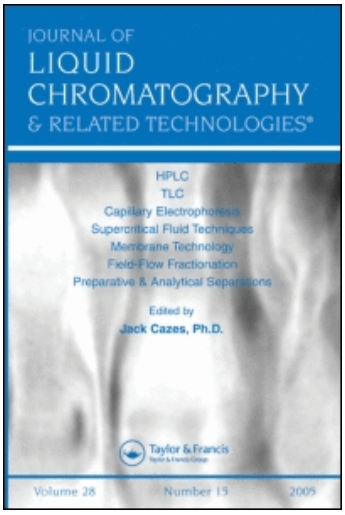

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of Methylated Purines by High Pressure Liquid Chromatography

Badaruddin Shaikh[a]; Shing-Kwan S. Huang[a]; Norbert J. Pontzer[a]; Walter L. Zielinski Jr.[a]
[a] Chemical Carcinogenesis Program, NCI Frederick Cancer Research Center, Frederick, Maryland

To cite this Article Shaikh, Badaruddin , Huang, Shing-Kwan S. , Pontzer, Norbert J. and Zielinski Jr., Walter L.(1978) 'Separation of Methylated Purines by High Pressure Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 1: 1, 75 — 88
To link to this Article: DOI: 10.1080/01483917808068379
URL: http://dx.doi.org/10.1080/01483917808068379

JOURNAL OF LIQUID CHROMATOGRAPHY, 1(1), 75-88 (1978)

SEPARATION OF METHYLATED PURINES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

Badaruddin Shaikh, Shing-Kwan S. Huang
Norbert J. Pontzer and Walter L. Zielinski, Jr.
Chemical Carcinogenesis Program
NCI Frederick Cancer Research Center, Frederick, Maryland 21701

ABSTRACT

This paper reports a method for the separation and measurement of methylated purines of interest to carcinogenesis studies by high-pressure liquid chromatography (HPLC) following their column chromatographic isolation from collected urine samples. HPLC was evaluated on three different cation-exchange columns, with optimum conditions obtained on a Partisil 10-SCX column employing isocratic elution with 0.25M $(NH_4)H_2PO_4$ at pH 4.0. This column was also found to be useful for the separation of monomethylguanine isomers. Application is shown to the analysis of rat urine following animal treatment with methyl methanesulfonate.

INTRODUCTION

Considerable attention has been given to the determination of alkylated purine bases in cellular DNA and RNA (e.g. (1-9)) following treatment of animals with both carcinogenic and non-carcinogenic alkylating agents. Studies have also been reported on the determination of methylated bases present in various RNA populations, and methylase activities associated with cells or tissues undergoing changes in growth and differentiation (10-12). Such studies have conventionally employed column cation-exchange methods for the separation and measurement of alkylated purines that are inherently tedious, time-consuming and resolution-limited.

In addition to column chromatography, paper and thin-layer chromatographic techniques have also been used (13-18). While HPLC methods have been reported for the separation of non-methylated nucleic acid bases, nucleosides, and nucleotides (19-22), little work has been reported on the use of HPLC for the separation and analysis of methylated purines of nucleic acids. Lakings et al. (23) recently reported an HPLC anion-exchange method for the separation of urinary methylated purine and pyrimidine bases, employing a specialized chromatographic system involving solvent and temperature gradients and an analysis time in excess of 16 h.

The study reported in this paper describes the development of a rapid HPLC method for the separation of specific methylated purines (3-methyladenine, and 7-methyl- and O^6-methylguanine) of significance to carcinogenesis studies as well as the non-methylated parent purines. A column chromatographic step is used for isolation of the individual urinary methyl purines prior to quantitative analysis by HPLC. The HPLC conditions also afford separations of a number of monomethyl guanine isomers. Application is shown for the analysis of changes in urinary levels of the methylated purines in rats treated with the alkylating agent, methyl methanesulfonate (MMS).

EXPERIMENTAL

Apparatus

An ALC 202 high-pressure liquid chromatograph having a gradient elution accessory (consisting of a 660 solvent programmer and two Waters 6000 high-pressure pumps), a U6K septumless injector, and a 254 nm ultraviolet absorption detector was used throughout this study (Waters Associates, Milford, MA). Peak areas and retention times were determined with a 3352A laboratory data system linked through a 18652A A/D converter to the UV detector output of the liquid chromatograph, with data output to a 9866A thermal-line printer (Hewlett Packard, Avondale, PA).

Columns

The columns evaluated were commercial stainless steel columns pre-packed with strong cation-exchangers. The column packings were 10μ Partisil 10-SCX (25 cm x 4.6 mm I.D., Whatman, Clifton, NJ), 8-12μ Aminex A-7 (25 cm x 2.1 mm I.D., Varian Associates, Palo Alto, CA), and 37-50μ Bondapak CX/Corasil (61 cm x 2 mm I.D., Waters Associates).

Chemicals

Aqueous buffers used as mobile phases were made up in deionized water using reagent grade chemicals. The pH was adjusted with either phosphoric acid or ammonium hydroxide. Guanine (and its 1, 3, 7, 9, and N^2-monomethyl derivatives), adenine, and 3-methyladenine were obtained from Sigma (St. Louis, MO) and Schwarz/Mann (Orangeburg, NY). O^6-Methylguanine and 8-methylguanine were synthesized by G. M. Muschik of our laboratory.

Mobile Phase Conditions

Following a preliminary evaluation of mobile phase variables (buffer type, pH, buffer concentration, column temperature), ammonium dihydrogen phosphate was selected as the mobile phase buffer under conditions of (a) 0.015 M (pH 4.7) at ambient temperature (Bondapak CX/Corasil); (b) 0.015 M (pH step change from pH 5.7 to pH 8.5) at 60°C (Aminex A-7); and (c) 0.025 M and 0.25 M (pH 4.0) at ambient temperature (Partisil 10-SCX).

Preparation of Purine Solutions

Standard solutions of the individual purines and their mixtures were prepared in 0.01N HCl and stored under refrigeration (5°C). Guanine, adenine, 7-methylguanine, and O^6-methylguanine were analyzed routinely to assure precision of the instrument and column conditions. The relative elution order of the solute peaks was found to be stable throughout this study, and small variations in absolute retention data were insignificant when normalized to the retention data of the standard bases.

Animal Experiment

Urine samples were collected from Fisher 344 male rats (8 weeks old) in metabolism cages for 24 h prior to MMS treatment (200 mg/kg body weight, administered by gavage), and 0-24 and 24-48 h following MMS treatment. Aliquots (5 ml) of the urine samples were filtered, adjusted to pH 2.0 with 1 M HCl, and chromatographed on a 20 x 2.0 cm column of -400 mesh Bio-Rad AG50W-X4 cation exchanger (NH_4^+ form) as reported previously (24) for selective collection of 7-methylguanine, 3-methyladenine, O^6-methylguanine and 1-methylnicotinamide. Aliquots (10-20 µl) of the individual concentrated fractions were taken for quantitative assay by HPLC.

RESULTS AND DISCUSSION

Evaluation of HPLC Cation-Exchange Columns

The standard purine mixture of interest for this evaluation contained guanine, 7-methylguanine, O^6-methylguanine, adenine and 3-methyladenine. This mixture was initially evaluated on the pellicular Bondapak CX/Corasil column. Sufficient solute retention and column selectivity to achieve base-line separation of this mixture was found lacking on this pellicular ion exchange column (Fig. 1).

Chromatography of the same mixture on Aminex A-7 indicated adequate peak separations (Table 1), yet a pH step-change (from 5.7 to 8.5, following elution of adenine) was required to elute 3-methyladenine. Attempts to improve the overall analysis on Aminex A-7 were unsuccessful, as noted by the findings that (a) with isocratic elution at pH 5.7, 3-methyladenine was retained on the column; (b) with isocratic elution at pH 8.5, 3-methyladenine was eluted at 19 min but the resolution of earlier peaks disappeared; and (c) with an exponential pH gradient from 5.7 to 8.5, 3-methyladenine still had an elution time in excess of 90 min.

The most satisfactory separation was obtained on Partisil 10-SCX, as indicated in the chromatograms shown in Figs. 2 and 3

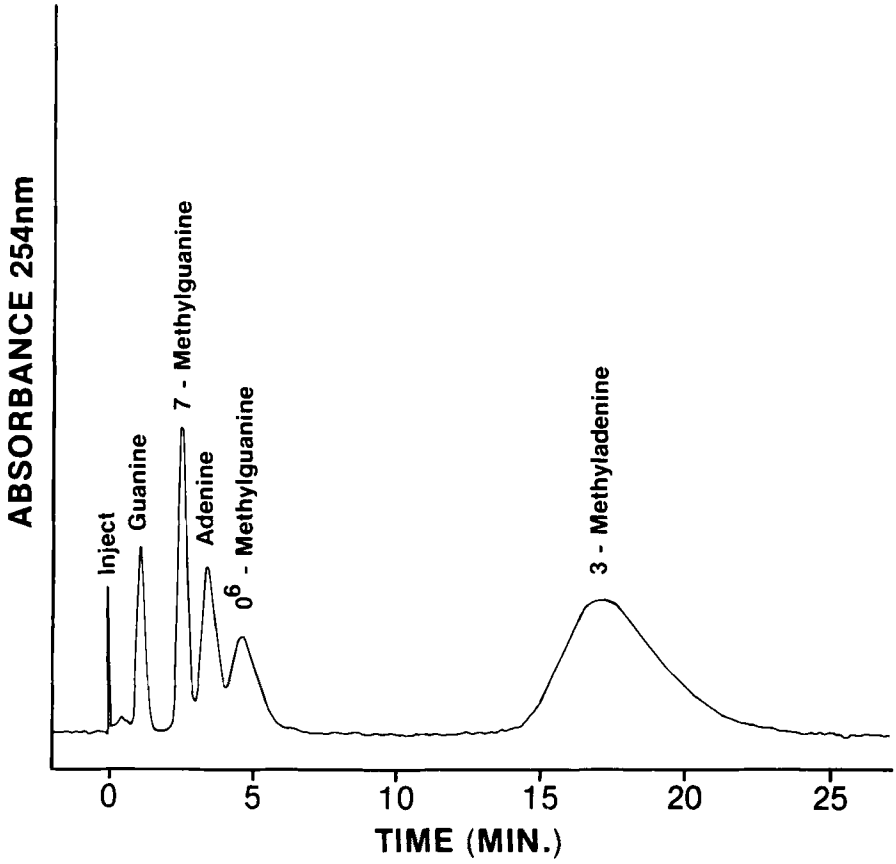

FIGURE 1

Chromatogram of standard purine mixture. Conditions: Column, Bondapak CX/Corasil, 61 cm x 2.0 m.m. stainless steel; temperature, ambient; detector sensitivity, 0.04 a.u.f.s.; mobile phase, 0.015 M $NH_4H_2PO_4$, pH 4.7; flow-rate, 1.0 ml/min; chart speed, 0.2 inch/min.

(using isocratic ambient elution with 0.025M and 0.25M ammonium dihydrogen phosphate buffer, respectively, at pH 4.0). Rapid separation of the five components comprising the standard purine mixture was obtained on Partisil 10-SCX (Fig. 3), in contrast to the inferior resolution (Bondapak CX/Corasil) and prolonged analysis time (Aminex A-7) obtained on the other two columns.

TABLE 1

Retention Data on HPLC Cation-Exchange Columns*

Purine	Bondapak CX/Corasil	Aminex A-7	Partisil 10-SCX	
			0.025M	0.25M
Guanine	1.2	15.9	5.4	4.1
7-Methylguanine	2.6	24.5	10.7	6.6
Adenine	3.5	46.3	13.1	5.8
O^6-Methylguanine	4.7	38.1	21.8	9.6
3-Methyladenine	17.3	98.7	52.1	12.6

* Conditions: Column dimensions and mobile phase (1.0 ml/min) conditions as given in experimental; pH step change from 5.7 to 8.5 on Aminex A-7 was made after elution of adenine (60 min after sample injection).

Furthermore, since the HPLC analysis time is short (Fig. 3), the smaller purine total elution volumes afford greater sensitivity for a given amount of purine than is feasible with long HPLC analysis times (e.g. 23). This is an attractive feature of this method in its application to the measurement of changes in urine concentrations of trace level purines (e.g. 3-methyladenine and O^6-methylguanine). A standard calibration curve covering a range of concentration from 35-140 ng for 7-methylguanine, 55-220 ng for O^6-methylguanine and 75-340 ng for 3-methyladenine showed excellent linearity with a correlation coefficient of 0.999 in all three cases. Finally, under the conditions used in Figure 2, separations are achievable for a number of other monomethylguanine isomers, as shown in Figure 4.

Recovery of Purines from Urine

Recoveries were determined for known amounts of purines added to 5 ml samples of control urine taken through the entire pro-

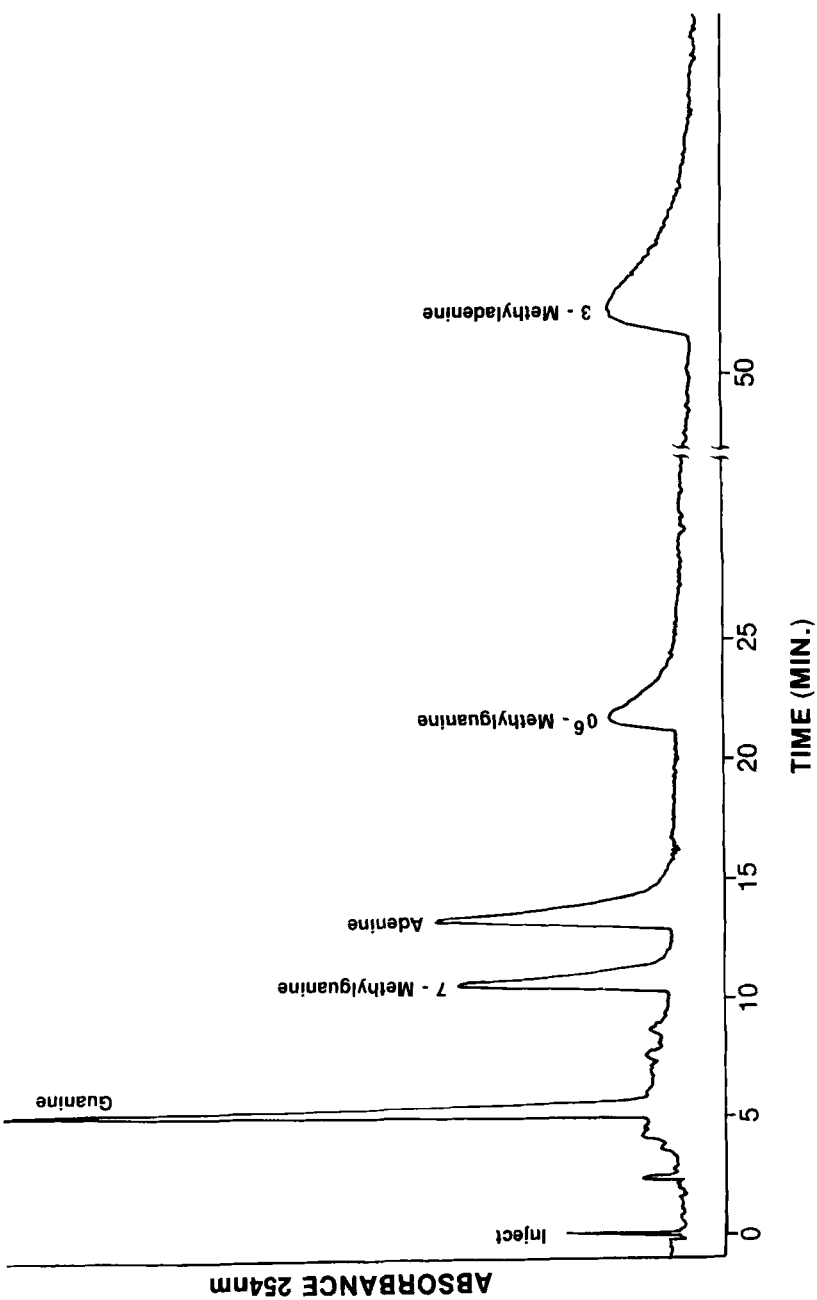

FIGURE 2. Chromatogram of standard purine mixture. Conditions: Column, Partisil 10-SCX, 25 cm X 4.2 m.m stainless steel; temperature, ambient; detector sensitivity 0.04 A.U.F.S.; mobile phase, 0.025 M $NH_2H_2PO_4$; pH 4.0; flow-rate, 1.0 ml/min; chart speed, 0.2 inch/min.

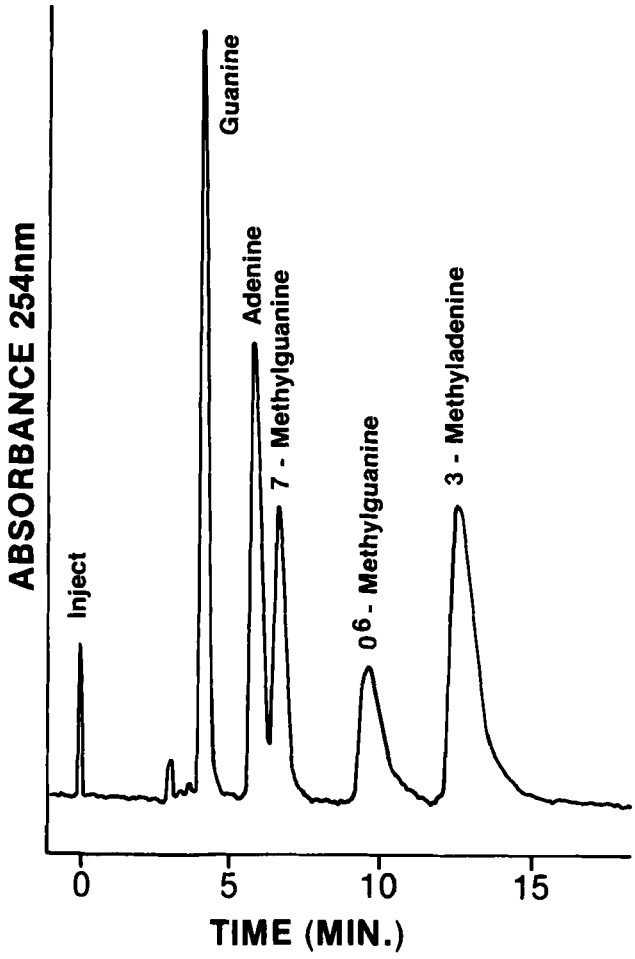

FIGURE 3

Chromatogram of standard purine mixture on Partisil 10-SCX column using 0.25 M mobile phase. Conditions same as in Fig. 2.

cedure (column chromatographic isolation, fraction collection, concentration, and HPLC assay). Recoveries for the addition of 300 and 500 μg of 7-methylguanine to urine were 86 ± 1.5% and 90 ± 3.5%, respectively; for 300 and 500 μg of O^6-methylguanine to urine were 74 ± 2.3% and 76 ± 2.5%, respectively; for 100 and

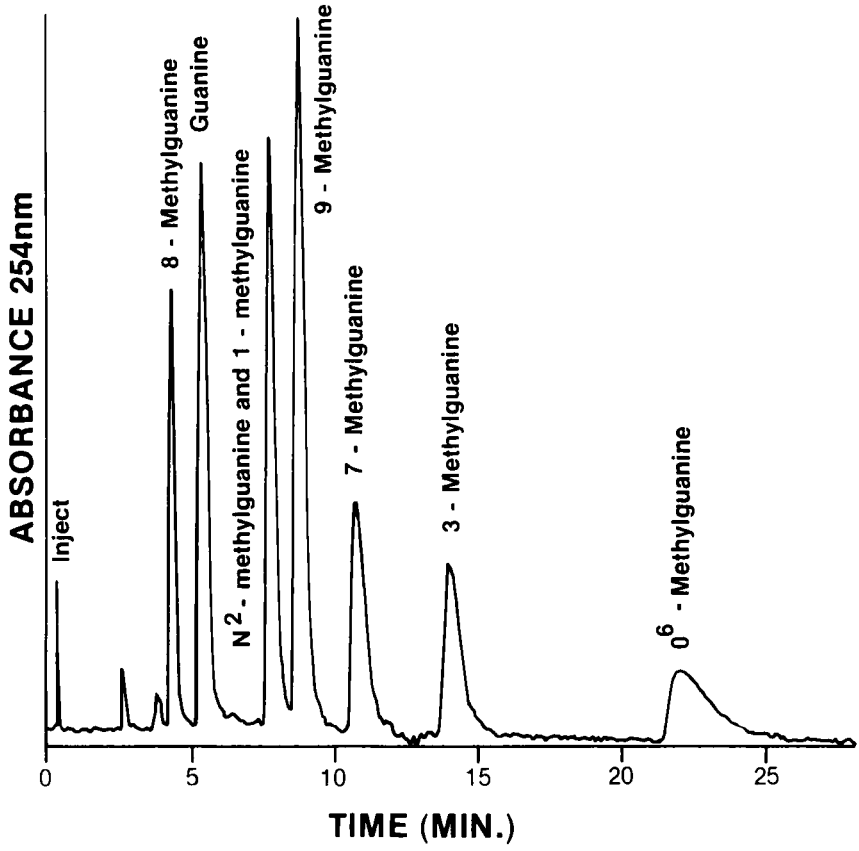

FIGURE 4

Chromatogram of mixture of monomethyl guanine isomers. Same conditions as in Fig. 2.

250 μg of 3-methyladenine to urine were 86 ± 0.9% and 90 ± 2.1%, respectively (and for 150, 250 and 500 μg of 1-methylnicotinamide to urine were 98 ± 2.9%, 104 ± 4.2% and 102 ± 2.6%, respectively).

Analysis of Rat Urine Following Administration with MMS

Urine samples obtained from rats treated with the methylating agent methyl methanesulfonate (MMS), prior to and following treatment, were passed through the column chromatography isolation

step and quantitation by HPLC analysis on the Partisil 10-SCX column. Quantitation by HPLC was achieved by comparison of integrated peak areas with those obtained from standard solutions of the individual purine (or 1-methylnicotinamide) in question, with injections of the standards prior to and immediately following HPLC injection of the individual components isolated from column chromatography. An illustration of the elution positions of the urinary components of interest for the column isolation step (using control urine spiked with the standards) is shown in Figure 5. In the MMS animal experiment, no O^6-methylguanine was detected in the urine. This is due in part to the report that O^6-alkylpurines do not survive acid treatment (as required for urine cleanup) and extremely little methylation is known to take place at the 6-oxygen position of guanine from MMS treatment (3). In contrast, a significant increase was observed in 1-methylnicotinamide excretion during the first 24 h following MMS treatment. This is consistent with the work reported by Chu and Lawley (25) on MMS. An elevation of 3-methyladenine was also observed during this period, however, which has not been previously reported. Since 7-methylguanine was not observed to change following MMS treatment (this work and Reference 25), plots of the ratios of 3-methyladenine/7-methylguanine and 1-methylnicotinamide/7-methylguanine can be used to reflect urinary changes during the course of the animal experiment (Figure 6).

CONCLUSIONS

A useful HPLC method for the analysis of specific methylated purines in rat urine has been developed and evaluated. Prior to HPLC analysis of methylated purines and 1-methylnicotinamide in urine, the samples of collected rat urine were passed through a single column chromatographic procedure to remove interfering compounds. Good recoveries through the entire method were achieved when urine was spiked with 7-methylguanine, O^6-methylguanine, 3-methyladenine and 1-methylnicotinamide by this method.

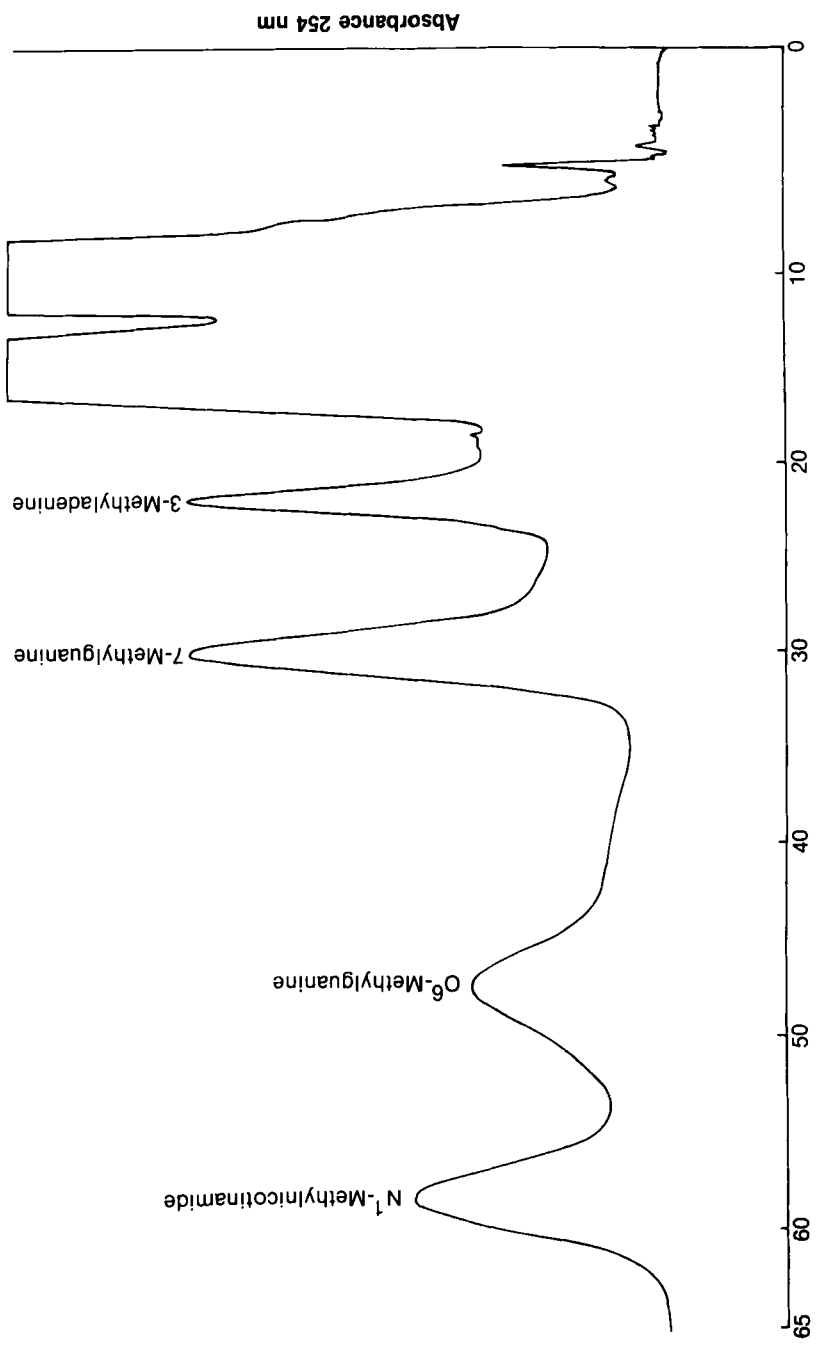

FIGURE 5. Chromatogram of control urine spiked with standards on AG50W-X4 (NH_4^+ form) minus 400 mesh (20 X 2 cm) Conditions: washed with about 250 ml H_2O and eluted with 1 M NH_4COOH, pH 8.9 after introduction of urine; detector sensitivity setting of 1.28 o.d. full scale; chart speed, 3 in/hr.

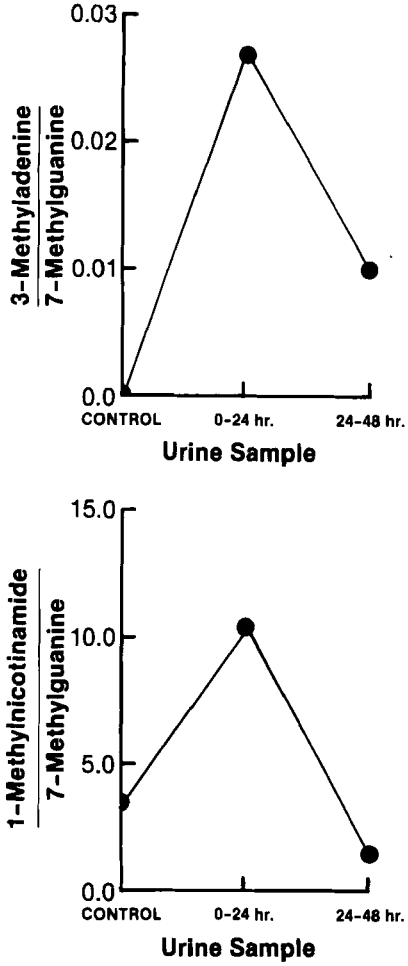

FIGURE 6

Changes in the urinary levels of 3-methyladenine and 1-methylnicotinamide relative to 7-methylguanine, following animal treatment with MMS. Column chromatography and HPLC conditions same as in Fig. 5 and 3, respectively.

Rat urine was analyzed after administration of MMS. An increased excretion of 1-methylnicotinamide and 3-methyladenine was observed 24 h after administration of MMS, while the level of 7-methylguanine remained relatively unchanged.

The methodology reported here should be useful for investigations of purine metabolism associated with certain diseases, including cancer. These methods have a potential to be extended in the studies of alterations of nucleic acid components caused by alkylating agent carcinogens and furthermore, elevated excretion of 1-methylnicotinamide and 3-methyladenine may be of use in monitoring DNA damage in patients undergoing alkylating agent therapy.

ACKNOWLEDGEMENTS

Research was sponsored by the National Cancer Institute under Contract No. NO1-CO-25423 with Litton Bionetics, Inc.

REFERENCES

1. Dunn, D.B., Smith, J.D. and Spahr, P.F., J. Mol. Biol. 2:113, 1960.
2. Mandel, L.R., Srinivasan, P.R. and Borek, E., Nature, 209:586, 1966.
3. Lawley, P.D., Topics in Chemical Carcinogenesis, Nakahara, W., Takayama, S., Sugimura, T. and Odashima, S., eds., University Park Press, Baltimore, Maryland, 1972, p. 237.
4. Lawley, P.D. and Shah, S.A., Biochem. J., 128:117, 1972.
5. Ahonen, J., Kivisaari, Vastamaki, M., Valimaki, M. and Kulonen, E., Biochim. Biophys. Acta, 262:233, 1972.
6. O'Connor, P.J., Capps, M.J. and Craig, A.W., Brit. J. Cancer, 27:153, 1973.
7. Margison, G.P., Capps, M.J., O'Connor, P.J. and Craig, A.W., Chem.-Biol. Interactions, 6:119, 1973.
8. Löfroth, G., Osterman-Golkar, S. and Wennerberg, R., Experientia, 30:641, 1974.
9. Löfroth, G. and Wennerberg, R., Z. Naturforsch., 29:651, 1974.
10. Hall, R.H., Methods Enzymol., 129:305, 1967.
11. Starr, J.L. and Sells, B.H., Physiol. Rev., 49:923, 1969.

12. Sharma, O.K. and Borex, E., Biochem., 9:2507, 1970.
13. Craddock, V.M., Biochem. Biophys. Acta, 195:351, 1969.
14. Iwanami, Y. and Brown, G.M., Arch. Biochem. Biophys., 124:472, 1968.
15. Fink, K. and Adams, W.S., J. Chromatog., 22:118, 1966.
16. Bjork, G.R. and Svensson, I., Biochim. Biophys. Acta, 138:430, 1967.
17. Grippo, P., Iaccarino, M., Rossi, M. and Scarano, E., Biochim. Biophys. Acta, 95:1, 1965.
18. Munns, T.W., Podratz, K. and Katzman, P.A., J. Chromatog., 76:401, 1973.
19. Burtis, C.A., J. Chromatog., 151:183, 1970.
20. Burtis, C.A., Munk, M.N. and MacDonald, F.R., Clin. Chem., 16:667, 1970.
21. Kirkland, J.J., J. Chromatog. Sci., 8:72, 1970.
22. Brown, P.R., Boblick, S. and Hanley, F.L., J. Chromatog., 99:557, 1974.
23. Lakings, D.B., Waalkes, T.P. and Mrochek, J.E., J. Chromatog., 116:83, 1976.
24. Shaikh, B., Pontzer, N.J., Huang, S.S. and Zielinski, W.L., J. Chromatog. Sci., 15:215, 1977.
25. Chu, B.C.F. and Lawley, P.D., Chem.-Biol. Interactions, 7:65, 1973.