ELSEVIER

Journal of Chromatography A, 793 (1998) 341–347

JOURNAL OF CHROMATOGRAPHY A

Capillary electrophoresis of urinary normal and modified nucleosides of cancer patients[1]

H.M. Liebich[a,*], G. Xu[a,b], C. Di Stefano[a], R. Lehmann[a]

[a]Medizinische Universitätsklinik, Abt. IV, Department of Clinical Chemistry, Otfried-Müller-Strasse, D-72076 Tübingen, Germany
[b]National Chromatographic R. & A. Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 116011 Dalian, China

Abstract

This paper gives a capillary electrophoretic method for the separation of 15 urinary normal and modified nucleosides from cancer patients in less than 40 min. A 500 mm×50 μm uncoated capillary column (437.5 mm to window) was used. The effects of the voltage and the sodium dodecyl sulfate (SDS) concentration in the buffer on the separation were studied. With reproducibilities of migration times better than 1.2% (R.S.D.) and determined concentrations better than 5–25%, depending on the concentrations of nucleosides in the urine, the analytical characteristics of the method were good. Using this developed method, the concentrations of 13 normal and modified nucleosides, extracted on a phenyl boronic acid affinity chromatography column, in 25 urines from patients of 14 kinds of cancer were determined. The levels (nmol/μmol creatinine) of modified nucleosides in urines from cancer patients were increased as compared with those in normal urines.
© 1998 Elsevier Science B.V.

Keywords: Nucleosides

1. Introduction

Methylated purine, pyrimidine and other modified nucleosides have been shown to be excreted in abnormal amounts in urine of patients with cancer [1–5]. By contrast, excretion of modified nucleosides by normal adults is relatively low [6,7]. These excretory products are predominantly minor components of transfer ribonucleic acid (tRNA) which originate from the degradation of macromolecules [8]. Evidence indicates that methylation of tRNA occurs only after synthesis of the intact molecule. Since no kinases have been found that will reincorporate the monomer units into tRNA, the modified bases and nucleosides are excreted following metabolic degradation of tRNA molecules [9]. Additional studies show that pseudouridine (Pseu) is not catabolized but excreted in urine as the intact molecule [10,11]. Efforts have been made to use modified nucleosides as biochemical markers for neoplastic diseases. Elevated concentrations have been suggested as possible markers [2,4,12–16] for leukemia [7,17], lymphoma [17,18], small cell lung cancer [19,20], oesophagus cancer [21], breast cancer [22,23], nasopharyngeal cancer [5], brain cancer [24], bronchogenic carcinoma [25], colorectal carcinoma [26], cancer of the urinary organs or female genital tract [27], Hodgkin's disease [28] and for the whole-body turnover of RNAs [2,12,21,24,29–32]. Urinary levels of modified nucleosides have been

*Corresponding author.

[1] Presented at the 9th International Symposium on High Performance Capillary Electrophoresis and Related Microscale Techniques, Anaheim, CA, 26–30 January 1997.

0021-9673/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
PII S0021-9673(97)00915-1

suggested to be useful for monitoring progress of disease and response to therapy [5,16,22,27,28].

Although modified nucleosides have been studied extensively in urine, only RP-HPLC following concentration by boronate gel [2,5,10,12,14,16,19, 20,27,29–34] and to some extent immunoassays [21,35] have been used as the main analytical methods. Recent technology developed in our laboratory has allowed the separation and quantification of nucleosides in normal urine using a capillary electrophoretic method [36]. Now, this technique was further optimized to make it suitable to the analysis of urine from cancer patients in a shorter time. Applying this method, normal and modified nucleosides in 25 urines from patients of 14 kinds of cancer were determined and the results compared with those from normal urine.

2. Experimental

2.1. Urine samples

Spontaneous urines were collected from 2 healthy men and 25 cancer patients (16 males, 9 females). After collection the samples were immediately frozen and stored at −20°C. For the analysis of the ribonucleosides the samples were thawed at room temperature. The patients were between 43 and 75 years of age. Fourteen kinds of cancer included breast, bronchial, oesophagus, rectum, hypopharynx, prostate, thigh, follicular, anaplastic, bladder, endocrine, floor of mouth cancer, as well as glioblastoma and oligodendroglioma. While urines were collected, most of the patients were being treated by chemotherapy and/or radiation. Some patients were operated several months or years ago, but the carcinoma recurred and existed at collecting time. Two of the patients died in the 5 months after urine collection.

2.2. Chemicals

Formic acid was purchased from Riedel-de Haen (Seelze, Germany). Ammonium acetate, methanol, ammonia, sodium dihydrogenphosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) were from Merck (Darmstadt, Germany). Borax (sodium tetraborate, $Na_2B_4O_7 \cdot 10H_2O$) and all nucleoside standards were obtained from Sigma (Deisenhofen, Germany). Sodium dodecyl sulfate (SDS) and Affi-gel 601 were obtained from Bio-Rad (Munich, Germany).

2.3. Nucleoside extraction from urine

The phenyl boronate gel affinity chromatographic method used to isolate the urinary nucleosides was the same as described before [33,36]. Ten ml of urine was mixed with 0.4 ml of 0.8 m*M* internal standard (3-deazauridine) solution and extracted on a phenyl boronate column (Affi-gel 601) possessing a specific affinity for *cis*-hydroxyl groups. The eluate from the phenyl boronate column was evaporated to dryness in a vacuum system at 39–40°C and dissolved in 1 ml of water producing an extract concentrated by a factor of 10 as compared to the original urine.

2.4. Capillary electrophoresis

All separations were performed on a Dionex (Idstein, Germany) capillary electrophoresis system (CES I) in an uncoated capillary (500 mm×50 μm I.D., 437.5 mm to window) from Grom (Herrenberg-Kayh, Germany). Fused-silica capillaries were conditioned before use by rinsing with 2 *M* NaOH and water for 15 min each. The electrophoretic separations were performed with 25 m*M* borax ($Na_2B_4O_7 \cdot 10H_2O$)–50 m*M* phosphate ($NaH_2PO_4 \cdot H_2O$) buffers of varying SDS concentrations. The buffers were obtained by dissolving different amounts of SDS in the aqueous solution, which contained 9.54 g (25 mmol) of $Na_2B_4O_7 \cdot 10H_2O$ and 6.90 g (50 mmol) of $NaH_2PO_4 \cdot H_2O$ per l and was adjusted to pH 6.7 by using 5% HCl. The buffers were filtered through a 0.45 μm membrane filter, and degassed in an ultrasonic bath for 10 min before use. The capillary was rinsed for 100 s with water, followed by 100 s with 0.1 *M* NaOH, 100 s with water, and finally 120 s with buffer after each run. Samples were introduced by gravity injection at 100 mm head height for 45 s (injected sample volume, 10–15 nl). On-column UV detections were performed at 260 and 210 nm in sequence. Data were collected using a dedicated computer system with a Dionex AC interface and Dionex AI-450 software.

2.5. Identification and quantification of nucleosides

The details of this procedure have been reported elsewhere [36]. The peaks were identified by: (1) comparing migration times of the unknown peaks with those of the standard nucleosides eluted under the same conditions, and (2) by spiking the sample with stock standard solutions of nucleosides. The calibration curves obtained by plotting the peak areas vs. concentrations of the nucleosides were obtained by eluting the three standard solutions with different concentrations. Because the standard solutions of the nucleosides were extracted under the same conditions as the urines, the concentrations of the nucleosides in urine could be calculated directly based on the calibration curves. The concentrations were then transformed into nmol/μmol creatinine. Urinary creatinine levels were determined by a modified Jaffe method [37].

3. Results and discussion

3.1. Optimization of operational conditions

A capillary electrophoretic method using an uncoated column with a length of 565 mm has been developed in our laboratory [36], and was applied to the analysis of urinary nucleosides extracted from healthy volunteers. The analysis was completed in less than 45 min. The results were similar to those from HPLC methods [33,36]. In this study new capillary columns with a length of 500 mm were used to further reduce the analytical time. The composition of the buffers and the voltage were varied to optimize the conditions for the separation of 15 normal and modified nucleosides of interest.

Fig. 1 shows that with the increase of the SDS concentration, migration orders and resolution of some nucleosides were changed. Pyrimidine nucleosides eluted before purine nucleosides in the order U<C<G<A<X and normal nucleosides eluted before their corresponding alkylated analogs. Using a buffer with 200 m*M* SDS, the nucleosides in the standard solutions can be separated. Unfortunately, nucleoside samples extracted from urine contain many other substances which disturb the determination of the nucleosides. Fig. 2 shows two typical electropherograms of nucleosides extracted from a urine. More peaks are obtained using the buffer with 300 m*M* SDS. The optimal buffer for the separation of nucleosides extracted from urine is 300 m*M* SDS–25 m*M* borate–50 m*M* phosphate (pH 6.7).

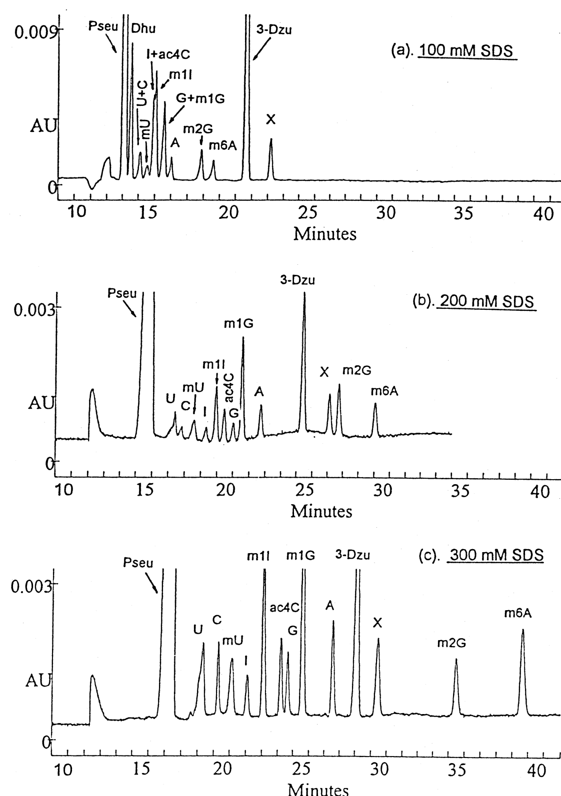

Fig. 1. The effect of SDS concentration on the separation of standard nucleosides solutions. Capillary: 500 mm×50 μm I.D. (437.5 mm to window); applied voltage: 7.0 kV; buffers and detection wavelength: (a) 100 m*M* SDS–25 m*M* borate–50 m*M* phosphate pH 6.7, 210 nm; (b) 200 m*M* SDS–25 m*M* borate–50 m*M* phosphate pH 6.7, 260 nm; and (c) 300 m*M* SDS–25 m*M* borate–50 m*M* phosphate pH 6.7, 260 nm; injection head height: 100 mm; load time: 45 s; peak identifications: Pseu=pseudouridine, Dhu=dihydrouridine, U=uridine, C=cytidine, mU=3-methyluridine +5-methyluridine, I=inosine, m1I=1-methylinosine, ac4C=N4-acetylcytidine, G=guanosine, m1G=1-methylguanosine, A=adenosine, 3-Dzu=3-deazauridine, X=xanthosine, m2G=2-methylguanosine, m6A=N6-methyladenosine.

Voltage control in capillary electrophoresis offers an important parameter affecting sample migration time. As field strength is increased, migration velocity increases, and, therefore, migration time de-

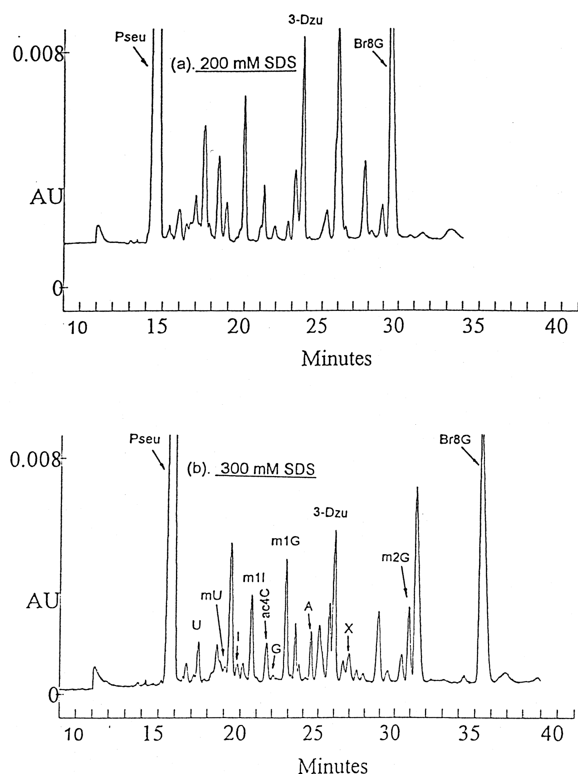

Fig. 2. The effect of SDS concentration on the separation of nucleosides extracted from urine. Sample: nucleosides extracted from a normal spontaneous urine; detection wavelength: 260 nm; buffers: (a) 200 m*M* SDS–25 m*M* borate–50 m*M* phosphate pH 6.7; and (b) 300 m*M* SDS–25 m*M* borate–50 m*M* phosphate pH 6.7; other conditions as in Fig. 1.

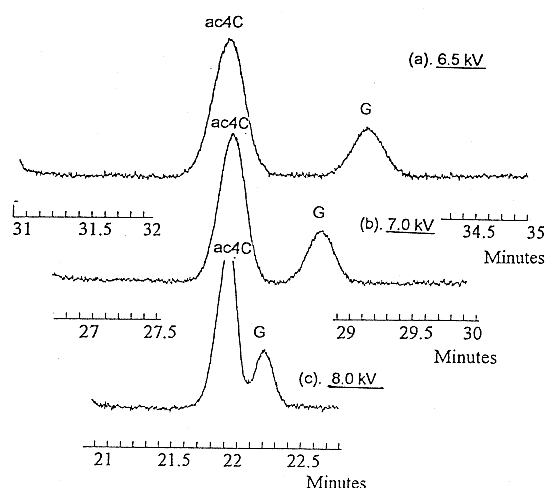

Fig. 3. The effect of the applied voltage on the separation of the 'critical pair' ac4C/G. Sample: standard nucleoside solution; detection wavelength: 210 nm; the buffer: 300 m*M* SDS–25 m*M* borate–50 m*M* phosphate pH 6.7; voltages: (a) 6.5 kV; (b) 7.0 kV with 49 μA of current; and (c) 8.0 kV; others as in Fig. 1.

creases. In general, peak efficiency, and, therefore, resolution increases as a function of voltage up to a point under a given set of run condition; above this point efficiency may diminish. For a given sample the applied voltage depends on the 'critical pair of substances', that is the pair of substances which are difficult to separate. In the nucleoside sample, the 'critical pair of substances' is N4-acetylcytidine (ac4C)/guanosine (G). When the applied voltages were increased from 6.5 kV to 8.0 kV (Fig. 3), the resolution between ac4C and G is reduced, but the analysis time is shortened. The optimal voltage is 7.0 kV with the current of 47–49 μA. Under these conditions, all nucleosides of interest could be baseline-separated (Figs. 1 and 2).

3.2. Reproducibility of quantification

Under the selected conditions, for a given sample, the within-day reproducibility of the migration times was better than 1.2%, of the peak areas better than 5%, which is similar to the results with a 565 mm×50 μm capillary column [36]. The analytical time was shortened to less than 40 min because of the shorter column.

To evaluate the reproducibility of the analytical method, five 10 ml aliquot samples, taken from a spontaneous urine, were extracted and analyzed. The coefficients of variation were 4–27% depending on the concentration of nucleosides and their migration position (Table 1). This reproducibility is sufficient for the analysis of nucleosides in urine.

3.3. Analysis of nucleosides in urine from cancer patients

By applying this capillary electrophoretic method and analysing 24 normal 24-h urine samples, the reference ranges of nucleosides in normal human urine have been established [36]. To avoid the inconvenience of 24-h collections, Gehrke et al. [10]

Table 1
Reproducibility of quantification of developed CE method[a]

	C (μM)	S.D.	R.S.D. (%)
Pseu	235.67	20.69	8.78
U	3.58	0.16	4.46
C	1.68	0.30	17.76
mU	2.09	0.18	8.74
I	2.14	0.59	27.42
m1I	9.57	1.11	11.58
ac4C	6.44	1.03	16.00
G	1.09	0.09	8.24
m1G	7.83	0.88	11.20
A	3.59	0.40	11.15
X	3.08	0.15	4.69
m2G	8.79	2.73	31.03

[a] Five aliquot samples from a spontaneous urine from a healthy volunteer were extracted and analyzed under identical conditions. C is the average value of 5 determinations.

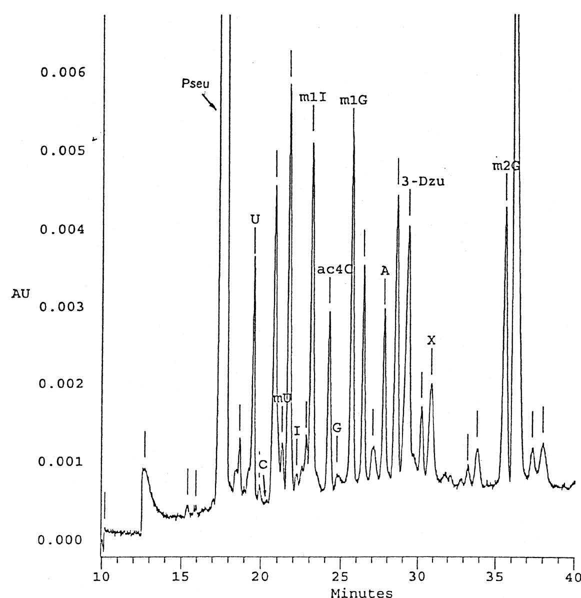

Fig. 4. Typical electropherograms of normal and modified nucleosides extracted from urine of a cancer patient. Sample: nucleosides extracted from a spontaneous urine from a patient with breast cancer; detection wavelength: 260 nm; buffer: 300 mM SDS–25 mM borate–50 mM phosphate pH 6.7; voltage: 7.0 kV with 49 μA of current; other conditions as in Fig. 1.

and Borek [4] undertook extensive studies to determine whether the level of excretion of modified nucleosides could be related to the creatinine level of random urine samples. From these studies, it became apparent that the ratio of nucleosides to creatinine in random samples was the same as that in 24-h urines. This means that random samples can be used, and the results are as valid as those from urine collected over a 24-h period, when nucleoside levels are expressed relative to creatinine. For this reason we could use spontaneous urine samples instead of 24-h collections in the study on the nucleosides excretion in cancer patients. Typical electropherograms of normal and modified nucleosides extracted from a spontaneous urine of a cancer patient are given in Fig. 4. The resolution is similar to that obtained with a longer column [36]. M6A was reported either to be present at a very low concentration in or to be absent from urine [2,36]. Its concentration in urine from cancer patients was not determined in this study. When UV detection was performed at 210 nm, dihydrouridine can also be determined [36].

Using the developed method, 25 spontaneous urines from patients with different kinds of cancer were analyzed. Fig. 5 demonstrates that the levels of nucleosides in urine from cancer patients were generally elevated, and that the increase of modified nucleosides is more pronounced than that of normal nucleosides. When a significant elevation of nucleosides is defined as a value higher than the average plus two standard deviations determined in normal urine, the concentrations of pseudouridine (pseu), 1-methylinosine (m1I), N4-acetylcytidine (ac4C), 1-methylguanosine (m1G) and 2-methylguanosine (m2G) in urine of patients with cancer were elevated significantly. Perhaps these nucleosides can be chosen as candidates as biochemical markers of cancer. These results are in agreement with the suggestions of several previous studies [5,15,27,33].

4. Conclusions

This study shows the applicability of capillary electrophoresis to the quantitative analysis of urinary modified nucleosides from cancer patients in less than 40 min with a good resolution and sufficient sensitivity. Compared with the method published in [36], the analytical time is shortened by 5 min

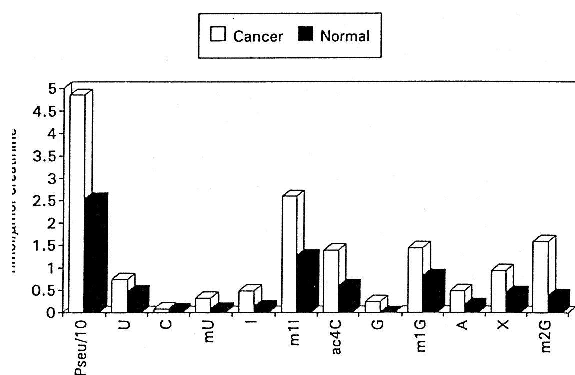

Fig. 5. Mean excretion of normal and modified nucleosides in urine from normal healthy volunteers and patients with cancer. Urinary nucleoside mean excretion levels (mean±S.D.) by healthy volunteers were: Pseu, 25.32±10.32; U, 0.47±0.190; C, 0.07±0.095; mU, 0.089±0.123; I, 0.14±0.10; m1I, 1.27±0.475; ac4C, 0.60±0.384; G, 0.01±0.021; m1G, 0.82±0.298; A, 0.18±0.172; X, 0.45±0.260; and m2G, 0.39±0.197. Urinary nucleoside mean excretion levels (mean±S.D.) by 25 cancer patients were: Pseu, 48.45±24.92; U, 0.745±0.344; C, 0.084±0.106; mU, 0.319±0.222; I, 0.480±0.509; m1I, 2.591±1.440; ac4C, 1.391±0.674; G, 0.247±0.318; m1G, 1.438±0.786; A, 0.486±0.566; X, 0.939±0.998; and m2G, 1.577±1.029.

because of a shorter column. With both methods the analytical results obtained are similar to those with HPLC [33]. Immunoassays with antibodies against modified nucleosides offer a possible alternative methodology with some advantages over HPLC and CE in the quantitation of one or few nucleosides. However, when a broad spectrum of modified nucleosides is studied, HPLC and CE are more suitable than immunoassays. We think that the CE method described in this paper is a complementary technique to HPLC for the analysis of nucleosides in urines and also offers some advantages over HPLC [36]. Because of the small number of samples, it is not yet possible to exactly correlate the nucleoside levels with the clinical data. It will also be necessary to correlate the levels of the modified nucleosides with those of conventional tumour markers.

Acknowledgements

G.X. gratefully acknowledges the fellowship from the Max-Planck-Institut of Germany.

References

[1] G. Nass, Modified Nucleosides and Cancer, Springer-Verlag, New York, 1983.
[2] G. Schoech, G. Sander, H. Topp, G. Heller-Schoch, in: C.W. Gehrke, K.C. Kuo (Eds.), Chromatography and Modification of Nucleosides, Part C, Elsevier, Amsterdam, 1990, p. C389.
[3] T.P. Waalkes, M.D. Abeloff, D.S. Ettinger, K.B. Woo, C.W. Gehrke, K.C. Kuo, E. Borek, Cancer 50 (1982) 2457.
[4] E. Borek, Tumour. Biol. 5 (1984) 1.
[5] R.W. Trewyn, R. Glaser, D.R. Kelly, D.G. Jackson, W.P. Graham, C.E. Speicher, Cancer 49 (1982) 2513.
[6] M.H. Gail, L. Munez, K.R. McIntire, B. Radovich, G. Braunstein, P.R. Brown, L. Deftos, A. Dnistrian, M. Dunsmore, R. Elashoff, N. Geller, V.L.W. Go, K. Hirji, M.R. Klauber, D. Pee, G. Petroni, M. Schwartz, A.R. Wolfsen, J. Nat. Can. Inst. 76 (1986) 805–815.
[7] R.W. Trewyn, M.R. Grever, Crit. Rev. Clin. Lab. Sci. 24 (1986) 71.
[8] T.P. Waalkes, S.R. Dinsmore, J.E. Mrochek, J. Natl. Cancer Inst. 51 (1973) 271.
[9] T.P. Waalkes, C.W. Gehrke, R.W. Zumwalt, S.Y. Chang, D.B. Lakings, D.C. Tormey, D.L. Ahmann, C.G. Moertel, Cancer 36 (1975) 390.
[10] C.W. Gehrke, K.C. Kuo, T.P. Waalkes, E. Borek, Cancer Res. 39 (1979) 1150.
[11] J. Speer, C.W. Gehrke, K.C. Kuo, T.P. Waalkes, E. Borek, Cancer 44 (1979) 2120.
[12] K. Nakano, T. Nakao, K.H. Schram, W.M. Hammargren, Nucleic. Acids Symp. Ser. (1991) 125.
[13] R.W. Zumwalt, T.P. Waalkes, K.C. Kuo, C.W. Gehrke, in: C.W. Gehrke, K.C. Kuo (Eds.), Chromatography and Modification of Nucleosides, Part C, Elsevier, Amsterdam, 1990, p. C15.
[14] K.C. Kuo, D.T. Phan, N. Williams, C.W. Gehrke, in: C.W. Gehrke, K.C. Kuo (Eds.), Chromatography and Modification of Nucleosides, Part C, Elsevier, Amsterdam, 1990, p. C41.
[15] J.I. Langridge, T.D. McClure, S. Shakawi, A. Fielding, K.H. Schram, R.P. Newton, Rapid. Commun. Mass. Spectrom. 7 (1993) 427.
[16] K. Nakano, T. Yasaka, K.H. Schram, M.L. Reimer, T.D. McClure, T. Nakao, H. Yamamoto, J. Chromatogr. 515 (1990) 537.
[17] T. Rasmuson, G.R. Bjork, Acta Oncol. 34 (1995) 61.
[18] T. Rasmuson, G.R. Bjork, L. Damber, S.E. Holm, L. Jacobsson, A. Jeppsson, B. Littbrand, T. Stigbrand, G. Westman, Recent Results Cancer Res. 84 (1983).
[19] B. Krattiger, A.E. Bruno, H.M. Widmer, R. Dandliker, Anal. Chem. 67 (1995) 124.
[20] T.P. Waalkes, M.D. Abeloff, D.S. Ettinger, K.B. Woo, C.W. Gehrke, K.C. Kuo, E. Borek, Eur. J. Cancer Clin. Oncol. 18 (1982) 1267.
[21] M. Masuda, T. Nishihira, K. Itoh, M. Mizugaki, N. Ishida, S. Mori, Cancer 72 (1993) 3571.
[22] T. Samuelsson, Nucleic. Acids. Res. 19 (1991) 6139.
[23] T. Rasmuson, G.R. Bjork, L. Damber, L. Jacobsson, A. Jeppsson, T. Stigbrand, G. Westman, Acta Oncol. 26 (1987) 261.

[24] C.C. Marvel, J. Del Rowe, E.G. Bremer, J.R. Moskal, Mol. Chem. Neuropathol. 21 (1994) 353.
[25] T. Rasmuson, G.R. Bjork, L. Damber, S.E. Holm, L. Jacobsson, A. Jeppsson, T. Stigbrand, G. Westman, Acta Radiol. Oncol. 22 (1983) 209.
[26] T. Rasmuson, G.R. Bjork, L. Damber, S.E. Holm, L. Jacobsson, A. Jeppsson, T. Stigbrand, G. Westman, Acta Radiol. Oncol. 23 (1984) 27.
[27] K. Koshida, J. Harmenberg, U. Stendahl, B. Wahren, E. Borgstrom, L. Helstrom, L. Andersson, Urol. Res. 13 (1985) 213.
[28] E. Borek, T.P. Waalkes, C.W. Gehrke, Cancer Detect. Prev. 6 (1983) 67.
[29] K. Nakano, T. Nakao, K.H. Schram, W.M. Hammargren, T.D. McClure, M. Katz, E. Petersen, Clin. Chim. Acta 218 (1993) 169.
[30] H. Topp, R. Duden, G. Schoch, Clin. Chim. Acta 218 (1993) 73.
[31] G. Schoch, H. Topp, A. Held, G. Heller Schoch, A. Ballauff, F. Manz, G. Sander, Eur. J. Clin. Nutr. 44 (1990) 647.
[32] G. Sander, H. Topp, G. Heller Schoch, J. Wieland, G. Schoch, Clin. Sci. 71 (1986) 367.
[33] H.M. Liebich, C. Di Stefano, A. Wixforth, H.R. Schmid, J. Chromatogr. A 763 (1997) 193.
[34] C.W. Gehrke, K.C. Kuo, J. Chromatogr. 471 (1989) 3.
[35] C. Reynaud, C. Bruno, P. Boullanger, J. Grange, S. Barbesti, A. Niveleau, Cancer Lett. 61(3) (1992) 255.
[36] H.M. Liebich, G. Xu, C. Di Stefano, R. Lehmann, H.U. Häring, P. Lu, Y. Zhang, Chromatographia 45 (1997) 396.
[37] H. Bartels, M. Boehmer, C. Heierli, Clin. Chim. Acta 37 (1972) 193.