Detection of Estrogen DNA-Adducts in Human Breast Tumor Tissue and Healthy Tissue by Combined Nano LC-Nano ES Tandem Mass Spectrometry

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For the first time estrogen DNA-adducts were identified in DNA human breast tumor tissue using nano-LC coupled to nano-Electrospray Tandem Mass Spectrometry. Normal breast tissue was analyzed analogously. The data obtained in the five breast tumor and five adjacent normal tissue samples were compared qualitatively, but no straightforward difference was observed. Prior to LC-MS analysis the DNA was enzymatically hydrolyzed to a nucleoside pool. The DNA-hydrolysates were directly injected onto a column switching system developed for on-line sample clean-up and subsequent analysis of the DNA-adducts. In four patients using Premarin, DNA-adducts of 4-hydroxy-equilenin (4OHEN) were detected. All except three samples contained DNA-adducts from 4-hydroxy-estradiol or 4-hydroxy-estrone. Also DNA isolated from eight alcohol fixed and paraffin embedded breast tumor tissue showed the presence of different estrogen DNA-adducts. Worthwhile mentioning is the presence of adducts responding to m/z 570 $>$ m/z 454 transition. This is a well-known SRM-transition indicative for the presence of the 2'-deoxyguanosine (dGuo) adduct of Benzo[a]pyrene. (J Am Soc Mass Spectrom 2003, 14, 482–491) © 2003 American Society for Mass Spectrometry

Hormone treatment is widespread for women of all ages. In the United States, for instance, 30% of post-menopausal women use hormone replacement therapy (HRT) [1], e.g., Premarin. Studies in which the development of breast and endometrial cancer was associated with estrogen therapy [2–6] were supported by a more recent follow-up study in which it was demonstrated that post-menopausal women have an increased risk of breast cancer when using estrogens, especially in combination with progestin [7]. In animals, too, a relationship between the administration of estrogens and the development of cancer was shown [8].

The carcinogenic properties of estrogens are explained by direct stimulation of cell proliferation via estrogen receptor mediated mechanisms [9, 10] and by mechanisms based on metabolic activation [11–14], leading to DNA damage such as oxidative damage [15–18] and the formation of DNA-adducts [19–28], which can cause mutations and induce cancer [29].

The latter pathways are the result of metabolic activation of estrogens by the cytochrome P-450 system leading to 2- and 4-hydroxy derivatives. The 2-hydroxy estrogens are excreted in the urine as a result of their fast transformation to water-soluble compounds [13, 14]. The 4-hydroxy form, however, has a longer half-life

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in the body and is further oxidized to the o-quinone-estrogen which can alkylate DNA [19–28]. In this respect, equilenin is noteworthy because of its fast transformation to the 4-hydroxy and quinone metabolites (Scheme **1**) [17, 18, 30].

The aim of our research is to elaborate LC-MS technology to such an extent that it can be used in the analysis of DNA-adducts present in humans, where a limited amount of sample is one of the limiting factors and sensitivity is one of the main issues. In this communication we wish to present our first, though preliminary results on the analysis of estrogen DNA-adducts isolated from small amounts of surgically removed breast tumor tissue by means of nano-LC/nano-electrospray tandem mass spectrometry in combination with column switching 2-dimensional (2-D)LC/nano ES MS/MS).

Following the report of D. Li et al. [31, 32] of the detection of aromatic DNA-adducts of Benzo[a]pyrene in adjacent breast tumor tissue, we also decided to search for Benzo[a]pyrene adducts of 2'-deoxyguanosine (dGuo) in both malignant and non-malignant tissue. Generally, DNA-adducts were detected in small amounts of DNA (38 μg or less).

Experimental

Products and Materials

The necessary enzymes Proteinase K, Nuclease P_1, Phosphodiesterase I from Croltalus adamanteus venom, alkaline phosphatase type III from Esherichia Coli, and water saturated buffered phenol were purchased from Sigma (Bornem, Belgium). Absolute ethanol was purchased from Riedel-de-Haën (Bornem, Belgium) and Tris-buffer from Fluka (Bornem, Belgium). All other chemicals used for DNA isolation or hydrolysis and HPLC analysis where purchased from Acros Organics (Eupen, Belgium).

Flow rates for nano-columns and pre-columns were delivered by a splitless CapLC system (Waters, Brussels, Belgium). This ternary system was used in a binary mode (A and B reservoirs were used to deliver the nano flow rates, C reservoir was used to load the pre-column). The CapLC system was connected to a FAMOS micro-injection system.

Nano-LC columns were purchased from LC-Packings (Amsterdam, The Netherlands). Two different stationary phases were used: Hypersyl C_{18} BDS, 3 μm particles (75 μm i.d. $*$ 150 mm) and Hypersyl C_8 BDS, 5μm particles (75 μm i.d. $*$ 150 mm). Another analytical column used in many experiments was a picofrit column (75μm i.d. $*$ 100 mm; New Objective, Cambridge, MA). The pre-column was a 300 μm i.d. $*$ 5mm column packed with C_{18}P3 as stationary phase (LC-Packings, Amsterdam, The Netherlands).

As detector, a Quattro II tandem mass spectrometer (Micromass, Manchester, UK) was used. The instrument was equipped with a Z-spray source and an electrospray NanoFlow probe. If the Picofrit column was used a Picosprayer (Micromass, Manchester, UK) was installed.

Preparation of Standards

Equilenin metabolite and its adducts were prepared according to the method Shen et al. [19], also described by us in an earlier paper [33]. Aliquots of 1 mL of sample were dried under reduced pressure and dissolved in 1 mL of methanol. An equal volume (10 μL) of each of the reaction mixtures was mixed together with 1 mL of water to get a solution of dGuo, 2'-deoxyadenosine (dAdo), and 2'-deoxycytidine (dCyd)-4OHEN adducts. This mixture was diluted 1000 times with water and after filtration, used for injection on the column switching system.

Two mg of Calf Thymus DNA dissolved in 1.5 mL of phosphate buffer (25 mM, pH 7,4), was treated with 1.5 mg of 4OHEN (in 500 μL methanol). After 4 h of reacting at 37 °C the DNA was precipitated with 4 mL of ice-cold 70% ethanol. After centrifugation, the supernatants could be removed and the DNA was ready for enzymatic hydrolysis.

Standards of 4-hydroxy-17-α-ethynyl-estradiol with dGuo, dAdo, and dCyd as well as 4-hydroxy-estrone with dGuo and dAdo were made by the procedure described by Cavalieri et al. [26]. Briefly, the 3,4-estrogen quinones were made from the 4-hydroxy analogs using activated manganese dioxide. The quinone was then reacted with the 2'-deoxynucleosides at 37 °C for 5 h in an acetic acid/water (50/50) environment. These samples were dried under reduced pressure and redissolved in methanol and diluted 1000 times with water before injection.

DNA Isolation from Tissue

Breast tumor tissue and normal tissue located adjacent to the tumor tissue were obtained for DNA adduct measurements from biopsies of five patients. The origin of the samples (patients) is summarized in Table 2. In Patient 1 the tumor was a moderately defined ductal adenocarcinoma, in Patient 2 the tumor was a well defined ductal adenocarcinoma, Patient 3 had an intracystic papillary carcinoma, Patient 4 had a poorly defined ductal adenocarcinoma, and from Patient 5 the type of tumor was unknown. From each sample at least 0.20 cm^3 was obtained. Larger samples were cut into pieces of 0.20 cm^3 prior to enzymatic digestion. With regard to sample preparation both malignant and healthy tissues were treated analogously. To each sample 1 mL of digestion buffer, 50 μL of DNA-ase and RNA-ase free sodium dodecyl sulfate (10% solution) and 0.15 mg proteinase K (dissolved in water) was added. This mixture was put in an oven at 50 °C overnight and was meanwhile gently shaken. The digestion buffer consisted of 100 mM sodium chloride, 25 mM EDTA, and 10 mM Tris/HCl buffered at pH 8. The

Table 2. Type of tissue with corresponding adducts

Patient and kind of tissue[a]	Injection volume (μL)[b]	Estrone–dCyd–adduct	Estradiol–dCyd–adduct	Equilenin–dCyd–adduct	Estrone–Thy–adduct	Estradiol–Thy–adduct	Estrone–dAdo–adduct	Estradiol–dAdo–adduct	Ethynyl-estradiol –dCyd–adduct	Equilenin–dAdo–adduct	Estrone–dGuo–adduct	Ethynyl-estradiol–Thy–adduct	Estradiol–dGuo–adduct	Ethynyl-estradiol–dAdo–adduct	Equilenin–dGuo–adduct	Benzo-[a]-pyrene–dGuo–adduct	Ethynyl-estradiol–dGuo–adduct	Known background of patient
1. tumor	100	·	·	·	·	+	·	·	·	·	+	·	+	·	·	+	·	unknown
1. normal	100	·	·	·	·	·	·	·	·	+	+	·	+	·	·	+	·	unknown
2. tumor	100	·	·	·	·	·	·	·	·	·	·	·	+	+	·	+	·	estradiol derivatives
2. normal	100	·	·	·	·	+	·	·	·	·	+	·	+	·	·	·	·	estradiol derivatives
3. tumor	100	·	+	+	·	·	·	·	·	·	·	·	+	+	·	+	+	unknown
3. normal	100	·	·	·	·	·	·	·	·	·	·	·	+	·	·	·	·	unknown
4. tumor	20	·	·	·	·	·	·	·	·	+	+	·	+	·	·	O[c]	·	Premarin
4. normal	20	·	·	·	·	·	·	·	·	·	·	·	·	·	·	O	·	Premarin
5. tumor	20	·	·	·	·	·	·	·	·	·	·	·	·	·	·	O	·	Premarin
5. normal	20	·	·	·	·	·	·	·	·	·	·	·	·	·	·	O	·	Premarin
6. tumor	100	·	·	+	·	·	·	·	·	·	·	·	+	+	·	·	·	No HST[d]
7. tumor	100	·	·	·	·	+	·	·	·	·	+	+	+	+	·	·	·	Premarin
8. tumor	100	·	·	·	·	+	·	·	·	·	+	+	+	+	·	+	·	Premarin
9. tumor	100	·	·	·	·	+	·	·	·	·	+	·	+	+	·	·	·	Probably no HST
10. tumor	100	·	·	·	·	+	·	·	·	·	+	·	+	+	·	·	·	no HST
11. tumor	100	·	·	·	·	+	+	·	·	·	+	·	+	+	·	·	+	no HST
12. tumor	100	·	·	·	·	·	·	·	·	·	+	+	·	·	·	·	+	no HST
13. tumor	100	·	·	·	·	·	+	·	·	·	+	+	·	·	+	·	·	Premarin

[a]Numbering refers to the origin of the sample, i.e., patient.
[b]100 μL injection volume is 38 μg DNA, 20 μL is 7.6 μg.
[c]O = not tested on the compound.
[d]HST = Hormone substitute therapy.

overnight digested sample was extracted with the same volume of extraction mixture [50% of water saturated phenol (pH 7.9), 25% of chloroform and 25% 3-methyl-1-butanol] and centrifuged for 10 min at 12,000 rpm. The upper aqueous layer containing the DNA and was collected. A 4 M sodium chloride solution (1/10 of the digestion buffer volume) was added to the DNA solution and the DNA was precipitated with ice-cold ethanol (at least 2 volumes). DNA samples were centrifuged for 30 min at 12,000 rpm and the liquid was removed from the precipitated DNA pellet. DNA pellets were washed with ice-cold 70% ethanol and centrifuged again. The wash liquid was removed with a small pipette and the pellet was further dried under a nitrogen-flow.

Eight breast tumor biopsies (Patients 6–13 in Table 2) were processed from alcohol fixed paraffin embedded material. The tumors were all invasive ductal carcinomas. 30–60 sections of 10 μm thickness were used of biopsies from 1 to 2 cm^2 diameter (±0.5 cm^3 of tumor tissue). No DNA could be isolated from formaldehyde-fixed tissue. Deparafination was done by shaking the 30–60 sections in 1 mL of toluene (37 °C, 15 min), followed by centrifugation and removal of toluene. This action was repeated. Then, the pellet was shaken twice in 1 mL of isopropanol, centrifuged and the isopropanol was removed. The pellet was further dried under nitrogen flow. Further digestion and isolation of DNA was carried out in the same way the fresh tissue was treated.

DNA quantification was by UV spectroscopy at 260 nm. If the A_{260}/A_{280} ratio was between 1.6 and 1.9, the DNA was considered to be pure.

The DNA extracted from 0.5 cm^3 of breast tumor tissue was in most cases between 350 and 900 μg, for normal tissue adjacent to the tumor tissue the yield was lower (60–100 μg).

Enzymatic DNA Hydrolysis

DNA hydrolysis was done according to the method of P. Crain [34]. 250 μg of DNA was dissolved in 500 μL of 1 mM Tris/HCl (pH = 7.4). The DNA was denatured by heating for 3 min at 100 °C and rapidly chilled in ice water slush. Then 1/10 volume of 0.1 M ammonium acetate (pH = 5.3) and 20 units nuclease P_1 (dissolved in 0.05 M ammonium acetate pH 5.3) was added and the mixture was kept at 45 °C. After 2 h, 1/10 volume of fresh ammonium bicarbonate (1 M, pH = 7.8) was added and 0.02 units of venom phosphodiesterase. This

mixture was put in an oven for 2 h at 37 °C and meanwhile gently shaken. Then 5 units of alkaline phosphatase were added and the mixture was kept for 1 h at 37 °C. The procedure resulted in a complete digestion of the DNA to 2'-deoxynucleosides. After ultrafiltration this solution was used immediately for analysis by LC/MS.

In order to check the validity of this enzymatic procedure, calf-thymus DNA was incubated with 4OHEN for 4 h and was enzymatically hydrolyzed according to the same procedure as described above. The completion of the reaction was checked by HPLC-UV and subsequent LC-MS analysis of this calf-thymus DNA hydrolysate showed the presence of the 12 4OHEN-2'-deoxynucleoside adducts (4 isomeric adducts each for dAdo, dCyd, and dGuo).

HPLC Conditions

Aliquots of 20 to 100 μL were injected on a pre-column (300 μm $*$ 5mm) in 10 mM ammonium acetate as mobile phase to flush the unmodified nucleosides to the waste. The flow rate over the pre-column was 7.5–10 μL/min. After 5 to 12 min, depending on the injection volume (5 min for 20 μL, 7.5 μL/min and 12 min for 100 μL, 10 μL/min), the pre-column was back-flushed to elute the DNA-adducts to the analytical nano-HPLC column. The flow rate over the analytical column was 300 nl/min (split-less).

The initial conditions were 10 mM ammonium acetate/methanol (13/87) for 22 min. Then a linear gradient was started going to 60% methanol in 15 min. These conditions were kept for another 3 min. Then, the column was flushed with 90% methanol.

When the picofrit column was used the aforementioned method was modified. The C_{18} pre-column was now loaded with 0.2% acetic acid containing 2% methanol. DNA-adducts were directed to the analytical picofrit column by a forward flush. The separation on the picofrit column was done using 0.2% of acetic acid and methanol (80/20) for 20 min. Then a linear gradient was started going to 60% of methanol in 15 min. This composition was kept constant for another 3 min. After this the column was flushed with 90% methanol to remove all impurities. Importantly, the column was equilibrated for 1 h before each injection and that between each sample one or more blank samples of water were injected.

Mass Spectrometric Conditions

The nano-HPLC column was directly coupled to the nano-electrospray probe (Micromass, Manchester, UK). All analyses were done under (+)ES conditions in SRM mode following the $[MH]^+ > [BH_2]^+$ transition. The different SRM transitions are summarized in Table 1. Dwell time, cone voltage (CV), and collision energy (CE), were the same for each SRM function and were set at 0.1 s, 40 V, and 15 eV, respectively. The ionization voltage was 3.50–3.65 kV, the collision gas was argon at $3.6 * 10^{-3}$ mbar, and the source temperature was kept at 45 °C. The flow rate of the drying gas was 30 L/hour and the cone gas flow rate was set at 10 L/hour.

Table 1. SRM-transitions

$[MH]^+ > m/z$	Component
512 > 396	Estrone–dCyd–adduct
514 > 398	Estradiol–dCyd–adduct
524 > 408	Equilenin–dCyd–adduct
527 > 411	Estrone–Thy–adduct
529 > 413	Estradiol–Thy–adduct
536 > 420	Estrone–dAdo–adduct
538 > 422	Estradiol–dAdo–adduct Ethynyl-estradiol–dCyd–adduct
548 > 432	Equilenin–dAdo–adduct
552 > 436	Estrone–dGuo–adduct
553 > 437	Ethynyl-estradiol–Thy–adduct
554 > 438	Estradiol–dGuo–adduct
562 > 446	Ethynyl-estradiol–dAdo–adduct
564 > 448	Equilenin–dGuo–adduct
570 > 454	Benzo-[a]-pyrene–dGuo–adduct
578 > 462	Ethynyl-estradiol–dGuo–adduct

Results and Discussion

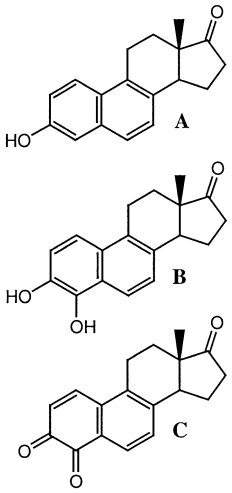
The collection of quantitative and qualitative information on DNA-adducts present in DNA from "in vivo" sources is important in order to get a better insight on tumor formation. Most importantly, data on adducts found in human DNA are extremely valuable because of their impact on human health. LC-MS has often been mentioned as a powerful method in this area because compared to ^{32}P-post labeling, it should be capable of identifying DNA-adducts regardless of the presence of reference material. Up to the present time, many of the literature data describe the results of in vitro experiments in which the issue of sensitivity is of minor importance. However, more data from in vivo experiments is becoming available on the detection of adducts found in animals treated with xenobiotics under controlled circumstances [22–25, 29, 35]. In some of these studies LC MS was used [36–41] as the method of choice.

LC-MS Analysis of Standard DNA Adducts

The coupling of a miniaturized 2-DLC system to tandem mass spectrometry is a powerful approach enabling the analysis of low quantities of DNA-adducts present in a matrix of mainly unmodified 2'-deoxynucleosides [33, 42–49].

The performance of the nano-LC-ES MS/MS system was studied with in vitro prepared DNA-adducts of 4OHEN with dGuo, dAdo, and dCyd (Scheme **2c**). As shown, all these 2'-deoxynucleosides gave four isomeric DNA-adducts [17, 18, 33] numbered 1 to 4 according to their elution order.

In vitro prepared reaction mixtures with 17-α-ethy-



Scheme 1. Structure of equilenin, 4-hydroxy-equilenin, and 3,4-equilenin-o-quinone.

nylestradiol-3,4-quinone gave different adducts (Scheme **2a**): Two dGuo adducts, two dAdo adduct isomers, and three 2'-deoxycytidine adducts. Also for 4-hydroxy-estrone adducts could be found in "in vitro" prepared mixtures: Two dGuo adducts and only one major dAdo adduct (Scheme **2b**). Analogously to the 4OHEN adducts also here the different isomers were numbered according to the elution order.

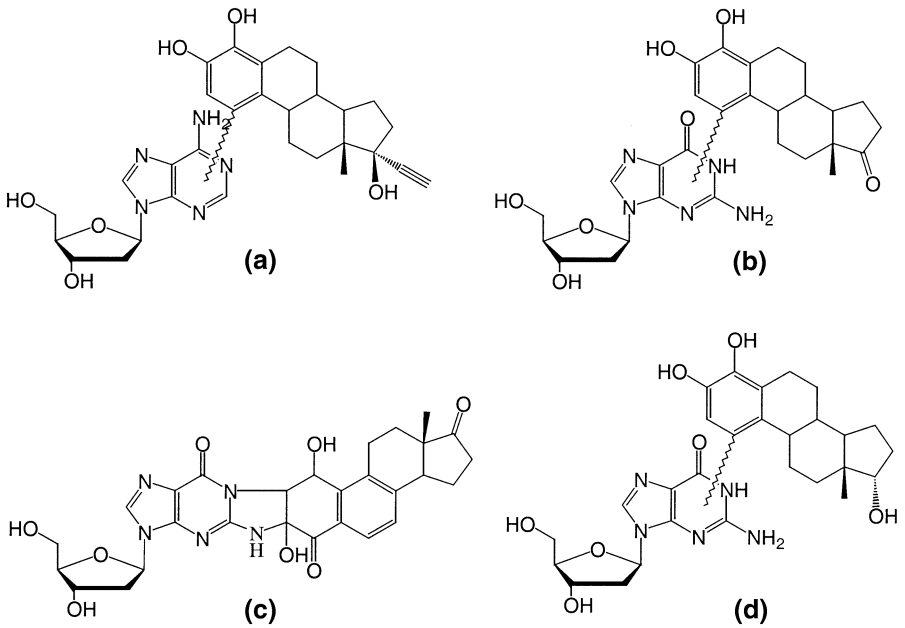
All DNA-hydrolysates were injected on a miniaturized 2-DLC MS/MS system in order to discard the major fraction, i.e., unmodified 2'-deoxynucleosides, prior to analysis of the adducts.

The DNA-adducts were pre-concentrated on the pre-column and were back-flushed after 5 to 12 min to the nano-LC-column for separation. Because of the more lipophylic character of the adducts it was possible to use high volume injections on the pre-column. It was shown that the injection of 10 μL of a $1.75 * 10^{-10}$ M solution of dG_4 [33] gave the same results as the injection of 100 μL of a $1.75 * 10^{-11}$ solution, i.e., all adducts were retained on the pre-column without any loss to the waste. Four aliquots of 100 μL of a $1.75 * 10^{-11}$ solution were injected. The relative standard deviation of the retention time was 2% and the standard deviation of the peak area was 24%.

All the isomers formed between the different 2'-deoxynucleoside and each of the estrogens were isobaric. However, analysis of the low energy collision activated dissociation (CAD)-spectra of the $[MH]^+$ did not allow us to elucidate the exact structures, i.e., the exact alkylation site at the heterocyclic moiety. Some of these product ion spectra were even identical. These are probably positional isomers formed by reaction at a different site of the heterocyclic moiety. In contrast to the 4OHEN-adducts, in this case no diasteromers can be formed.

Chromatographic Behavior

Depending on the different gradient conditions and the different solvent systems, the retention times changed slightly for all reference compounds. Reference compounds were available for most of the adducts except for the adduct of 4-hydroxy-estradiol. The adduct with the smallest k'-value was dG_1 (t_r = 24.5 min for the system with acetic acid and a picofrit column). The last eluting adduct (highest k'-value) was the dGuo-4-hydroxy-17-α-ethynylestradiol adduct (t_r = 41.6 min for the system with acetic acid and a picofrit column). All



Scheme 2. (**a**) 17α-ethynylestradiol-2'-deoxyadenosine adduct. (**b**) Estrone-2'-deoxyguanosine adduct. (**c**) Equilenin-2'-deoxyguanosine adduct. (**d**) Estradiol-2'-deoxyguanosine adduct.

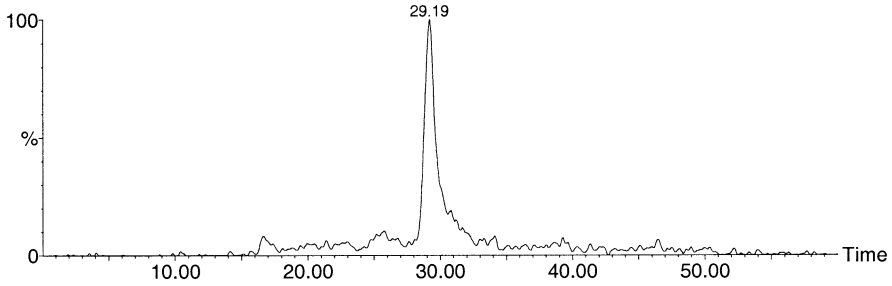


Figure 1. Chromatogram of SRM channel 554 > 438 (estradiol-2'-deoxy guanosine adduct) of a breast tumor DNA (patient 3) analyzed with nano LC-nano (+)ES MS (picofrit column 75 μm * 10 cm, Aquasyl C18) with column switching (300 μm * 5mm, C18, 10 μm) after 12 min, 100 μL injection volume (ca. 38 μg DNA).

the in vitro synthesized adducts of 4OHEN (4 dAdo isomers, 4 dCyd-isomers, and 4 dGuo-isomers), 4-hydroxy-estrone (1 dAdo isomers and 2 dGuo-isomers) and 4-hydroxy-17-α-ethynylestradiol (2 dAdo isomers, 3 dCyd-isomers, and 2 dGuo-isomers), 22 in total, elute within this time frame. Due to the highly apolar character of estrone, estradiol, ethynylestradiol, and equilenin, all adducts of these estrogen are likely to behave similar on a reversed phase LC system. Therefore they are expected to all elute in the same time frame, fixed by the two mentioned reference adducts (dG_1: 24.5 min and dGuo-4-hydroxy-17-α-ethynylestradiol adduct: 41.6 min). Benz[a]pyrene adducts are probably even more apolar than the estrogen adducts and therefore are expected to have a higher retention time compared to the estrogen-adducts.

Although different gradients were used, the retention of the compounds varied only slightly (e.g., dG_1: from 24.5 to 19.0 min and dG_4: from 47.4 40.9 min).

Mass Spectrometric Detection

Because it was expected that DNA-adducts in both malignant and non-malignant tissue would be present in very low quantities, the DNA hydrolysates were analyzed under (+)ES using the specific $[MH]^+ > [BH_2]^+$ SRM transition (see Table 1). These transitions were selected from the low energy CAD product ion scans of the different standards. The typical SRM transition for the benzo[a]pyrene adduct was found in the literature [50]. The $[MH]^+ > [BH_2]^+$ transition is a well known fragmentation pattern for (2'-deoxy)nucleosides because of the preferred cleavage of the anomeric bond [26, 27, 33, 43, 44, 51, 52]. For a CV of 40 V and a CE of 15 eV the loss of the 2'-deoxyribofuranosyl group from the protonated molecule $[MH]^+$ was the main fragmentation pattern for all our reference compounds. The above mentioned energy settings were optimized for the 4OHEN adducts and kept constant during the LC-MS run. If the S/N ratio was higher than two in a certain SRM channel, the adduct with the corresponding SRM transition was stated as present. (As described by us in an earlier paper [33], the sensitivity of the method was investigated: 197 fg of a dG_4 adduct could still be detected under SRM conditions with S/N of 8.4.)

Analysis of DNA-Hydrolysates from DNA Isolated from Breast Tissue

A total of 18 samples was analyzed: 5 malignant breast tissue samples, 5 samples of adjacent tumor tissue, and 8 alcohol fixed and paraffin embedded malignant breast tumor tissue samples. Fifteen out of these eighteen DNA samples showed a SRM respons for estrogen adducts (Table 2) and almost every DNA sample showed the presence of a dGuo adduct of 4-hydroxy-estradiol (Figure 1>, Scheme **2d**) and 4-hydroxy-estrone (4-hydroxy-estrone adduct 1) respectively at levels practically up to five times the detection limit [33]. Both adducts have very comparable retention times in the middle of the earlier mentioned retention time frame. Furthermore, the identified dGuo-4-hydroxy-estrone adduct has the same retention time as the corresponding standard. Since estradiol and estrone are both endogenous estrogens the presence of the corresponding adducts was not surprising.

Also, endogenous estrogens adducts with thymidine (sample 1, 2, 7–11) and dAdo (samples 11 and 13, Figure 2) were found. Noteworthy was that adducts were found in the DNA of both normal and malignant tissue but no straightforward differences could be noticed. An interesting result of the nano-LC MS/MS analysis was the response for SRM transition typical for the adducts of exogenous administrated estrogens like equilenin and 17-α-ethynylestradiol. 4OHEN adducts with dAdo (patients 1, 4, Figure 3), dGuo (patient 13, Figure 4) and dCyd (patients 3, 6) were detected at retention times at which the in vitro made 4OHEN standards were eluting. More specific dA_3, dG_4 and dC_3 or dC_4 were detected. Since in earlier nano-LC MS experiments using gradient elution conditions (see also above) sometimes a shift in retention time of the reference compounds was noted (sometimes up to 1 min) no real distinction between diastereomers dC_3 and dC_4 could be made because the t_R of both compounds differed by only 1.4 min. In Figures 3 and 4, the upper traces show the SRM transition 548 > 432 and 564 > 448 of dA and dG standards. In the lower trace the response of the breast tumor tissue of patient 4 (Figure 3) and patient 13 (Figure 4) is given. In the tumor sample signals could be seen at a retention time of 42, 2, and 37.3 min, respectively. This

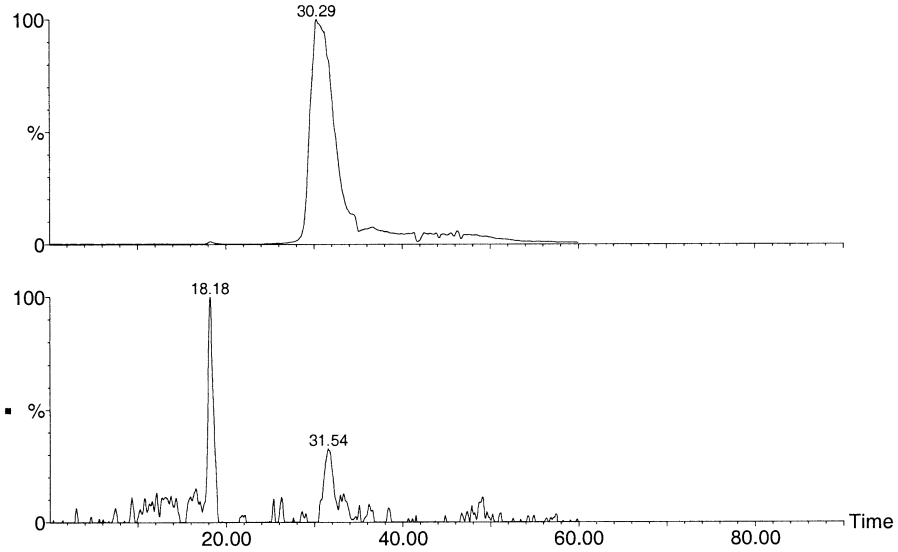


Figure 2. Chromatogram of SRM channel 538 > 432 (equilenin-2'-deoxy adenosine adduct). Upper: In vitro prepared reference adducts. Lower: Breast tumor DNA of patient 9 treated with equilenin. Both were analyzed with nano LC-nano (+)ES MS (picofrit column 75 μm * 10 cm, aquasyl C18) with column switching (300 μm * 5 mm, C18, 10 μm) after 12 min, 100 μL injection volume (= 38 μg DNA). The peak at 18.18 min is not an adduct; it is a component that is even present in a blank injection.

corresponded exactly with the same retention time as the dA_3 and dG_4 reference adduct. Remarkebly, 4OHEN adducts were found in either tumorous or healthy tissue, but never in the both tissues at the same time.

Patients 5, 7, and 8 received Premarin (equilenin) but no adducts were detected. However, in these cases the amount of tissue was extremely small (smallest sample was 0.2 cm^3) which could explain that even under SRM conditions no conclusions could be drawn. As far as we know patient 6 did not receive Premarin, nevertheless an equilenin adduct was found. At the moment no plausible explanation for this observation can be given unless the medical background of the patient was incomplete. Such uncertainties should be avoided in the future by implementing a good protocol with regard to the patient's history.

In many of the samples, adducts of 4-hydroxy-17-α-ethynylestradiol were detected such as adducts of dAdo (patients 3, 6–11), dGuo (patients 10, 11, 13) and thymidine (patients 7, 8, 12, 13). This is not entirely surprising because of its use as a contraceptive. Figure 5 shows the detection of a standard of a dAdo-4-hydroxy-17-α-ethynyl-estradiol adduct in the upper trace and the corresponding SRM transi-

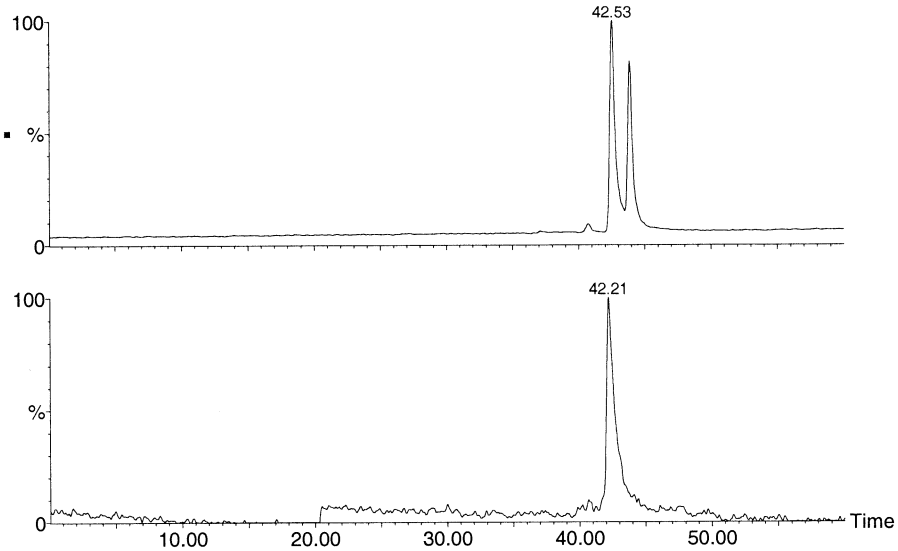


Figure 3. Chromatogram of SRM trace 548 > 432 (equilenin-2'-deoxy adenosine adduct). Upper: In vitro prepared reference adducts. Lower: Breast tumor DNA of patient 4 treated with equilenin. Both were analyzed with nano LC-nano (+)ES MS (Column 75 μm * 15 cm, BDS C8) with column switching (300 μm * 5 mm, C18P3, 10 μm) after 7 min, 20 μL injection volume (= 7.6 μg DNA).

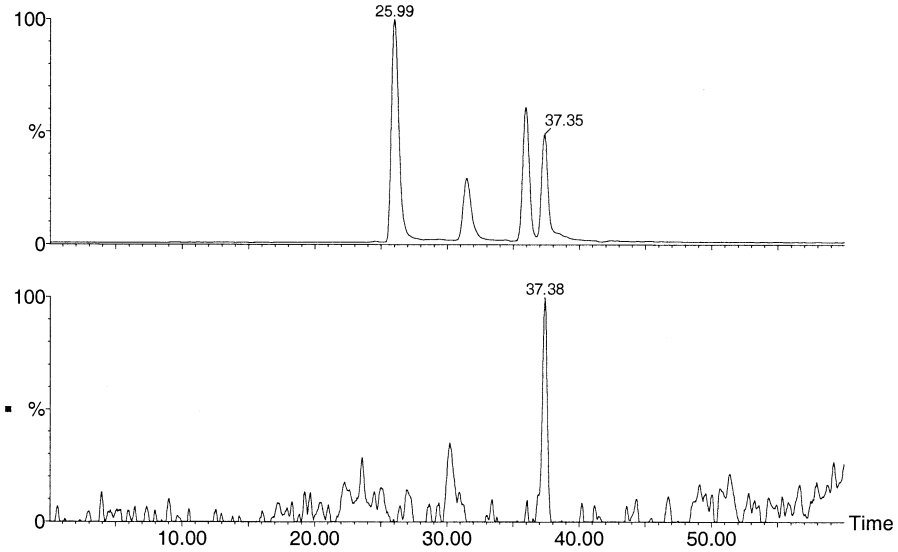


Figure 4. Chromatogram of SRM trace 564 > 448 (equilenin-2'-deoxy guanosine adduct). Upper: In vitro prepared reference adducts. Lower: Breast tumor DNA of patient 13 treated with equilenin (Premarin). Both were analyzed with nano LC-nano (+)ES MS (picofrit column 75 μm * 10 cm, aquasyl C18) with column switching (300 μm * 5 mm, C18, 10 μm) after 12 min, 100 μL injection volume (= 38 μg DNA).

tion found in a breast tumor sample in the lower trace. The specific SRM transition, together with the comparable retention time as the synthesized standard, again identified the adduct in the breast tumor sample.

Finally, it is worthwhile mentioning that in some samples an adduct was present responding to the 570 > 454 SRM transition. This response was indicative for the presence (patients 1–3, 7) of a dGuo adduct of benzo-[a]pyrene 7,8-diol 9,10-epoxide. This adduct eluted approximately 3 min after the last eluting equilenin reference adduct, depending on the solvent system used.

At the moment no quantitative measurements have been taken, but a rough estimation can be made on the basis of the detection limit of 200 fg for a 4OHEN-dGuo adduct [33]. In most of the 2-DLC-MS runs around 38 μg of DNA (hydrolysate) was injected. This means that 2.8 adducts in 10^9 nucleosides were detected. Since the signal of most DNA-adducts detected here was close to the detection limit, we may

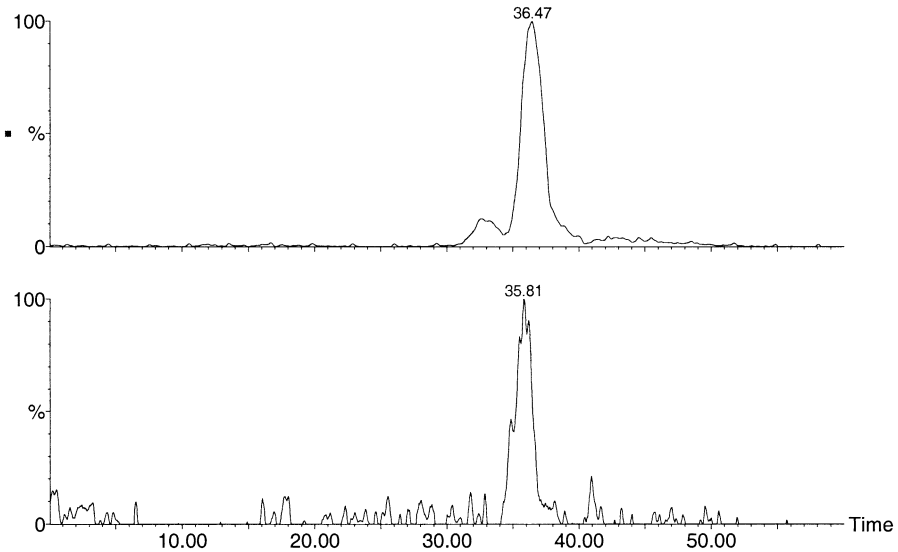


Figure 5. Chromatogram of SRM trace 562 > 446 (17-α-ethynylestradiol-2'-deoxy adenosine adduct). Upper: In vitro prepared reference adducts. Lower: Breast tumor DNA of patient 3 treated with equilenin. Both were analyzed with nano LC-nano (+)ES MS (picofrit column 75 μm * 10 cm, aquasyl C18) with column switching (300 μm * 5 mm, C18, 10 μm) after 12 min, 100 μL injection volume (= 38 μg DNA).

assume the estrogen DNA-adducts to be present in the same order of magnitude.

Conclusion

From the data presented above we can conclude that nano-LC coupled to nano-electrospray tandem mass spectrometry can be used for DNA-adduct analysis in human DNA. Adducts of endogenous and exogenous estrogens like equilenin, which is proven to be carcinogenic [7, 8], could be found in breast-tumor tissue and adjacent normal breast tissue. However we hereby want to stress that these results are very preliminary and that in order to (1) validate the complete procedure and (2) draw biochemical conclusions it is obvious that many more samples need to be analyzed, quantitative data included, and a tight protocol covering the patient's medical history should be designed. Furthermore, a careful selection of blank tissue will be important for future measurements.

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