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Analysis of tamoxifen-induced DNA adducts by ^{32}P-postlabelling assay using different chromatographic techniques

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Abstract

DNA isolated from livers of rats receiving tamoxifen was analysed by the ^{32}P-postlabelling method. The postlabelled DNA hydrolysis mixture was analysed both by reversed-phase HPLC with ^{32}P on-line detection and by TLC on polyethyleneimine plates followed by autoradiography. Using the HPLC method, five well separated adduct peaks could be detected, while by the TLC method, two groups of adduct spots were observed. The detection limit of the TLC assay was lower (0.5 adducts/10^{10} nucleotides) than that of the HPLC assay (3 adducts/10^{10} nucleotides). Thus, the TLC assay is more sensitive but also more laborious. The advantages of the HPLC assay were, in addition to better resolution, the ease of quantification and operation.

Keywords: ^{32}P-Postlabelling method; DNA adducts; Tamoxifen

1. Introduction

Tamoxifen [(Z)-1-{4-[2-(dimethylamino)ethoxy]-phenyl}-1,2-diphenyl-1-butene] is widely used in the adjuvant treatment of breast cancer among postmenopausal women [1]. The use of tamoxifen as a chemopreventative agent for women having a risk of developing breast cancer has also been proposed [2]. Tamoxifen has been shown to induce DNA adduct formation in the livers of different rodent species in vivo, and it is considered a liver carcinogen in animal models [3–11]. The use of this antiestrogen has also been associated with an increased risk of endometrial cancer in humans [3]. However, in humans no tamoxifen-induced DNA adducts have so far been reported in vivo [12], but are detected in vitro in a human microsomal system and in cultured lymphocytes [6,13]. Metabolic activation is needed for DNA adduct formation. The metabolic pathway leading to tamoxifen adducts, as well as the chemical nature of these adducts are still largely unknown [14–22]. It appears that activation of tamoxifen in rat and human microsomes produces several different types of DNA adducts [6].

The principal method used in the analysis of DNA adducts in vivo, including those formed by tamoxifen, has been the highly sensitive ^{32}P-postlabelling method [4–13,15–22]. In this method, the separation of the adducts, after labelling the DNA digest, is carried out by thin-layer chromatography (TLC) on ion-exchange polyethyleneimine-cellulose (PEI). In

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some studies, high-performance liquid chromatographic (HPLC) assays have been applied to separate further the resolved TLC adduct spots [15,18,20,22]. Some HPLC methods have been described for the analysis of the free drug and its major metabolites in plasma and tissues [23,24], but no reports have appeared on the direct application of HPLC for the separation of ^{32}P-labelled DNA adducts of tamoxifen in the hydrolysis mixture. We have now developed an HPLC method with on-line radioisotope detection for the determination of the tamoxifen adducts in the ^{32}P-postlabelling mixture and we have analysed tamoxifen-treated rat liver samples by this method and by a previously reported TLC method [13].

2. Experimental

2.1. Chemicals

RNase A, RNase T1, micrococcal nuclease, spleen phospodiesterase and apyrase from potato were obtained from Sigma (St. Louis, MO, USA). Proteinase K and P1 Nuclease were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). T4 polynucleotide kinase was from United States Biochemicals (Cleveland, OH, USA) and [gamma-^{32}P]ATP from Amersham International (Little Chalfont, UK). Methanol was HPLC grade (J.T. Baker, Deventer, Netherlands). All other chemicals were of analytical grade and were either from Sigma or from E. Merck (Darmstadt, Germany).

2.2. Isolation and hydrolysis of DNA. Labelling of the nucleotides

Rats were given tamoxifen p.o. 45 mg/kg per day for two weeks. The DNA isolation was carried out as previously described [13]. In brief, the rat liver (0.6–1.2 g) was homogenised in 5 ml of 1 m*M* $MgCl_2$, 10 m*M* Tris–HCl, 0.15 *M* NaCl (pH 8), after which the suspension was treated with 5 ml of 0.5% Triton X-100. The nuclei were collected by centrifugation at 900 *g* for 10 min at 2°C. DNA was isolated with RNase A and RNase T1 treatment followed by proteinase K digestion. The digest was extracted with phenol and chloroform–isoamyl alcohol (24:1) and DNA was precipitated with ethanol.

A 5-μg amount of DNA was enzymatically digested to 3′-mononucleotides as described [13], first by incubating DNA for 2 h at 37°C with micrococcal nuclease (80 mU/ug DNA in 3 m*M* bicine pH 9.0, 0.5 m*M* $CaCl_2$) and then for 2 h at 37°C with spleen phosphodiesterase (1.6 mU/ug DNA) in added 20 m*M* ammonium acetate, pH 5.0. P1 nuclease (1 mg/ml; 1 h at 37°C) was used for dephosphorylation of normal nucleotides. Larger amounts of DNA can be hydrolysed by increasing equally the amounts of enzymes.

The modified nucleotides were converted to ^{32}P-postlabelled diphosphates in labelling mixture (2 μl) containing 2.4 U T4 polynucleotide kinase and 2.3 pmol ATP (7 uCi [g-^{32}P]ATP, 3000 Ci/mmol). The reaction was carried out at pH 9.6.

Some experiments were performed, where a solid-phase extraction (SPE) clean-up procedure was applied for the hydrolysis mixtures before labelling (Bond-Elut, Varian, Walton-on-Thames, UK). The hydrolysis mixture was washed with 5 ml of water and 3 ml of 25% methanol. The sample was then collected by eluting first with 1 ml of 25% methanol after which with 3 ml of 90% methanol and finally with 2 ml of 100% methanol. Samples were dried in a vacuum centrifuge and dissolved in water (1 μl).

Before the samples were analysed by TLC, the excess [gamma-^{32}P]ATP was degraded by adding apyrase (30 mU). However, this step is not necessary and because of the danger of introducing unknown contaminating enzymes, it has been deleted. The whole labelling mixture was applied to the TLC plate. For HPLC analysis the mixtures were diluted to 20 μl with water, and the whole sample was injected into the HPLC system.

2.3. Instrumentation

Beckman HPLC system Gold (Berkeley, CA, USA) was used with a Phenomenex Kromasil C_{18} (150×2 mm, particle size 5 mm) column. A small guard-column (Opti-guard, 1.5 cm×1 mm, packed bed) was installed in the front of the analytical column. The volume of the sample loop was 20 μl.

Radioactivity was measured on-line with a Beckman 171 radioisotope detector. In most of the chromatographic runs the size of the teflon sample loop in the flow-cell was 50 μl. In order to increase

sensitivity, the size of the sample loop was increased to about 150 μl, which was then folded into a scintillation tube containing scintillation liquid (Ready Safe, Beckman). The adducts were quantified by integration of the peak area with subtraction of background radioactivity.

TLC analyses were performed on 10×15 cm PEI-cellulose TLC plates (Macherey–Nagel, Duren, Germany) as described [13]. The adducts were detected in a Fuji XBAS 2000 phosphoimager (Japan).

2.4. Chromatographic conditions

Separations were carried out at ambient temperature by using a binary gradient with methanol and 0.2 *M* ammonium formate (pH 5.4) adjusted to pH 4.2 with phosphoric acid (resulting in a final concentration of 20 m*M* with respect to phosphoric acid). Labelled samples were analysed using three different gradients. Gradient A had initial conditions of 2% methanol for 5 min after which the proportion of methanol increased linearly to 60% in 40 min and then further to 100% in 10 min. 100% methanol was maintained for 5 min followed by a linear decrease to 2% in 10 min. Some of the rat liver samples were eluted with a gradient in which the increase of the proportion of methanol to 60% took place in 30 min (gradient AA). A third gradient was devised to elute the main adduct in an isocratic part of the program for maximal separation. This gradient (gradient AAA) started at 0.5 *M* ammonium formate–20 m*M* phosphoric acid, pH 4.6 containing 2% methanol for 5 min. Methanol concentration increased linearly to 43% in 45 min and remained isocratic for 25 min, followed by a linear increase to 100 in 15 min. The flow-rate was 0.25 ml/min.

TLC separations were carried essentially as before [13]. The pre-washed plates were first (D1) developed with 1.7 *M* NaH_2PO_4 (pH 6.0) for 6 h using a paper wick on top. The upper part of the plates with the wick was cut off and discarded, after which the plates were developed in opposite direction (D3) with 2.7 *M* lithium formate, 6.4 *M* urea (pH 3.5) for 1 h. The next direction (D4) was developed 90 degrees from D3 in 0.4 *M* lithium chloride, 0.25 *M* Tris–HCl, 4.25 *M* urea (pH 8) into a paper wick for 3 h. The paper wick was cut off. D5 was developed into the same direction as D4 in 1.7 *M* NaH_2PO_4 (pH 6.0) with a paper wick for 5 h. After each direction the plates were washed by flotation in water and dried.

3. Results and discussion

3.1. TLC analysis

TLC separation was performed according to a previously published method developed for the analysis of tamoxifen induced adducts in human microsomal system and lymphocytes [13]. When rat liver DNA samples were analysed in the present study, two groups of adduct spots were resolved (Fig. 1). Each group contained one major and one minor component.

3.2. HPLC analysis

In the HPLC analysis of the ^{32}P-labelled rat liver DNA samples with gradient A, it was possible to detect different adduct peaks at the retention times between 38 min and 48 min (Fig. 2). Four peaks were well separated and in these peaks shoulders were observed. These shoulders could possibly be separated into distinct peaks by a different gradient as shown below.

We also studied the possibility of increasing the sensitivity of the HPLC method by modifying the flow-cell of the detector. In order to increase sensitivity, a long residence time is required for the sample in the flow cell of the radioisotope detector. We increased the sample loop size from 50 μl up to about 150 μl. With a flow-rate of 0.25 ml/min the peaks became quite broad leading to overlapping of adjacent peaks (data not shown). The loss of resolution in the cost of sensitivity is not harmful if the total amount of tamoxifen adducts is of interest and no background material elutes at same retention times.

The sensitivity can also be increased by using a steeper increase in the proportion of methanol in the mobile phase (gradient AA). With higher methanol concentration the adducts elute faster and when the increase in methanol proportion occurs in fairly short periods of time the different tamoxifen adducts concentrate into one or two peaks. This steep step in

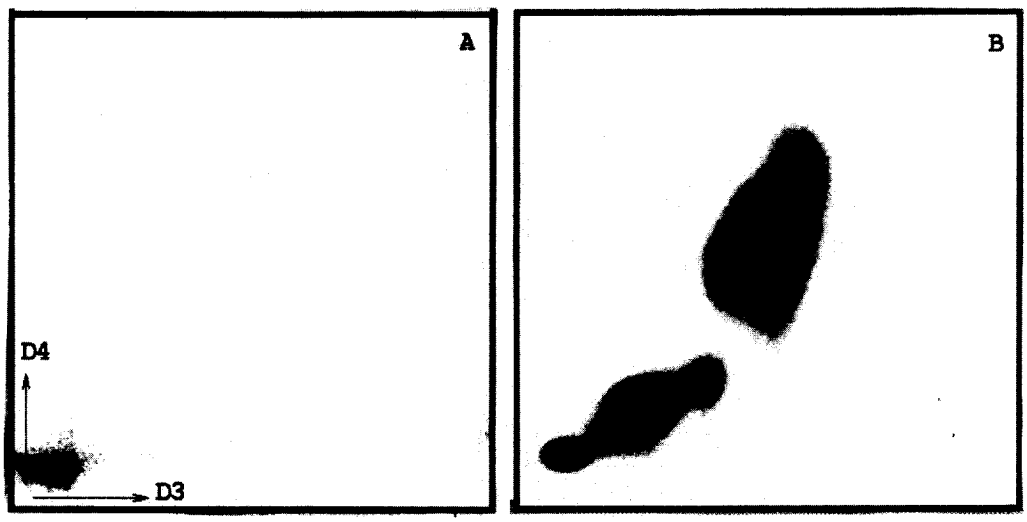

Fig. 1. TLC separations of the postlabelled digests of DNA (5 μg) from rat liver. (A) Control and (B) rat treated with tamoxifen, containing ca. 31 adducts/10^6 nucleotides. The directions of the TLC elution systems are shown. D1 was run opposite to D3 (no D2) and D5 parallel to D4. The origin is in the left bottom corner. Separation conditions described in Section 2.4.

the gradient should occur after the other radioactive material in the sample has eluted (at 30 min in the gradient AA). Thus, the chromatograms of the samples analysed with gradient AA consisted of one large peak (at about 34 min) and a smaller one (at about 39 min) (Fig. 3).

A third way to increase the sensitivity is to use more than 10 μg of labelled DNA in the HPLC analysis. When more than 30 μg of DNA is used, a purification by a SPE procedure is needed before the labelling to remove unwanted components from the labelling mixture and to avoid overloading of the chromatographic system. In the chromatograms of the extracted samples the early eluting peaks were washed away and also the background radioactivity was lower (Fig. 3B). We found out that even 150 μg of DNA hydrolysate can be injected without overloading the chromatographic system if SPE is employed prior to labelling. The ^{32}P-labelling seemed to be slightly more efficient after the purification step, the recovery of the samples being 109% (data not shown).

Maximal resolution is achieved by gradient AAA, employing an isocratic solvent mixture at the time when the main tamoxifen adducts are eluting (Fig. 4). Five large peaks are well separated and some small peaks are additionally noted.

3.3. Detection limits

In the TLC analysis the detection limit for tamoxifen adducts was estimated to be 0.5 adducts/10^{10} normal nucleotides in a 10-μg DNA sample (2 amol of adducts), while the detection limit in the HPLC analysis with gradient AA was about 3 adducts/10^{10} normal nucleotides when 150 μg of DNA was analysed (0.1 fmol of adducts). The detection limits were calculated assuming the labelling efficiency to be 100%. However, as the adducts remain chemically unidentified, and no standards can be used to calculate the true labelling efficiency, the true adduct levels may vary.

3.4. Comparison of TLC and HPLC methods

The resolution of the reversed-phase HPLC system is better than that of the ion-exchange TLC system. Using the TLC method we were able to detect two large adduct spots containing several minor components in the rat liver DNA samples. Using TLC, Pathak and Bodell have been able to see a total of eight well-separated tamoxifen adducts, produced in a rat liver microsomal activation system [6]. The HPLC method with an isocratic step allowed separation of five main tamoxifen adduct peaks and

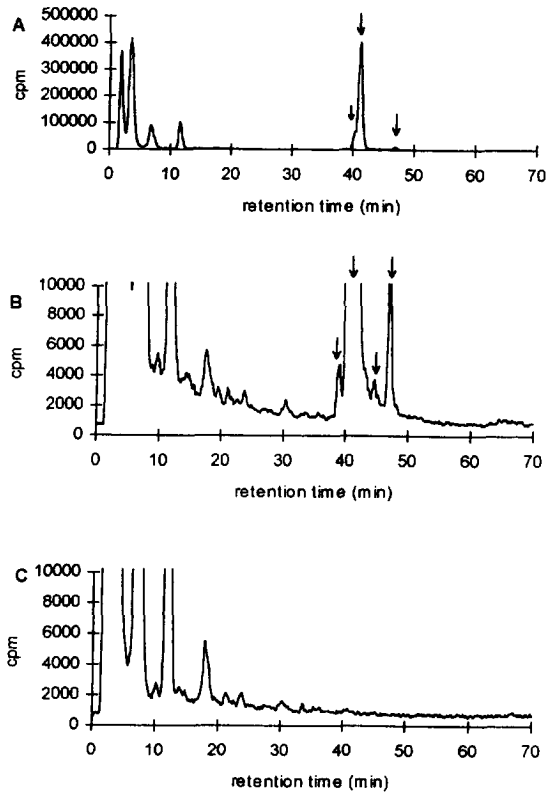

Fig. 2. HPLC separation of the postlabelled digests of DNA (5 μg) from rat liver with gradient A. The peaks eluting after 38 min are the tamoxifen-induced DNA adducts. The early eluting radioactive peaks contain ATP and orthophosphate. (A,B) Rat liver samples containing ca. 31 adducts/10^6 nucleotides in two different cpm scales; (C) control. Volume of the flow-cell was about 50 μl. Separation conditions as in Section 2.4.

several minor peaks. HPLC would provide an easy means of isolating these peaks preparatively for further chemical characterisation.

The detection limit in the TLC assay used was markedly better than that of the HPLC assay, even when the sensitivity of the HPLC method was increased by increasing the size of the sample loop, and by injecting high amounts of DNA hydrolysate into the system. The possibility for longer exposures makes the sensitivity of the TLC method such, that, very low levels of adducts can be detected. Yet in the analysis of biological samples the practical sensitivity depends on many parameters including absolute sensitivity, resolution and reproducibility.

There are marked advantages in HPLC over TLC

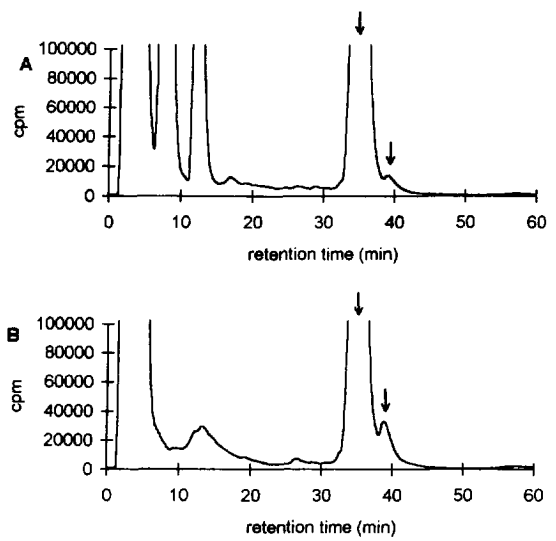

Fig. 3. HPLC separation of the postlabelled digests of DNA (10 μg) from rat liver with gradient AA and the effect of the C_{18} purification prior the labelling, shown in panel (B). The sample contained ca. 31 adducts/10^6 nucleotides. Tamoxifen-induced DNA adducts elute after about 32 min. The volume of the flow-cell was about 150 μl.

when the adduct levels are in excess of $1/10^9$ normal nucleotides, as in the case of tamoxifen-treated rat liver samples and, perhaps, in human tissue specimens, e.g., resolution is better in HPLC. Quantification is easier in the on-line HPLC system as compared to counting the radioactivity in the TLC plates. The performance of the TLC plates varies from batch to batch, while the HPLC columns have a better reproducibility over a long period of time. With

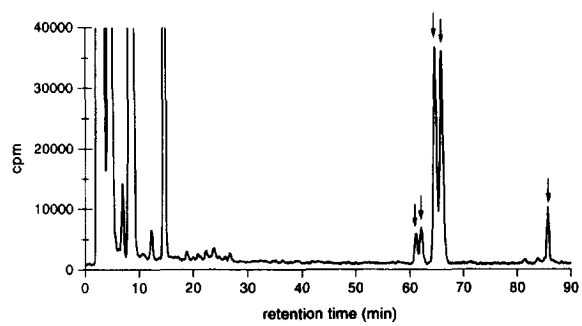

Fig. 4. HPLC separation of rat liver DNA adducts with gradient AAA, in which the eluent is isocratic between 50 and 75 min. The arrows mark the main tamoxifen adducts. Only 1/5 of the labelled sample was injected; the flow-cell was 75 μl.

HPLC it is also possible to isolate reaction products in a preparative scale. The time factor is also of practical importance: HPLC is completed in one hour while TLC takes two days. Yet several parallel samples can be processed in TLC.

One of the disadvantages of the HPLC system we can mention is the contamination of the system. In order to inhibit ^{32}P-activity accumulation in the system we have added orthophosphoric acid to the mobile phase to avoid the interaction of ^{32}P-orthophosphate from [gamma-^{32}P]ATP and free silanols in the stationary phase. Still the contamination of the chromatographic system or the flow-cell of the detector may be a problem, as the background in the chromatograms increases. Also, cross-contamination was found to be a problem when rat liver samples with high amounts of adducts were analysed. The addition of orthophosphoric acid to the mobile phase has also other advantages. The pH of the mobile phase should be kept low in order to ensure that the protonation equilibrium of the phosphate groups, present in the labelled nucleotide adducts, will favor uncharged species. Also, a high ionic strength of the mobile phase seems to be an advantage to achieve optimal peak shapes and suitable resolution [25].

The present study shows that TLC analysis is more sensitive in the detection of tamoxifen adducts as compared to HPLC analysis. However, the HPLC separation with on-line detection is a good alternative to TLC separation in the case of biological samples, the best advantages being the better resolution and the ease and speed of operation due to the on-line detection and quantification. The resolution is essential when interference by unknown background material is present. Thus only the HPLC technique was capable of detecting tamoxifen-induced DNA adducts in endometrial samples from breast cancer patients [26].

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