ELSEVIER

Journal of Chromatography B, 751 (2001) 87–92

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of urinary methylated purine pattern by high-performance liquid chromatography

M. Cristina Di Pietro, Daniela Vannoni, Roberto Leoncini, Giulia Liso, Roberto Guerranti, Enrico Marinello*

Institute of Biochemistry and Enzymology, University of Siena, Via Aldo Moro, 53100 Siena, Italy

Received 8 May 2000; received in revised form 4 August 2000; accepted 8 August 2000

Abstract

We describe the group selective separation and quantification of unmodified and modified purines in human urine by high-performance reverse phase liquid chromatography. The pattern of oxypurines and methylated purines: hypoxanthine (Hx), xanthine (X), 1-methyl hypoxanthine (1-MHx), 1-methyl guanine (1-MG), 3-methyl guanine (3-MG), 7-methyl guanine (7-MG), 1-methyl xanthine (1-MX), 3-methyl xanthine (3-MX), 7-methyl xanthine (7-MX), 1,7-dimethyl guanine (1,7-*d*MG), 1,3-dimethyl xanthine (1,3-*d*MX), 1,7-dimethyl xanthine (3,7-*d*MX) and 1,3,7-trimethyl xanthine (1,3,7-*t*MX) were determined in a single run in urine of a healthy subject and a gout patient before and after treatment with allopurinol. This method may be useful to investigate the urinary pattern of methylated bases in diseases involving purine metabolism.

Keywords: Purines; Methylated

1. Introduction

Modified nucleosides and bases have been reported in DNA and RNA, especially *t*-RNA [1–5]. Methylation is a frequent modification of bases, and is a mechanism regulating gene expression and DNA replication. Methylated bases are formed by specific enzymes which modify the primary sequence of *t*-RNA inserting methyl groups at specific sites. These enzymes are constant in homogeneous biological systems, but in organisms or tissues undergoing some process of differentiation, they show profound quantitative and qualitative alterations [6–10].

Altered methylation patterns may be implicated in cancer [11–13]. Abnormally high *t*RNA methylase capacity has been reported in crude extract of 30 different neoplastic tissues. The capacity was increased by a factor of 2 to 10 with respect to the corresponding normal tissues [6,14–16].

There has been much recent interest in analysis of modified purines in DNA [11–13,17–24] and biological fluids [19,25,26] by HPLC, especially monitoring of DNA damage due to endogenous oxidants and radical forming agents or as an index of cytotoxic activity. Methylated purines are also present in urine [27–32]. Variations in the excretion of these metabolites have been demonstrated in pathologies such as immunodeficiencies, leukemia and other

*Corresponding author. Tel.: +39-577-234-286; fax: +39-577-234-285.

E-mail address: marinello@unisi.it (E. Marinello).

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00471-0

tumors [32], probably related to altered nucleic acid turnover, altered methylase capacity [6], or renal reabsorption. The first studies on urinary methylated purine bases such as 1-methylxanthine, 7-methylxanthine and 1,7-dimethylxanthine were believed to be exogenous and were found to be absent in subjects avoiding coffee, tea and chocolate [29,30,33,34]. Weissman et al. [27] identified other methylated bases (8-OH-7-MG, 1-methylguanine, N_2-methylguanine and 1-methylhypoxanthine) in urine, and classified them as endogenous metabolites; however the method of analysis (two dimensional paper chromatography) was not very sensitive.

Here we report a simple and reproducible procedure for the analysis of methylated bases, using a slight modification of Weissman's method of extraction of purines from urine, but completely substituting the last part of the procedure with the much more selective and specific high-performance liquid chromatography (HPLC). In a single run we were able to monitor many methylated purine bases present in human urine.

The aim of this study was to develop a useful method of obtaining more data on the urinary excretion of purine compounds in control subjects and patients with diseases affecting purine metabolism. Here we report the typical urinary purine pattern of a healthy control and a gout patient, before and after treatment with allopurinol.

2. Experimental

2.1. Chemicals

Standards of hypoxanthine, xanthine, 1-methylhypoxanthine, 1-methylxanthine, 3-methylxantine, 7-methylxanthine, 1,7-dimethylxanthine, 1,3-dimethylxanthine, 1,3,7-trimethylxanthine, 1-methylguanine, 3-methylguanine, 7-methylguanine and 1,7-dimethylguanine, were purchased from Fluka Chemie (Bucks, Switzerland) and Sigma (St. Louis, MO, USA). Monobasic potassium phosphate, potassium hydroxide, ammonium hydroxide, sodium hydroxide, perchloric acid, hydrochloric acid, silver nitrate and methanol (HPLC grade) were purchased from Baker (Phillipsburg, NJ, USA). Dowex 50W-X8 20-50 mesh was obtained from Bio-Rad (Richmond, CA, USA).

2.2. Standard solutions

Stock solutions of all compounds (several concentrations) were prepared by dissolving pure standards in Milli-Q water or in 0.5 N KOH to a final concentration of 50 m*M*.

2.3. Sample preparation

Twenty-four-hour urine was obtained from a control subject, a volunteer of our laboratory, and a gout patient before and after allopurinol administration. The patient was hospitalised and on a balanced diet without limitation of purine. The control subject was healthy, not overweight and on the same diet as the patient.

According to the Weissman procedure [27], an aliquot (200 ml) of urine was diluted, acidified and run on an ion-exchange chromatograph column (Dowex 50W-X8) eluting with 1 *M* ammonium hydroxide; the elute was treated with silver nitrate, a specific precipitant for purines, resuspended with 0.05 N HCl, heated to 100°C for 5 min, centrifuged and reduced under vacuum before HPLC.

Urine samples underwent various manipulations during extraction and concentration that could cause loss of material. The recovery of methylated purines was calculated by comparison with the recovery of xanthine and hypoxanthine, previously evaluated in untreated urine diluted 1:5 and analysed by the same HPLC procedure. Recovery was calculated to be 91± 4%. The reproducibility of the extraction procedure was tested by processing five aliquots of the same urine sample. Coefficients of variation (C.V.%) for peak areas were in the range 0.5–3.0% for all metabolites.

2.4. Apparatus and chromatographic conditions

A Vista 5500 high-performance liquid chromatograph (Varian, Sunnyvale, CA, USA) equipped with a variable wavelength UV detector (Varian, Model 2550) and an electronic integrator (Varian, Model 4290) was used. For diode assay analysis of the peaks we used a P.E. 1020 LC system (Perkin–Elmer

Co., Norwalk, CT, USA) consisting of a mod. 250 binary pump, a mod. 235C D.A. detector and a mod. 1020 Nelson LC-plus controller.

A ready-to-use prepacked Supelcosil LC-18 column (Supelco, 250×4.6 mm I.D. 5μm) (Bellefonte, PA, USA), protected by a precolumn (20×4.6 mm I.D.) filled with the same packing (Supelguard, Supelco) completed the analytical system.

Elution was carried out with a linear gradient of 0.01 *M* potassium phosphate buffer (adjusted to pH 5.5 with 0.5 *M* potassium hydroxide) and methanol from 0 to 20% in 45 min. The flow-rate was 1.5 ml/min.

3. Results and discussion

3.1. Optimal conditions

HPLC separation of the standard methylated purine bases was optimised at room temperature. The effects of different concentrations and pH of the mobile phase are shown in Figs. 1 and 2 respectively. 10 m*M* KH_2PO_4 and pH 5.5 were chosen as the best. The relative proportions of buffer and methanol were also varied to optimise separation with respect to time as shown in Fig. 3. Good linearity was obtained in the range of 0.1–10 nmoles. The detection limit was 0.02 nmoles. Correlation coefficients and regression equations are reported in Table 1. The overall between-run and between-day precision of the retention times and peak areas was evaluated: the standard deviation (SD) and coeffi-

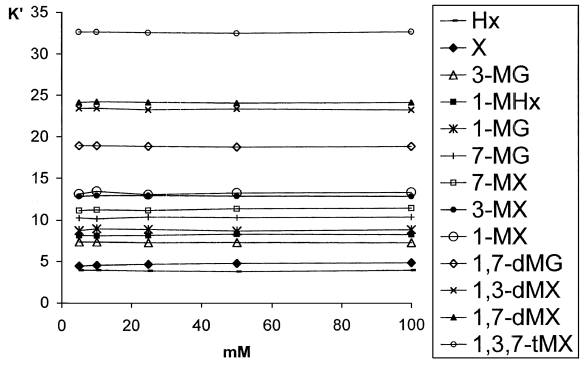

Fig. 1. Effect of different ionic strengths of buffer (pH 5.5) on K′ values of oxypurines and methylated purine bases.

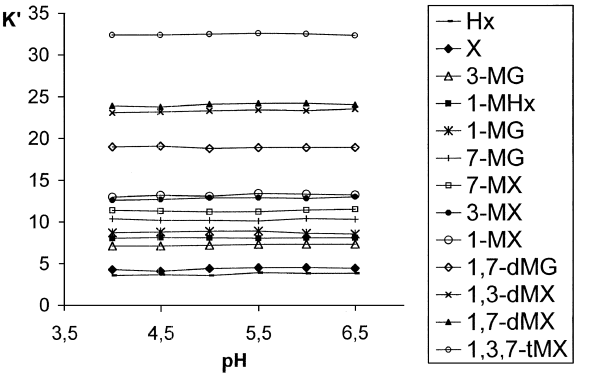

Fig. 2. Effect of different pH of phosphate buffer (10 m*M*) on K′ values of oxypurines and methylated purine bases.

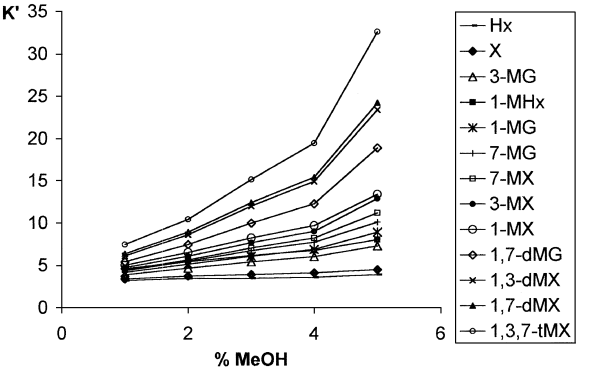

Fig. 3. Effect of methanol concentration on K′ values of oxypurines and methylated purine bases.

Table 1
Regression equations and correlation coefficients for standard oxypurines and methylated purines

Compound	Regression equation	Correlation coefficient
Hx	$y=3338.1x$	$R^2=0.989$
X	$y=2560.6x$	$R^2=0.995$
3-MG	$y=1809.0x$	$R^2=0.994$
1-MHx	$y=3180.7x$	$R^2=0.994$
1-MG	$y=3222.2x$	$R^2=0.996$
7-MG	$y=1865.1x$	$R^2=0.994$
7-MX	$y=2073.1x$	$R^2=0.999$
3-MX	$y=1979.1x$	$R^2=0.992$
1-MX	$y=2310.3x$	$R^2=0.991$
1,7-*d*MG	$y=1522.9x$	$R^2=0.891$
1,3-*d*MX	$y=2135.7x$	$R^2=0.893$
1,7-*d*MX	$y=2025.6x$	$R^2=0.995$
1,3,7-*t*MX	$y=1535.9x$	$R^2=0.991$

cients of variation (C.V.%) for all the analytes combined was 0.13–0.53 min and 0.25–2.65% for the retention times and 1424–30 753 arbitrary units and 0.21–2.44% for peak areas respectively.

3.2. Behaviour of methylated purines in urinary extract

In urine chromatograms, we tested the identity and the purity of the peaks by their retention times, diode array analysis and coelution with standards spiked in the sample. The coelution was also verified changing the run conditions (different pH or buffer ionic strength and methanol percentages in the gradient) Fig. 4 shows a chromatogram of a typical urine extract alongside standards. Table 2 shows the urinary levels of purine bases obtained in the subjects considered in this study.

4. Conclusions

This method can be used to evaluate the urinary methylated purine pattern in control subjects and patients with disorders of purine metabolism: it is fast, taking less than 45 min, and detects a large number of purines, including those substituted in position 3 (3-methyl xanthine and 3-methyl guanine) which are not detectable by paper chromatography.

The determination of these metabolites may be useful for better characterisation and monitoring of pathologies involving purine metabolism, such as

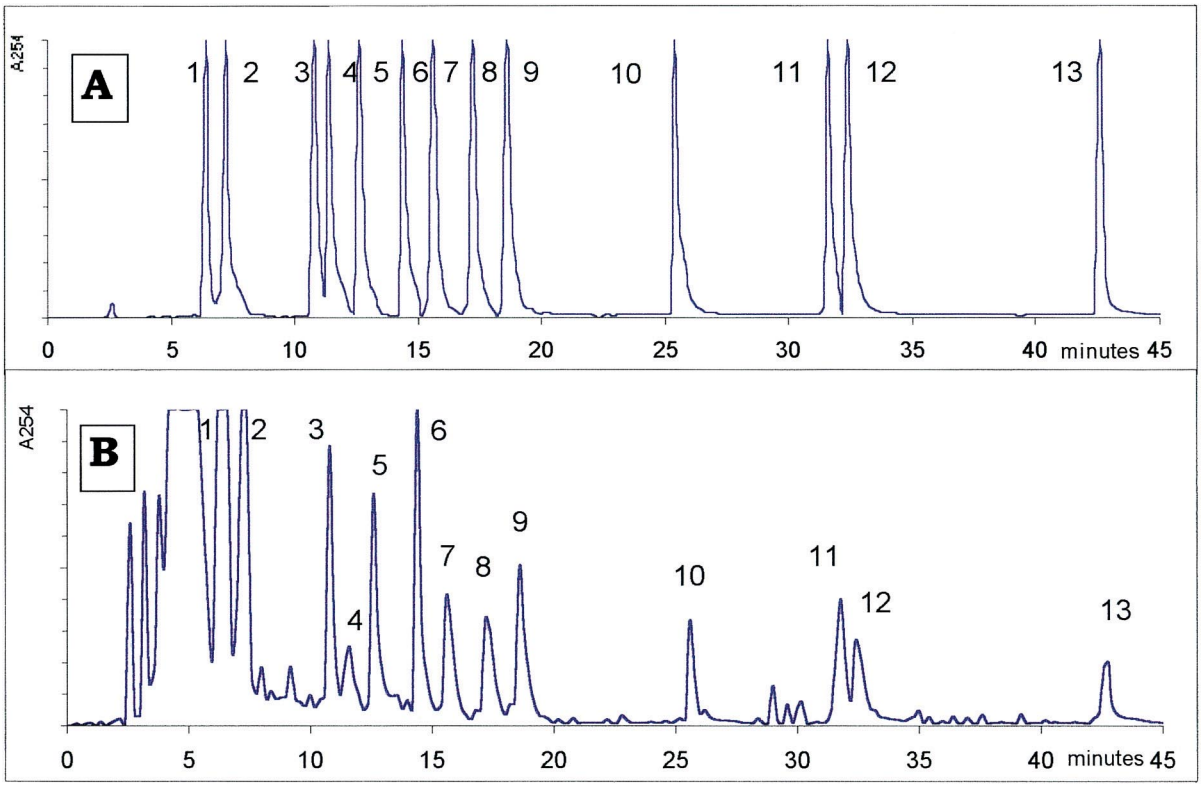

Fig. 4. A typical HPLC chromatogram of standards (A) and urine sample (B) eluted according as reported in Section 3.1. Peaks: (1) Hx; (2) X; (3) 3-MG; (4) 1-MHx; (5) 1-MG; (6) 7-MG; (7) 7-MX; (8) 3-MX; (9) 1-MX; (10) 1,7-*d*MG; (11) 1,3-*d*MX; (12) 1,7-*d*MX; (13) 1,3,7-*t*MX.

Table 2
Levels of oxypurines and methylated purines (μmol/day) in a typical urinary extract of a control subject and a gout patient before and after allopurinol treatment[a]

Compound	Retention time (min)	Control	Gout patient untreated	Gout patient treated
Hx	6.34	32.87	137.01	290.03
X	7.44	28.53	50.79	168.90
3-MG	10.77	2.88	27.55	229.40
1-MHx	11.41	n.d.	3.05	9.02
1-MG	12.44	6.32	20.45	38.76
7-MG	14.26	15.61	30.21	46.31
7-MX	15.40	2.32	7.76	23.77
3-MX	17.03	n.d.	6.31	18.27
1-MX	18.44	2.71	18.65	11.60
1,7-*d*MG	25.08	3.12	12.35	22.67
1,3-*d*MX	31.21	1.98	9.04	61.96
1,7-*d*MX	32.06	n.d.	3.03	6.76
1,3,7-*t*MX	42.95	3.19	4.03	11.96

[a] n.d.=not detectable.

gout, and it may be correlated with the severity of the disease or the diet of the patient

The results of the gout patient showed an increase in most bases as previously demonstrated for hypoxanthine and xanthine [35]. It would be interesting to confirm these promising results in a larger number of patients.

References

[1] M. Uziel, C. Koh, J. Chromatogr. 59 (1974) 1553.
[2] J. Mrochek, S.R. Dinsomore, T.P. Waalkes, Cancer. Inst. 53 (1974) 1553.
[3] L.A. Culp, E. Dore, G.M. Brown, Arch. Bioch. Biophys. 136 (1970) 73.
[4] G.E. Davis, W. Gehrke, C. Kuok, P.F. Agris, J. Chromatogr. 173 (1979) 281.
[5] H. Yuki, T. Yajma, H. Kawasaki, A. Jamaji, Anal. Biochem. 97 (1979) 203.
[6] E. Borek, Cancer Res. 31 (1971) 596.
[7] R.W. Turkington, J. Biol. Chem. 244 (1969) 5140.
[8] S.J. Kerr, Z. Dische, Ophtalmologie 9 (1970) 286.
[9] R.L. Hancock, P. McFarland, R.R. Fox, Experientia 23 (1967) 806.
[10] S.J. Kerr, Biochemistry 9 (1970) 690.
[11] H. Kaur, B. Halliwell, Biochem. J. 318 (1996) 21.
[12] J.W. Park, B.N. Ames, Proc. Nat. Acad. Sci. USA 85 (1988) 7467.
[13] L. Leclerq, C. Laurent, E. De Pauw, Anal. Chem. 69 (10) (1997) 1952.
[14] E. Tsutsui, P.R. Srinivasan, E. Borek, Proc. Natl. Acad. Sci. USA 56 (1969) 1003.
[15] G.L. Viale, Cancer. Res. 31 (1971) 605.
[16] Y. Kuchino, E. Borek, Nature 271 (1978) 126.
[17] A. Wahllander, E. Renner, G. Karlagans, J. Chromatogr. 338 (1985) 369.
[18] W. Voelter, K. Zech, P. Arnold, G. Ludwig, J. Chromatogr. 199 (1980) 345.
[19] H. Mawatari, Y. Kato, S. Nishimura, N. Sakura, K. Ueda, J. Chromatogr. B 716 (1998) 392.
[20] C. Bory, C. Chantin, R. Boulieu, Adv. Exp. Med. Biol. 431 (1998) 765.
[21] T. Nakahara, A. Shiraishi, M. Hirano, T. Matsumoto, T. Kuroki, Y. Tatebayashi, T. Tsutsumi, K. Nishiyama, H. Ooboshi, K. Nakamura, Anal. Biochem. 180 (1989) 38.
[22] J.H. van Delft, E.J. van Weert, M.J. van Winden, R.A. Baan, Chem. Biol. Interact. 80 (3) (1991) 281.
[23] H.J. Helbock, K.B. Beckman, M.K. Shigenaga, P.B. Walter, A.A. Woodall, H.C. Yeo, B.N. Ames, Proc. Natl. Acad. Sci. USA 95 (1) (1998) 288.
[24] K.E. Herbert, M.D. Evans, M.T. Finnegan, S. Farooq, N. Mistry, I.D. Podmore, P. Farmer, J. Lunec, Free Radic. Biol. Med. 20 (3) (1996) 467.
[25] M.K. Shigenaga, C.J. Gimeno, B.N. Ames, Proc. Natl. Acad. Sci. USA 86 (24) (1989) 9701.
[26] D. Germadnik, A. Pilger, H.W. Rudiger, J. Chromatogr. B 689 (2) (1997) 399.
[27] B. Weissman, P.A. Bromberg, A.B. Gutman, J. Biol. Chem. 224 (1957) 407.
[28] K. Fink, W.S. Adams, F.W. Davis, M. Nakatani, Cancer. Res. 23 (1963) 1824.
[29] A.H. Van Gennip, J. Grift, E.J. Van Bree-Blom, D. Ketting, J. Chromatogr. 163 (1979) 351.

[30] N.R. Scott, J. Chakraborty, V. Marks, J. Chromatogr. 375 (1986) 321.
[31] B. Porcelli, D. Vannoni, A. Di Stefano, R. Leoncini, Atti Accad. Fisiocritici 8 (1989) 135.
[32] S.S. Mirvish, J. Medalie, C.A. Linsell, E. Yousuf, S. Reyad, Cancer 27 (3) (1971) 736.
[33] A. Colonna, T. Russo, F. Esposito, F. Salvatore, A. Cimino, Anal. Biochem. 130 (1983) 19.
[34] B.R. Dorrbecker, S.H. Mercik, P.A. Kramer, J. Chromatogr. 336 (1984) 293.
[35] D. Vannoni, B. Porcelli, R. Leoncini, A. Di Stefano, M. Pizzichini, E. Marinello, Riv. It. Biol. Med. 10 (1990) 11.