ELSEVIER

Journal of Chromatography B, 732 (1999) 375–381

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Importance of high-performance liquid chromatographic analysis of serum *N*-acylneuraminic acids in evaluating surgical treatment in patients with early endometrial cancer

S. Diamantopoulou[a], K.D. Stagiannis[b], K. Vasilopoulos[c], P. Barlas[c], T. Tsegenidis[a], N.K. Karamanos[a,*]

[a]*Section of Organic Chemistry–Biochemistry and Natural Products, Department of Chemistry, University of Patras, 261 10 Patras, Greece*

[b]*Department of Obstetrics and Gynecology, School of Medicine, University of Thessaly, Larissa, Greece*

[c]*State General Hospital of Patras, Department of Obstetrics and Gynecology, Patras, Greece*

Received 10 May 1999; received in revised form 23 June 1999; accepted 23 June 1999

Abstract

The objectives of this study were the quantification of the two major sialic acid (Sia) forms – *N*-acetylneuraminic (Neu5Ac) and *N*-glycolylneuraminic acids (Neu5Gc) – in serum before and after surgical treatment of early endometrial cancer and the relation of their levels with the progress of surgical therapy. The major Sia forms were liberated from sera glycoconjugates by mild acid hydrolysis, separated as per-*O*-benzoylated derivatives by a highly sensitive reversed-phase HPLC method and detected at 231 nm. Total Sia content in sera of healthy women was not related to age and body weight. Neu5Ac was identified as the major Sia in sera from both cancer patients, healthy individuals as well as in tissue specimens ($\geq$94% of total Sia). In patients with endometrial cancer the total Sia level before surgical treatment (709.5$\pm$306.5 mg/l) was significantly higher ($p \leq 0.0001$) than that of the control group (213.5$\pm$88.7 mg/l). The elevation in Sia level was exclusively due to Neu5Ac. Following surgical therapy, serum Neu5Ac levels (699.4$\pm$305.6 mg/l) were significantly decreased (305.9$\pm$114.5 mg/l). In one case, where Neu5Ac level was increased 15 days and eight months after surgery (1.8 and 2.5 times as compared to control, respectively), a metastasis not detected during surgery was recorded. The obtained results suggest that Neu5Ac level in serum may be used as a tumor marker in evaluating the suitability of surgical treatment in early endometrial cancer.

Keywords: *N*-Acetylneuraminic acid; *N*-Glycolylneuraminic acid

1. Introduction

Sialic acids constitute a family of more than 30 derivatives of a monosaccharide called neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosic acid), most of them being *O*-acetylated [1]. *N*-Acetylneuraminic (Neu5Ac) and *N*-glycolylneuraminic acids (Neu5Gc) are the most commonly occurring derivatives. Because of their terminal location in glycoproteins and glycolipids and their negative charge at physiological pH, sialic acids play key biological roles. The most important role is their ability to act as biological masks preventing

*Corresponding author. Tel./fax: +30-61-997-153.
E-mail address: N.K.Karamanos@upatras.gr (N.K. Karamanos)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00304-7

recognition of receptors by ligands and antigenic sites by components of the immune-defense system.

Highly sialylated surfaces protect tumor cells from immune defense and thus facilitate metastatic spread [2,3]. Sialic acid (Sia) levels are elevated in a number of different types of cancer, such as breast, ovary and lung cancer [4–6] and this has been attributed to the increased amount and/or activity of sialyltransferases [7]. The most common type of Sia in serum of human is Neu5Ac. The metastatic cancer in rat was identified to be closely associated with a significantly increased synthesis of this major tissue sialic acid [8]. Sia levels are of limited use for the initial diagnosis of cancer because of a remarkable elevation can also be seen in other diseases, such as myocardial infraction [9], diabetes [10,11], bacterial infection [12], inflammatory and other diseases [13,14]. The most tumor markers are specifically associated with histological types of cancer, while Sia do not [15]. Furthermore, Sia as glycoprotein components are present in larger quantities on the surface of cancer cells than in normal ones and they expressed faster than other tumor markers in case of metastases [16]. It has been proposed, therefore, that serial measurements of sialic acids can be proved valuable in detecting the development of metastases or monitoring the tumor bulk in response to treatment [17].

Endometrial cancer is the most commonly found tumor of the female genital tract, which when is early diagnosed has good prognosis and low death rates [18,19]. It has been recently reported [20] that mean value of serum Sia in women with endometrial cancer increases significantly as compared to control group. The elevation of total Sia was associated to the burden of the tumor and especially with stages I and II+III [20]. However, no information on how serum Sia levels could be related to the progress of the disease after surgery is available.

A few methods, which are based on high-performance liquid chromatography (HPLC), are available for Neu5Ac and Neu5Gc analysis in minute amounts [21,22]. The aim of this study was to estimate whether the content of Neu5Ac and Neu5Gc in serum could be used as a marker controlling the efficiency of the surgical treatment at early stages of the disease. Neu5Ac and Neu5Gc contents were determined following a simple clean-up procedure for the removal of interfering substances present in serum and tissue specimens [23] and a highly sensitive and accurate HPLC method [21].

2. Experimental

2.1. Chemicals and biologic material

Neu5Ac, Neu5Gc and *N*-acetylneuraminyl-α-(2,3)-lactose from human milk were purchased from Sigma (St. Louis, MO, USA). Sep-Pak C_{18} cartridges were obtained from Waters (Milford, MA, USA). All other chemicals used were of analytical grade. Benzoylation mixture consists of 10% (w/v) benzoic anhydride, 5% (w/v) *p*-dimethylaminopyridine in pyridine. The solution was stored at 4°C, and remained stable for more than a week.

Blood sera from 30 healthy women and 16 women with endometrial cancer were used. Members of the healthy group were aged from 40 to 67 years (12 pre- and 18 post-menopausal). A further grouping was attempted according to body weight (50–65 and 65–90 kg). Patients were aged from 42 to 65 years. Two of the patients were at the peri-menopausal stage, the remaining being beyond the menopause. All patients presented with abnormal uterine bleeding and dilatation curettage (D&C) confirmed the diagnosis of endometrial carcinoma. All 16 women included in this study were found to have endometrial adenocarcinoma. Histological grading was evaluated according to classification adopted by the International Federation of Gynecology and Obstetrics (FIGO). In 11 cases moderately differentiated adenomatous carcinoma with partly solid areas was identified (G2). Five cases identified as highly differentiated adenomatous carcinoma (G1). The clinical staging of the disease was stage I according to the staging classification for carcinoma of the uterine corpus adopted by the FIGO (carcinoma confined to the corpus including the isthmus). In all cases included in the study a total abdominal hysterectomy and bilateral salpingo-oophorectomy was performed and a wide vaginal cuff was extracted. No radical hysterectomy was attempted. After surgery all women were treated with medroxy-progesterone acetate for the studied period. The sera from patients were obtained before and 15 days to eight months

after surgical treatment. Tissue specimens obtained from the operations were immediately rinsed with 0.2% NaCl and kept at −40°C until use.

2.2. Sample treatment

2.2.1. Serum

A 100-μl volume of serum was mixed with two volumes of saturated ammonium sulfate, pH 10, and the mixture was kept at 0°C for 5 min. Following centrifugation at 11 000 *g* for 5 min, the precipitate was dissolved in 200 μl of water and the solution was chromatographed on a Sephadex G-25 column (3.0×1.3 cm I.D., PD-10 prepacked column, Pharmacia, Uppsala, Sweden). The column was washed with 0.8 ml of water. Following elution of macromolecules eluted with the void volume of the column with 1.5 ml of water, the elute was collected and kept at −40°C for further analysis.

2.2.2. Tissue

A 50–100-mg dry weight amount of endometrial tissue specimens was homogenized at 0°C for 1 min with 3 ml of 2×distilled water. Following washing of the homogenizing system with 2 ml of water, the obtained suspension was centrifuged at 11 000 *g* for 15 min and the supernatant was kept at −40°C until use.

2.3. Sample hydrolysis and purification

Glycoconjugates were hydrolyzed in 20 m*M* trifluoroacetic acid (TFA) at 80°C for 2 h in capped centrifuge tubes. Standards were prepared by treating known amounts of Neu5Ac and Neu5Gc under the same conditions. Hydrolysates were freeze–dried and the obtained residues were dissolved in 1 ml of water. To remove cationic substances, the solutions were then chromatographed on a Dowex 50X8 column (40×3 mm, I.D., H^+ form). Sialic acids were recovered by eluting the column with 1 ml of water. The elute was partly neutralized to a of pH 3–5 with 0.5 *M* NH_4OH and were then chromatographed on a $-NH_3^+$ column (Dowex 1AG, 40×3 mm, I.D. 200–400 mesh, $HCOO^-$ form). Neutral monosaccharides were removed by washing the column with 1 ml of water and sialic acids were eluted with 2 ml of 2 *M* formic acid. The latter fraction was collected, lyophilized and taken for per-*O*-benzoylation.

2.4. Derivatization procedure

Per-*O*-benzoylated derivatives were prepared by a previously described procedure [21]. Briefly, 100 μl of benzoylation mixture was added to the dry hydrolysates and the mixture was heated at 80°C for 20 min. The reaction was terminated by adding nine volumes of water and shaking vigorously on a vortex mixer. For complete destruction of the remaining benzoic anhydride, the mixture obtained was heated for a further 5 min at 80°C. Excess of reagents and under-benzoylated derivatives were removed by passing the mixture through a Sep-Pak C_{18} cartridge, which had been equilibrated with 5 ml of methanol and 10 ml of water. After sample addition to the cartridge, it was washed with 5 ml of water and the per-*O*-benzoylated derivatives of sialic acids were eluted with 5 ml of acetonitrile. After evaporation of the latter fraction, the dry residue was dissolved in 200 μl of acetonitrile and centrifuged at 10 000 *g* for 1 min in a Beckman Microfuge. Aliquots of 10–20 μl were taken for HPLC analysis.

2.5. HPLC procedure

The HPLC system used was consisted of an LKB pump (Pharmacia) equipped with a Reodyne Model 7125 injector with a 50-μl loop (Cotati, CA, USA) and an LDC 1204 A UV detector with an 8-μl flow cell (LDC, FL, USA). Separation was performed on a Supelcosil LC-18 column (250×4.6 mm I.D.), particle size 5 μm (Supelco, Bellfonte, PA, USA) equipped with a RP-18 pre-column (30×4.6 mm I.D., Brownlee Labs., Santa Clara, CA, USA). The samples were chromatographed with 67% (v/v) aqueous acetonitrile at room temperature. The flow-rate of the eluent was 1.5 ml/min. The eluted peaks were recorded at 231 nm. Quantitation was performed by comparing the peak areas obtained from the samples with those of standards solutions.

2.6. Statistical analysis

Statistically significant differences in serum sialic acid levels between healthy individuals and patients

with early endometrial cancer as well as of those before and after surgical treatment were evaluated using *t*-test and the two-way completely randomized analysis (ANOVA) using the origin software (version 5.0).

3. Results

3.1. Method parameters and applicability in biologic samples

A typical high-performance liquid chromatogram of the standard per-*O*-benzoylated derivatives of Neu5Ac and Neu5Gc is given in Fig. 1A. The two major sialic acid forms were completely separated from each other. Method quality parameters (sensitivity, linearity and precision) have been examined for aqueous solutions of Neu5Ac and Neu5Gc following the entire procedure, i.e., hydrolysis, purification and per-*O*-benzoylation. The relationship between peak area and concentration of aqueous solutions of Neu5Ac and Neu5Gc was linear within the range of 5 μg/ml to 5 mg/ml with correlation coefficients of 0.9995 and 0.9992 ($n=12$), respectively, in excellent agreement with previously published values [24]. The equations of linearity for Neu5Ac and Neu5Gc were $y=-1.13+15.6x$ (SD=2.12) and $y=-0.75+11.6x$ (SD=1.38), where y=peak area and x=concentration of sialic acids in μg/ml. The detection limit estimated at a signal-to-noise ratio equal to three corresponded to 0.8 μg/ml. The precision of the method was determined by 12 repeated analyses of each sialic acid from an aqueous solution containing Neu5Ac and Neu5Gc at concentrations of 10 and 18 μg/ml, respectively. Injections of 10 μl of per-*O*-benzoylated derivatives gave relative standard deviations of 2.4% for Neu5Ac and 2.1% for Neu5Gc.

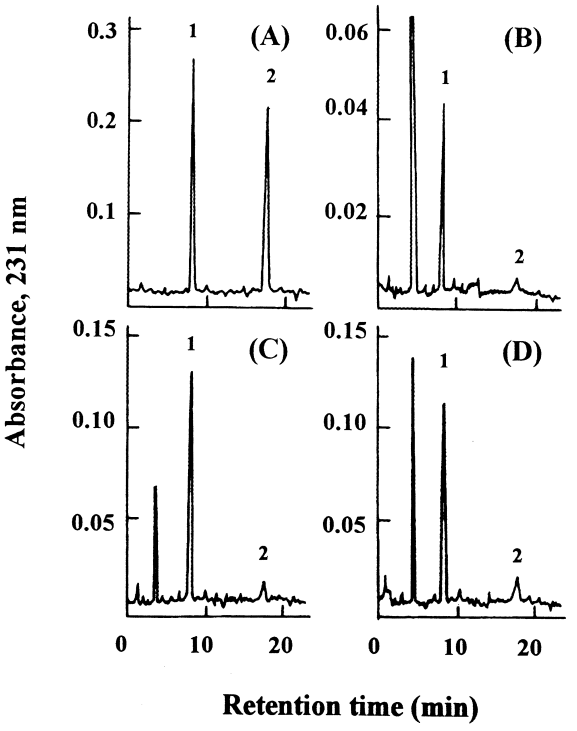

Fig. 1. High-performance liquid chromatographic profiles of Neu5Ac and Neu5Gc in a standard mixture (A), sera from a healthy woman and with endometrial cancer (B and C, respectively) and a tissue specimen (D). Peaks 1 and 2: per-*O*-benzoylated derivatives of Neu5Ac and Neu5Gc, respectively.

The HPLC profiles obtained for the serum of a healthy woman (control), a woman with endometrial cancer and an endometrial specimen are given in Fig. 1B–D, respectively. The retention times obtained for the per-*O*-benzoylated derivatives of Neu5Ac and Neu5Gc in sera and tissue samples analyzed were identical with those of standard compounds (7.5±0.5 min and 17.5±0.5 min, respectively). This suggested that the HPLC separation may be accurately applied for identification of the two major sialic acid forms in tissue and sera samples. To evaluate the applicability of the method to serum and tissue samples, sensitivity, linearity and precision were also tested by spiking sera samples and tissue homogenates with various concentrations of standard solutions of Neu5Ac and Neu5Gc. Standard solutions and spiked samples were hydrolyzed separately with 20 m*M* TFA at 80°C for 2 h. Measuring the peak areas in spiked samples and comparing with those obtained with standards treated under the same hydrolytic conditions it was found that more than 98% of both sialic acids were recovered. Furthermore, both detection limit and linearity of the methods were not affected when spiked samples were analyzed. The obtained results were in accordance with those reported when the HPLC method was applied for the sialic acid analysis in isolated glycoconjugates [21,24]. The precision of the pro-

cedure was also determined by six repeated determinations of both sialic acids from spiked serum and tissue samples. When equal volumes of per-*O*-benzoylated sialic acids were injected (10 μl injections from spiked samples containing Neu5Ac and Neu5Gc at 10 and 18 μg/ml, respectively), the relative standard deviations were 2.6% and 2.3% for Neu5Ac and Neu5Gc, respectively. These results suggested that the clean-up procedure as well as the HPLC analysis may accurately be used for quantitative analysis of Neu5Ac and Neu5Gc in sera samples and tissue specimens.

3.2. Quantitation of sialic acids in serum samples and tissue specimens

Analysis of sialic acids in sera samples and tissue specimens showed that Neu5Ac is the major sialic acid (≥94% of total Sia in control group, patients with endometrial cancer and tissue specimens) (Table 1).

The control group was used to determine possible variation in Sia content according to age and body weight. No statistically significant differences were identified in Neu5Ac and Neu5Gc levels in 12 pre- and 18 post-menopausal women as well as between the two body weight groups of controls (not shown). The average±SD concentration of total serum Sia and Neu5Ac in sera of healthy women were 213.5±88.7 and 196.6±86.8 mg/l, respectively, whereas those found in sera of women with endometrial cancer were 709.5±306.5 and 699.4±305.6 mg/l, showing a statistically significant ($p \leq 0.0001$) increase (3.3 times on the basis of average values) (Table 1). The increase was exclusively due to increased synthesis of the major sialic acid, the Neu5Ac. The average±SD level of Neu5Ac in sera from 15 women out of 16 following randomized analysis for a period from 15 days to eight months after surgical treatment was 305.9±114.5 mg/l, which is not statistically different from control value at the level of $p \leq 0.05$. In all 15 patients the decrease of Neu5Ac concentrations was considered large enough as to be significant.

In one case, one day after surgery the serum Neu5Ac level decreased to a value close to that of the control group. A significant increase ($p \leq 0.0001$) of Neu5Ac was also observed 15 days after the surgery (1.8 times higher as compared to control), whereas eight months later the increase was 2.5 times (Fig. 2). This case was found to have a metastasis in pelvic lymph nodes. Since most of the conditions that could affect the Neu5Ac level in sera

Table 1
Sialic acid content in serum and tissue specimens from healthy individuals and women with endometrial cancer[a]

	Neu5Ac	Neu5Gc	Total Sia
Serum			
Control	196.6±86.8	16.9±6.3	213.5±88.7
Before surgery	699.4±305.6*	13.2±5.8	709.5±306.5*
After surgery	305.9±114.5	10.9±2.2	317.1±114.3
Tissue	639.1±176.4	17.9±8.1	657.1±175.1

[a] Results are expressed as mg/l of serum and μg/g of dry tissue. Values are means±SD. Asterisks show the statistically significant differences ($p<0.0001$) as compared to respective control values.

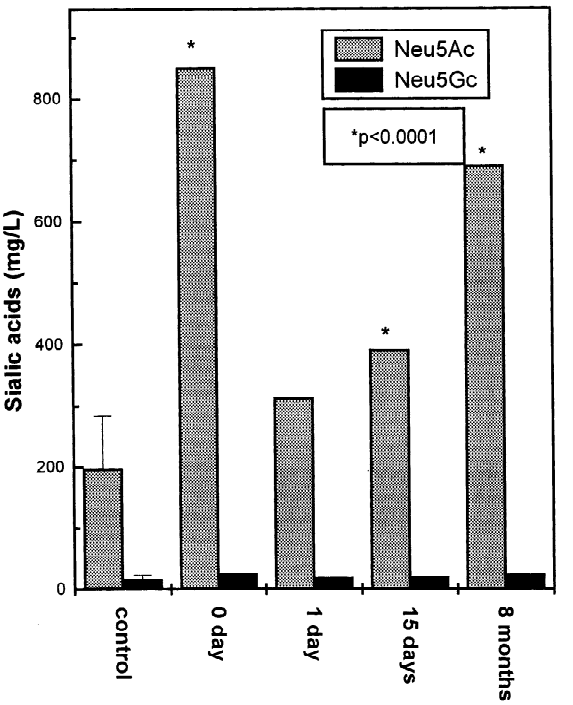

Fig. 2. Concentration of Neu5Ac and Neu5Gc in a women before (0 day) and after (one day, 15 days and eight months) surgical treatment. This case was identified to have a metastasis in pelvic-lymph node not detected, however, during surgery. Asterisks show statistically significant differences as compared to respective control values.

were excluded after careful clinical and laboratory data evaluation, the increase of Neu5Ac level was well related with this metastasis. It is worth noticing that the statistically significant elevation in the concentration of Neu5Ac was noted 15 days after the surgery.

4. Discussion

The majority of sialic acids in sera and tissues are bound to glycoproteins. The sialic acid-containing water soluble macromolecules were almost completely precipitated (recovery 98.5%) with saturated ammonium sulfate, pH 10, at 0°C. Sialic acids were released from their macromolecules by mild hydrolysis, which ensures the lowest possible degradation of liberated sialic acids [21]. With this hydrolysis procedure all *O*-acetyl groups are removed, whereas *N*-acyl linkages are stable. Glycosidic linkages of neutral hexoses are mainly stable under the mild hydrolytic conditions used and minute amounts of them are completely removed with the ion-exchange chromatographic steps.

The finding that there are no statistical differences in Sia content between pre- and post-menopausal healthy women as well as between the two body weight shared groups suggests that the functional condition of the ovary and the amount of body lipid tissues do not affect the levels of both sialic acids. Therefore, all healthy women were used for the estimation of normal range values.

Comparing the mean concentrations of total serum sialic acids in serum from women with endometrial cancer with controls, a significant elevation in total Sia was identified. The average value of Sia level was 3.3-times higher than the control group. This was in excellent agreement with recent results [20], where total serum Sia levels were also compared. In this study it was further elucidated that the elevation in Sia is exclusively due to Neu5Ac. The average values of Neu5Ac after the surgical treatment were significantly decreased. In some cases Neu5Ac level in sera, after the tumor removal, reached normal level. In one case, eight months after surgery, a dramatic increase in the concentration of Neu5Ac was found. This patient was found to have pelvic-lymph node metastasis. The decrease in Neu5Ac level following surgical treatment may well be explained by the removal of tumor that is rich in cell bound sialic acid-containing glycoproteins/glycolipids. In case, however, where a metastasis is in progress the increase in serum sialic acids could be attributed to increased cell proliferation of malignant cells masked with the highly sialylated glycoproteins/glycolipids.

Surgical therapy alone was the original treatment method for endometrial carcinoma [25]. More radical surgical approaches seems to be unnecessary for early stages since only 11% of patients with stage I endometrial carcinoma will be found to have pelvic-lymph node metastasis [26]. Moreover, the morbidity after radical hysterectomy is considerably increased [27–29]. In our study, all included cases have been characterized as early adenocarcinoma of the endometrium (up to stage I, G1&G2 according to FIGO). Clinical data [26,29,30] support the idea of avoiding radical therapeutic approaches for stage I endometrial cancer. Therefore, a tumor marker that could early detect the cases of recurrent or metastatic disease would be of great importance, as it will early detect cases that need further treatment with the above mentioned available therapeutic alternatives.

The results of this study suggest that Neu5Ac level in serum of women with endometrial cancer was elevated before the operation and decreased significantly after the removal of the tumor bulk, reaching the normal values. In case of metastasis, however, the Neu5Ac level is increased significantly. Therefore, although sialic acids could not be used for the initial diagnosis of endometrial cancer as they are also increased in some other diseases, the quantitative measurement of Neu5Ac may greatly help in monitoring the progress of surgical therapy of early endometrial cancer and diagnosis of metastasis.

References

[1] G. Reuter, R. Schauer, Methods Enzymol. 230 (1994) 168.
[2] R. Schauer, Trends Biochem. Sci. 10 (1985) 357.
[3] R. Schauer, Adv. Carbohydr. Chem. 40 (1982) 131.
[4] H.K.B. Silver, K.A. Karim, J. Chromatogr. 224 (1981) 381.
[5] A.J. Moss, N.K. Bissada, C.M. Boyd, W.C. Hunter, Urology 13 (1979) 182.
[6] H.K.B. Silver, K.A. Karim, F.A. Salinas, K.D. Swenerton, Surg. Gynecol. Obstet. 153 (1981) 209.

[7] S. Narayanan, Ann. Clin. Lab. Sci. 24 (1994) 376.
[8] E. Makatsori, A. Aletras, N.K. Karamanos, T. Tsegenidis, Biomed. Chromatogr. 13 (1999) 57.
[9] G. Lindberg, G.A. Eklund, B. Gullberg, L. Rastam, Br. Med. J. 302 (1991) 143.
[10] B. Radhakrishnamurthy, G.S. Berenson, P.S. Pargaonar, Lab. Invest. 34 (1976) 159.
[11] Y. Tomino, W. Inoue, M. Yagame, Y. Namoto, H. Sakai, K. Ito, K. Nagaoka, N. Ikeda, J. Diab. Complications 2 (1988) 175.
[12] N. Stefenelli, H. Klotz, A. Engel, P. Bauer, J. Cancer Res. Clin. Oncol. 109 (1985) 55.
[13] T. Ozben, Ann. Clin. Biochem. 28 (1991) 44.
[14] R. Shamberger, J. Clin. Chem. Clin. Biochem. 22 (1984) 647.
[15] K.M. Erbil, J.D. Jones, G.G. Klee, Cancer 55 (1985) 404.
[16] Y. Shimizu, K. Hasumi, K. Masubuchi, Y. Okudaira, Gynecol. Oncol. 33 (1989) 231.
[17] S.L. Tewarson, V.P. Mittal, M. Singh, G.P. Gupta, Indian J. Cancer 30 (1993) 125.
[18] K.M.C. Greven, B.W. Corn, Curr. Prob. Cancer 21 (1997) 65.
[19] A.G. Shumsky, P.M.A. Brasher, G.C.E. Stuart, J.G. Nation, Gynecol. Oncol. 65 (1997) 379.
[20] A. Paszkowska, H. Berbec, A. Semczuk, M. Cybulski, Eur. J. Obstet. Gynecol. 76 (1998) 211.
[21] N.K. Karamanos, B. Wikstrom, C.A. Antonopoulos, A. Hjerpe, J. Chromatogr. 503 (1990) 421.
[22] A.E. Manzi, S. Diaz, A. Varki, Anal. Biochem. 188 (1990) 20.
[23] T. Tsegenidis, N.K. Karamanos, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 793.
[24] E. Makatsori, K. Fermani, A. Aletras, N.K. Karamanos, T. Tsegenidis, J. Chromatogr. B 712 (1998) 23.
[25] J.A. Stallworthy, Ann. Royal College Surg. Engl. 48 (1971) 293.
[26] B.V. Lewis, J. A Stallworthy, R. Cowdell, J. Obstet. Gynaecol. Br. Commun. 77 (1970) 343.
[27] R. Park, W. Patow, W. Petty, E. Zimmermann, Gynecol. Oncol. 2 (1974) 60.
[28] D. Keller, R.L. Kempson, G. Levine, C. McLennan, Cancer 33 (1974) 1108–1116.
[29] G.C. Lewis Jr., R. Mortel, N.H. Slack, Cancer 39 (1997) 959.
[30] G.D. Malkasian Jr., D.G. Decker, E. Mussey, C.E. Johnson, Am. J. Obstet. Gynecol. 110 (1971) 15.