Recovery (50 pmol, n = 3, %)		Recovery (500 pmol, n = 3, %)	
	Intra-day	Inter-day	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
ATP	0.1	0.3	87.2	5.1	89.6	2.3	104.6	1.0	108.9	0.2
ADP	0.1	0.5	96.3	1.9	94.4	1.2	105.4	0.8	108.7	0.3
AMP	0.2	0.5	91.2	1.3	91.3	0.4	104.7	0.8	108.9	0.3
cAMP	0.1	0.4	92.9	1.0	89.8	0.6	108.6	1.9	110.0	0.4
Ado	0.1	0.6	91.1	1.0	89.8	0.8	102.2	1.2	106.6	0.3
A	0.2	1.7	91.7	1.4	92.8	0.3	106.5	0.9	111.8	0.4
GTP	0.1	0.8	97.2	1.5	94.1	1.1	104.9	0.9	108.0	0.4
GDP	0.3	1.0	87.7	5.3	92.1	3.2	104.6	0.9	108.3	0.5
GMP	0.3	1.3	90.8	0.6	91.8	1.4	106.0	0.9	108.9	0.3
Guo	0.2	0.4	91.9	1.6	89.5	1.2	105.3	0.5	109.4	0.5
G	0.2	1.6	91.3	0.2	92.5	1.2	106.1	0.8	108.5	1.2
IMP	0.6	3.5	88.5	1.7	89.3	2.9	100.2	3.0	104.2	0.5
Ino	0.1	0.4	91.6	1.0	88.3	3.6	105.8	0.7	110.1	0.6
HX	0.1	1.0	91.2	0.3	92.2	1.1	109.7	0.7	114.9	0.8
XMP	0.2	1.3	91.5	0.2	92.4	1.1	106.0	1.5	108.5	0.4
Xao	0.1	0.9	91.1	1.4	91.4	0.8	104.9	1.3	109.6	0.3
X	0.1	1.2	92.5	0.8	94.4	0.4	109.1	0.8	113.0	0.4
UA	0.5	5.4	85.2	0.9	88.5	6.2	105.2	2.4	104.5	0.3
dAdo	0.3	2.9	85.1	1.4	84.7	0.6	94.0	0.4	104.5	0.3
dGuo	0.1	0.4	90.6	1.3	87.7	0.8	100.9	0.7	105.4	0.7
dIno	0.3	0.8	93.6	3.0	92.3	1.1	101.2	0.7	110.3	0.4
NAD^+	0.1	2.9	92.4	0.8	92.2	0.8	106.7	0.9	91.0	0.4

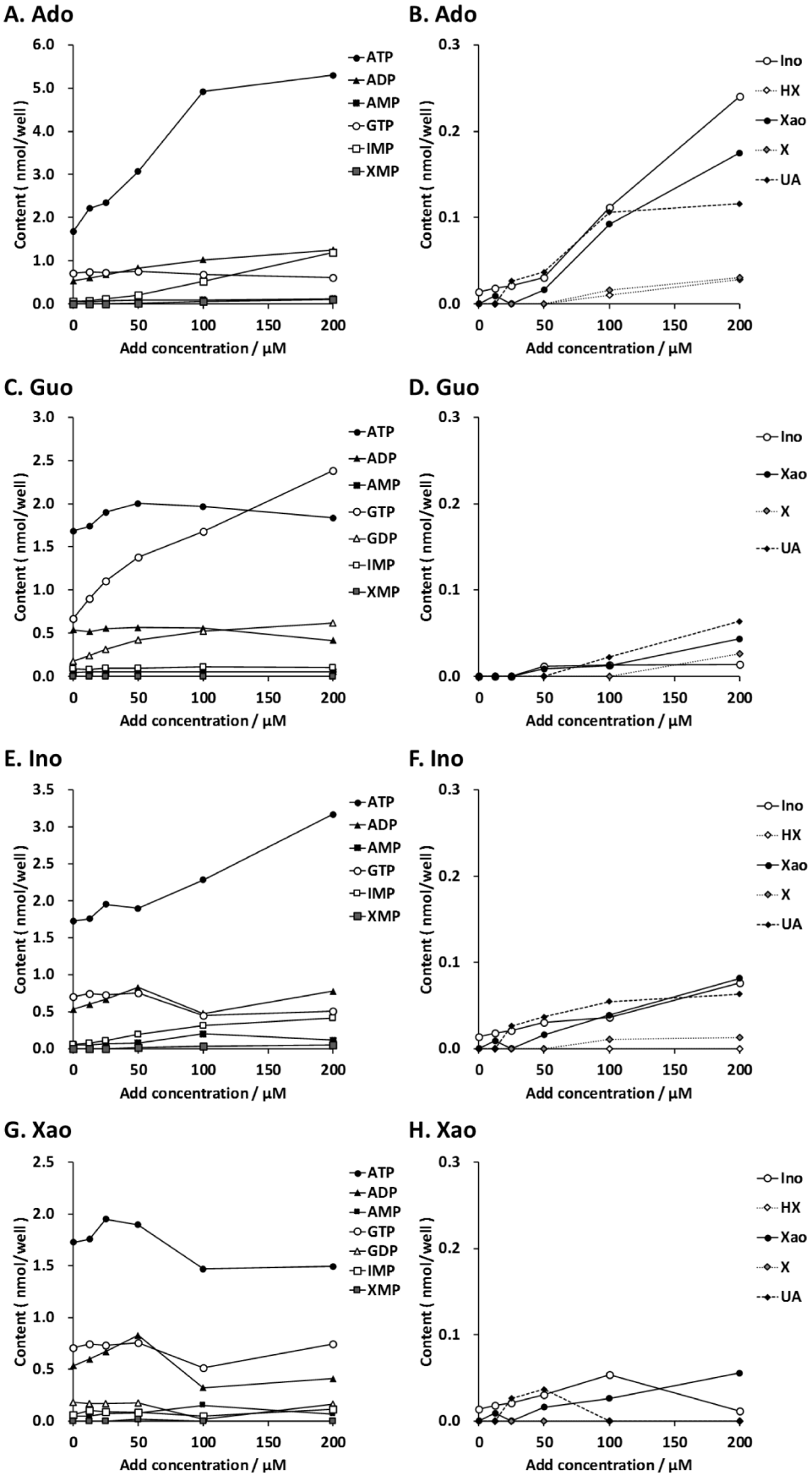

Fig. 3 Nucleoside uptake study in HepG2 cells. (A, B) Ado, (C, D) Guo, (E, F) Ino, and (G, H) Xao was supplied to HepG2 cells cultured in 24-well plates, and the cells were harvested after 2 h at each concentration of 0, 12.5, 25, 50, 100, and 200 μM in HBSS. Each assay was performed in duplicate or triplicate wells, and average values of wells were plotted.

Fig. 3 except for 0 (nmol/well) was more than the LOQ when determined on a chromatogram. Supplying Ado increased ATP, AMP, IMP, XMP, Ino, Xao, HX, X, and UA levels (Figs. 3A and 3B). Increased intracellular ATP and IMP levels in HePG2 cells were also observed after supplying Ino (Figs. 3E and 3F). We consider that the *salvage* pathway involving adenine phosphoribosyltransferase and hypoxanthine-guanine phosphoribosyltransferase was activated by exposure to Ado and Ino. Supplying Guo to the HepG2 cells increased GTP, GDP, GMP, Xao, X, and UA levels in a dose-dependent manner (Figs. 3C and 3D). These results suggest that metabolism by guanase enzymes proceeds early and that hepatocytes have strong xanthine oxidase activity.

In the cell culture medium, we observed the metabolism from adenosine to inosine, from Guo to G further than X, and from Ino to HX (data not shown). An increase in the levels of salvaged purine nucleotide and the final metabolite, UA, was not observed. With the examination time being 2 h, we believe that transport of the nucleotide and final metabolite outside the cells rarely occurs.

In the case of Xao, a small percentage of the supplied amount was incorporated in the cells as an intact form (Figs. 3G and 3H). To exclude variations in the cell culture results, individual wells must be normalized for protein concentration and cell number. In this study, it was not possible to conduct the normalization by protein or DNA quantification because protein and DNA were immediately denatured after a certain time to minimize the influence on purine metabolism. Further work is underway to conduct accurate evaluation through the normalization using WST-1 formazan salt that is directly correlated with the protein assay and cell number and is nondestructive.

Conclusions

We developed a novel HPLC method for simultaneous determination of intra- and extracellular purines. Our method had good sensitivity and excellent resolving power when applied to complex mixtures, demonstrating that it is useful for studying the overall pattern of purine metabolism. This method is expected to be broadly applicable for the determination of purine content in various samples such as cells and biological specimens. This method should also be useful for evaluating the intestinal absorption and metabolic dynamics of purines under a variety of conditions.

Acknowledgements

This work was supported by JSPS KAKENHI Grant No. 25750055.

References

1. H. K. Choi, S. Liu, and G. Curhan, *Arthritis Rheum.*, **2005**, *52*, 283.
2. D. Brule, G. Sarwar, and L. Savoie, *J. Am. Coll. Nutr.*, **1992**, *11*, 353.
3. H. K. Choi and G. Curhan, *Arthritis Rheum.*, **2004**, *51*, 1023.
4. E. N. Liberopoulos, G. A. Miltiadous, and M. S. Elisaf, *Lancet,* **2004**, *364*, 246.
5. S. Ryu, Y. Chang, Y. Zhang, S. G. Kim, J. Cho, H. J. Son, H. Shin, and E. Guallar, *Am. J. Epidemiol.*, **2012**, *175*, 133.
6. T. Yamamoto, Y. Moriwaki, and S. Takahashi, *Clin. Chim. Acta*, **2005**, *356*, 35.
7. T. Yamamoto, Y. Moriwaki, S. Takahashi, Z. Tsutsumi, T. Ka, M. Fukuchi, and T. Hada, *Metabolism*, **2002**, *51*, 1317.
8. C. J. Eastmond, M. Garton, S. Robins, and S. Riddoch, *Br. J. Rheumatol.*, **1995**, *34*, 756.
9. K. Nishioka, T. Sumida, M. Iwatani, A. Kusumoto, Y. Ishikura, H. Hatanaka, H. Yomo, H. Kohda, T. Ashikari, Y. Shibano, and Y. Suwa, *Alcohol.: Clin. Exp. Res.*, **2002**, *26*, 20S.
10. A. Contreras-Sanz, T. S. Scott-Ward, H. S. Gill, J. C. Jacoby, R. E. Birch, J. Malone-Lee, K. M. Taylor, C. M. Peppiatt-Wildman, and S. S. Wildman, *Purinergic Signalling*, **2012**, *8*, 741.
11. J. Czarnecka, M. Cieslak, and K. Michal, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, **2005**, *822*, 85.
12. T. Fukuuchi, M. Yasuda, K. Inazawa, T. Ota, N. Yamaoka, K. Mawatari, K. Nakagomi, and K. Kaneko, *Anal. Sci.*, **2013**, *29*, 511.
13. B. D. Gill and H. E. Indyk, *J. AOAC Int.*, **2007**, *90*, 1354.
14. K. Kaneko, T. Yamanobe, and S. Fujimori, *Biomed. Chromatogr.*, **2009**, *23*, 858.
15. S. Zur Nedden, R. Eason, A. S. Doney, and B. G. Frenguelli, *Anal. Biochem.*, **2009**, *388*, 108.
16. S. Cohen, L. P. Jordheim, M. Megherbi, C. Dumontet, and J. Guitton, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, **2010**, *878*, 1912.
17. R. L. Cordell, S. J. Hill, C. A. Ortori, and D. A. Barrett, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, **2008**, *871*, 115.
18. E. J. Coolen, I. C. Arts, E. L. Swennen, A. Bast, M. A. Stuart, and P. C. Dagnelie, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci*., **2008**, *864*, 43.
19. J. Wang, T. Lin, J. Lai, Z. Cai, and M. S. Yang, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, **2009,** *877*, 2019.
20. K. Kaneko, Y. Aoyagi, T. Fukuuchi, K. Inazawa, and N. Yamaoka, *Biol. Pharm. Bull.*, **2014**, *37*, 709.
21. K. Kaneko, Y. Kudo, T. Yamanobe, K. Mawatari, M. Yasuda, K. Nakagomi, and S. Fujimori, *Nucleosides, Nucleotides Nucleic Acids*, **2008**, *27*, 628.
22. P. Vinas, N. Campillo, G. F. Melgarejo, M. I. Vasallo, I. Lopez-Garcia, and M. Hernandez-Cordoba, *J. Chromatogr. A*, **2010**, *1217*, 5197.
23. N. Yamaoka, K. Kaneko, Y. Kudo, M. Aoki, M. Yasuda, K. Mawatari, K. Nakagomi, Y. Yamada, and T. Yamamoto, *Nucleosides, Nucleotides Nucleic Acids*, **2010**, *29*, 518.
24. N. Yamaoka, Y. Kudo, K. Inazawa, S. Inagawa, M. Yasuda, K. Mawatari, K. Nakagomi, and K. Kaneko, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, **2010**, *878*, 2054.
25. S. Hou and M. Ding, *Anal. Sci.*, **2010**, *26*, 1111.
26. F. Q. Yang, D. Q. Li, K. Feng, D. J. Hu, and S. P. Li, *J. Chromatogr. A*, **2010**, *1217*, 5501.
27. J. Klawitter, V. Schmitz, D. Leibfritz, and U. Christians, *Anal. Biochem.*, **2007**, *365*, 230.
28. T. Fukuuchi, A. Morimura, M. Kawatani, K. Yamamoto, N. Yamaoka, and K. Kaneko, *Nucleosides, Nucleotides Nucleic Acids*, **2014**, *33*, 445.