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Tamoxifen Forms DNA Adducts In Human Colon After Administration Of A Single [^{14}C]-Labeled Therapeutic Dose.

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Abstract

Tamoxifen is widely prescribed for the treatment of breast cancer and is also licensed in the U.S. for the prevention of this disease. However, tamoxifen therapy is associated with an increased occurrence of endometrial cancer in women and there is also evidence that it may elevate the risk of colorectal cancer. The underlying mechanisms responsible for tamoxifen-induced carcinogenesis in women have not yet been elucidated but much interest has focussed on the role of DNA adduct formation. We investigated the propensity of tamoxifen to bind irreversibly to colorectal DNA when given to ten women as a single [^{14}C]-labeled therapeutic (20 mg) dose, ~18 h prior to undergoing colon resections. Using the sensitive technique of accelerator mass spectrometry, coupled with HPLC separation of enzymatically digested DNA, a peak corresponding to authentic dG-N^2-tamoxifen adduct was detected in samples from three patients, at levels ranging from 1-7 adducts/10^9 nucleotides. No [^{14}C]-radiolabel associated with tamoxifen or its major metabolites was detected. The presence of detectable CYP3A4 protein in all colon samples suggests this tissue has the potential to activate tamoxifen to α-hydroxytamoxifen, in addition to that occurring in the systemic circulation, and direct interaction of this metabolite with DNA could account for the binding observed. Although the level of tamoxifen-induced damage displayed a degree of inter-individual variability, when present it was ~10-100 times higher than that reported for other suspect human colon carcinogens such as PhIP. These findings provide a mechanistic basis through which tamoxifen could increase the incidence of colon cancers in women.

Introduction

The antiestrogen tamoxifen is the most widely prescribed drug worldwide for the treatment of breast cancer[1]. In 1998 it was also approved by the US FDA for the prevention of breast cancer in healthy high risk women on the basis of trial data indicating an overall 49 % reduction in the occurrence of invasive breast cancer with tamoxifen treatment compared to placebo in this population (1, 2). It has been estimated that currently 2 million women in the US would experience a net benefit from taking tamoxifen as a chemopreventive agent (3). However, tamoxifen use is associated with a 2-8 fold increased incidence of endometrial cancer (4) and there is some evidence that tamoxifen also elevates the risk of gastrointestinal cancers by as much as 3-fold (5). A specific increase in the frequency of colorectal cancers has also been described in some studies (6, 7). In addition, a meta-analysis of randomized controlled trials reported tamoxifen was correlated with a modest but significant increase in gastrointestinal cancers (8), although another recent overview of randomised trials in early breast cancer does not support this contention (9). Whilst tamoxifen does not appear to cause liver cancer in women (10), in rats, hepatic tumours develop with chronic dietary administration of doses greater than 5 mg/kg (11). These liver tumours are considered to arise through a genotoxic mechanism. Tamoxifen activation involves conversion to α-hydroxytamoxifen, catalyzed primarily by CYP3A4 in humans (12, 13). This metabolite can then be further conjugated, generating the more reactive α–sulfate-ester but whether this step actually occurs in human tissues has not yet been confirmed (14). The resulting reactive

1. http://ntp.niehs.nih.gov/ntp/roc/eleventh/profiles/s167tamo.pdf

carbocations are able to bind predominantly to the N^2-position of guanine, forming adducts including α-(N^2-deoxyguanosinyl)tamoxifen (dG-N^2-tam) (15, 16), which are proven mutagenic lesions in mammalian cells (17, 18). Analogous reactions may also occur with other Phase I metabolites of tamoxifen (19, 20).

The underlying mechanisms responsible for tamoxifen induced carcinogenesis in women have not yet been elucidated. Studies investigating the presence of tamoxifen DNA adducts in human tissues have reported conflicting results and there is much debate over this issue and whether tamoxifen induced DNA damage might be a contributing factor in the development of cancers in women (4). Low levels of the major dG-N^2-tam lesion formed in rat liver have been detected in leukocytes and endometrial tissue of tamoxifen treated women by two groups using ^{32}P-postlabeling analysis (21-23). However, in each case adducts were only observed in samples from a fraction of the patients, indicating variability in tamoxifen metabolic activation, the formation or repair of DNA adducts. Other investigators have failed to detect adducts in human samples, applying similar methods and alternative HPLC-electrospray ionization tandem mass spectrometry approaches (24, 25). Part of the reason for these discrepancies is the fact that if damage is induced, it is likely to be close to or below the limits of detection achievable with the assays typically employed. With this is mind we have previously utilized the technique of accelerator mass spectrometry (AMS), one of the most sensitive adduct detection methods available (26), to demonstrate that [^{14}C]-tamoxifen binds to endometrial and myometrial DNA in women at extremely low levels (~400 adducts/10^{12} nucleotides) after administration of a therapeutic dose (27).

Nothing is known regarding the ability of tamoxifen to induce DNA damage in gastrointestinal tissue of women, therefore we have now investigated the propensity of tamoxifen to bind to colorectal DNA when given as a single [^{14}C]-labeled dose (20 mg) prior to patients undergoing colon resections. We demonstrate evidence for inter-individual variation in the ability of tamoxifen to act as a genotoxin and also describe efforts to characterise the nature of the binding detected, which highlights some important issues relating to the use of AMS for DNA adduct detection.

Materials and Methods

Chemicals. Chemicals and solvents were purchased from Sigma Chemical Co, Poole, UK, unless stated otherwise. Tamoxifen citrate was a gift from Dr. J. Topham, Zeneca plc, Macclesfield, UK. [1-Phenyl-U-^{14}C]tamoxifen (2.03 GBq/mmol) of >98 % radiochemical purity, was from Cambridge Research Biochemicals (Cleveland, UK). *N*-Desmethyltamoxifen and α-hydroxytamoxifen were synthesized according to published methods (19, 28). Gelatin capsules containing 1.85 MBq of [^{14}C]-tamoxifen and 20 mg unlabeled drug were prepared in the Radiopharmacy Department at Leicester Royal Infirmary. The committed effective radioactive dose equivalent was less than 180 μSv, which is below the natural background radiation to which people are exposed in daily life during the course of a month (29).

Patients and sample collection. Study protocols were approved by the Human Research Ethics Committee, Leicester Health Authority, the Department of Health Committee on the Administration of Radioactive Substances to Persons – the Medicines (administration of radioactive substances) Regulation of 1978, United Kingdom, and the Institutional Review Board at the Lawrence Livermore National

Laboratory (LLNL). Informed consent was obtained from all patients prior to studies commencing and only those who had never previously been treated with tamoxifen were eligible. Women recruited to the study (mean age 60.4 years, range 26 to 81 years) were non-smokers and were undergoing colon resections associated with inflammatory bowel disorders. Patients were not known to have colon cancer and were not receiving any other drug therapies at the time. Each patient was given 20 mg tamoxifen citrate containing 1.85 MBq [^{14}C]-tamoxifen ~18 h before surgery. Venous blood (10 mL) was collected in heparinised tubes at the time of surgery. Colon samples (full thickness) were taken from normal tissue and rinsed in ice cold phosphate buffered saline and after transfer from theatre, weighed and snap frozen in liquid nitrogen. The exact location of each tissue sample within the colon was not defined. Control tissue and blood from a patient who did not receive [^{14}C]-tamoxifen was also obtained for determination of background ^{14}C levels in DNA and plasma.

CYP3A4 protein quantitation

Tissue samples (~0.5 g) were powdered using a pestle and mortar cooled in liquid N_2 then suspended in an equal volume of ice-cold lysis buffer (50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 5 mM EDTA, 5% (v/v) glycerol, 1% (v/v) Triton X-100, 25 mM NaF and 2 mM Na_2VO_4). Before use, one complete protease inhibitor cocktail tablet (Roche) was added to 10 mL buffer. Samples were centrifuged (10,000 g, 5 min, 4 ^{o}C) and the supernatant stored for up to 1 week at –80 ^{o}C. Protein concentrations were determined by a Bicinchoninic acid procedure (Sigma) following the manufacturer's instructions. Equal amounts of protein (50 µg) were separated on pre-cast SDS 7.5% polyacrylamide gels (Ready Gel, Biorad). Human CYP3A4 (0.1 pmoles) (Supersomes™ Cat. No. P207, BDBiosciences, Oxford, UK) was included as

a standard. After electrophoresis, proteins were transferred onto nitrocellulose filters (Amersham Hybond, ECL) and the filters incubated overnight at 4 ^{o}C with anti-human CYP3A4/3A7 antibody (Cat.No. B458234 BD Biosciences) diluted 1:3000. Immunoreactive proteins were detected using anti-mouse peroxidase-coupled secondary antibody diluted 1:2500 (Sigma, Cat No. F5897) and enhanced chemoluminescence (ECL system, Amersham, UK). Chemiluminescence was quantitated using Syngene Gnome image analysis (Syngene, Cambridge, UK).

Measurement of tissue and plasma concentrations

Plasma and tissue concentrations of [^{14}C]-tamoxifen equivalents were measured by liquid scintillation counting. Tissues (100 mg) were solubilised in 1 mL NCS II tissue solubiliser (Amersham [Canada] Ltd) overnight at 37 °C. Scintillant (Optiphase HiSafe 2, Fisher Chemicals) (9 mL) was added and after allowing for chemiluminescent decay, samples were counted in a Wallac 1410 scintillation counter (LKB Instruments).

DNA isolation

DNA was extracted using Qiagen column chromatography (Qiagen Ltd, Crawley, UK) according to the manufacturer's instructions. DNA was dissolved in water and the purity determined by the A_{260}/A_{280} ratio. DNA samples (in solution) were shipped to the LLNL National Resource for Biomedical AMS (NIH project RR13416) for analysis.

Measurement of protein content in DNA samples

Where sufficient DNA was available (100-150 μg) the protein content was determined using the QuantiPro High Sensitivity Protein Assay Kit (Sigma-Aldrich

Co., Dorset, U.K.). Total protein concentration was calculated from the absorbance measured at 562 nm.

Digestion and HPLC separation of DNA samples.

Where colon DNA was available, samples from [^{14}C]-tamoxifen treated patients (numbers 1, 3, 4, 5, 6, 7 and 8) and from the control patient were digested to nucleosides and subject to HPLC separation. Briefly, each DNA sample was split into four aliquots of 50 μg and hydrolysed in 30 μL digestion buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5) using DNase I (10 U), shrimp alkaline phosphatase (2 U) and snake venom phosphodiesterase (0.05 U) for 2 h at 37°C. Enzymes were removed by passing each digest through a Microcon filter (3000 molecular weight cutoff, Millipore, Billerica, MA), then for each patient the samples were combined and concentrated to dryness.

Each DNA digest (typically 200μg) was separated using these conditions on a Jasco HPLC system (Jasco, Chelmsford, Essex, UK) designated for AMS samples only, consisting of Intelligent HPLC pumps (PY-1580) and dynamic mixer (HG-1580-32), an Intelligent autosampler (AS-1555) and a UV/Vis multiwave detector (MD-1510) set at 254 nm. Samples were injected onto a Hypersil BDS C_{18} column (4.6 x 250 mm, 5 μ, Phenomenex, Macclesfield, UK), and eluted with 0.5 M ammonium acetate (A) and methanol (B) at a flow rate of 1 mL/min over 40 min (0-5 min, 60% B, 5-7.5 min, 35%B, 7.5-12.5 min, 35% B, 12.5-15 min, 60% B, 15-20 min, 75%B, 20-25 min, 75%B, 25-27.5 min, 80% B, 27.5-35 min, 80% B, 35-40 min, 60% B). Two blank runs were performed in between samples to prevent cross contamination and fractions

were collected at 30 second intervals for the duration of each run. Individual HPLC fractions were then concentrated to dryness under vacuum and shipped to LLNL.

A sample containing authentic standards of tamoxifen, α-hydroxytamoxifen, 4-hydroxytamoxifen and *N*-desmethyltamoxifen along with digested control human DNA, was analysed under the same conditions. The dG-N^2-tamoxifen adduct was prepared by reaction of α-acetoxytamoxifen with salmon sperm DNA (15) and also subject to HPLC analysis for comparison of retention times. The adduct structure was verified by mass spectrometry using a Waters Quattro Ultima Pt (Waters, Manchester, UK), operated in the positive ion mode. The molecular mass was determined using a total ion scan, then tandem MS/MS was employed to further confirm the structure (Figure 4C, inset). Although it was not possible to determine the exact isomer, it is likely to be a *trans* form since the α-acetoxytamoxifen used in the reaction was predominantly the *trans* isomer (15).

AMS measurement

Prior to analysis, each HPLC sample was redissolved overnight in 500 μL methanol:water (70:30). DNA samples (500 μg) and HPLC fractions were supplemented with a precise amount of carrier (1μL tributyrin, equivalent to 615 μg carbon), to provide sufficient carbon mass for optimal sample preparation and AMS analysis. Samples were then converted to elemental carbon using standard protocols, by combustion to CO_2, followed by reduction to filamentous graphite (30). The resulting graphite was analysed by AMS, with each graphite sample analysed up to seven times for radiocarbon content or until the measurement variation was within ± 5%. Data are presented as attomoles ^{14}C per mg DNA or per HPLC fraction and

results from tamoxifen treated patients are compared with those from the control, untreated patient.

Results

In patients administered a single therapeutic dose of [^{14}C]-tamoxifen the mean plasma concentration of [^{14}C]-tamoxifen equivalents was 14.7 ± 2.5 ng/mL or ~40 ± 7 fmoles/mg (mean ± SEM, n = 10), which is consistent with other published reports, including our previous study in hysterectomy patients given an equivalent dose under an analogous treatment protocol (22 ± 3 ng tamoxifen equivalents/mL plasma or ~60 ± 8 fmoles/mg) (27). The mean level of total [^{14}C]-tamoxifen equivalents measured in colon tissue was 653 ± 135 fmols/mg tissue (Figure 1), which exceeds plasma levels by a factor of ~16. Concentrations of total [^{14}C]-radiolabel displayed a degree of variability amongst individuals with the highest, detected in patient 10, being 8.5-fold greater than the lowest amount measured in patient 7.

Western blot analysis of tissue samples revealed CYP3A4 protein was expressed in all patients to varying extents, with levels ranging from 0.37 - 1.71 pmols/mg protein (Figure 2). The presence of this enzyme suggests tamoxifen could be converted to metabolites within colorectal tissue, including α-hydroxytamoxifen (13), which may then be available for direct reaction with DNA.

Binding of [^{14}C]-tamoxifen to human colon DNA

The ^{14}C content of DNA samples from the ten tamoxifen treated patients is shown in Figure 3, and can be compared to the background level of ^{14}C naturally occurring in colon DNA of an untreated patient (46.3 attomoles/mg DNA). In all patients the level of ^{14}C was significantly elevated above background concentrations, ranging from 3.6 - 18.0-fold higher, which is consistent with the presence of bound [^{14}C]-tamoxifen.

There was a strong positive association between the tissue concentration of [^{14}C]-tamoxifen equivalents and DNA radiocarbon content (Pearson's product moment correlation coefficient, r = 0.88). However, there was no correlation between DNA binding and CYP3A4 protein expression for individual patients.

Extracted DNA from two patients was analysed for the presence of residual protein. Low amounts of contamination were detected, with 1.97 μg and 2.24 μg of protein present per 100 μg of DNA in samples from patients 2 and 9 respectively.

HPLC-AMS analysis of colon DNA

To establish if bound [^{14}C]-radiolabel was covalently associated with cellular DNA, where sufficient extracted DNA was still available, samples were digested to 2'-deoxynucleosides using a method proven capable of efficiently hydrolysing DNA to its individual nucleosides, as indicated by HPLC analysis with UV detection. Prior to injection the digest was passed through a Micron filter which removes material with a molecular weight greater than 3000, thereby excluding any residual protein or peptide contamination along with complexes of DNA containing tightly bound protein. Initially, a total of 200 μg DNA from both Patient 1 and a control patient was digested and subjected to HPLC separation, with fractions collected at 30 second intervals to maximise peak resolution. The HPLC system was developed to achieve adequate separation between tamoxifen, its major metabolites, the dG-N^2-tamoxifen adduct and 2'-deoxynucleosides as shown in Figure 4, panels C and D.

As would be expected, the reconstructed chromatogram from the analysis of DNA from the control, untreated patient, illustrates a low background level of ^{14}C across the

run (Figure 4B). This is consistent with the natural abundance of ^{14}C in HPLC solvents and general reagents as well as that present in the DNA digest. The lowest limit of detection for ^{14}C by HPLC-AMS analysis, calculated as the mean plus two times the standard deviation of ^{14}C in HPLC fractions from the control DNA digest, was ~2.75 attomoles, which translates to approximately 400 adducts/10^{12} nucleotides in a single fraction.

The radiochromatogram produced for [^{14}C]-tamoxifen treated Patient 1 reveals two main peaks eluting at ~11.5 and 23.5 min (designated peaks 1 and 2 respectively, Figure 4A). Importantly, no peaks were observed at retention times corresponding to free tamoxifen or any of its major metabolites, which indicates the DNA extraction procedures employed are capable of efficiently removing any non-bound tamoxifen derivatives. In addition, there is no excess ^{14}C associated with the 2'-deoxynucleosides, which would elute around 3-4 min, confirming that the ^{14}C detected in DNA samples is not due to metabolic incorporation of this isotope into the nucleotide pool. Importantly, peak 2 elutes at a time consistent with the dG-N^2-tamoxifen adduct standard. The identity of the more polar species in peak 1 is not presently known as it does not coincide with any of the available tamoxifen derivatives analysed.

Based on results from the analysis of intact DNA from Patient 1 (described above), ~70 attomoles of ^{14}C was digested and loaded onto the HPLC column. The total amount of ^{14}C recovered from the column, calculated by summation of the ^{14}C content in all 40 HPLC fractions, was ~80 attomoles, which is in good agreement with the theoretical value and indicates the two peaks observed account for virtually all the excess ^{14}C detected by AMS analysis of the intact DNA, suggesting if samples were

contaminated with adducted protein it does not significantly contribute to the binding measured. Of this 80 attomoles, it can be estimated that ~ 9% was contained in the peak eluting where dG-N^2 tamoxifen would be expected, which translates to a damage level in the region of 1050 adducts/10^{12} nucleotides.

In order to ascertain whether the results obtained for patient 1 were representative of the response to [^{14}C]-tamoxifen administration, a number of 30 second fractions corresponding to the areas of interest (10-12.5 min and 22-25 min) from six of the other patients were also analysed. Due to the cost associated with AMS measurements it was not possible in this case to submit all 80 fractions for each patient. The partial chromatograms in Figure 5 illustrate considerable variation among individuals. Patient 4 has a single large peak corresponding in retention time to the dG-N^2-tam adduct; this was also a major peak in the digest from Patient 8, who had an additional unidentified peak eluting a couple of minutes before the adduct. The amount of ^{14}C contained in the adduct peak for patients 4 and 8 was ~84 and 58 attomoles, which equates to approximately 7000 and 4300 dG-N^2-tamoxifen adducts/10^{12} nucleotides respectively. In patients 3 and 7 no distinct peaks were evident, whilst very small peaks were apparent in patients 5 and 6 but these did not coincide with the dG-N^2-tamoxifen adduct standard, suggesting if such adducts were present they must be below the limit of detection. The discrepancy between excess total ^{14}C measured in intact DNA and that detected by HPLC-AMS for some patients may be due to the presence of multiple minor [^{14}C]-labeled components, eluting in areas of the chromatogram not selected for AMS analysis. These findings support the need to conduct such HPLC separations of adducted DNA for each compound under investigation when using AMS for adduct quantitation (24). Interestingly, none of the

other patients exhibited a peak eluting at the same time as the polar peak 1 detected in Patient 1, signifying this is not consistently formed as a result of tamoxifen intervention.

Discussion

We have shown for the first time, using the high sensitivity of AMS, the ability of tamoxifen to form DNA adducts in human colon tissue following a standard therapeutic or chemopreventive dose. It is known that postmenopausal estrogen treatment results in a reduced risk of colorectal cancer (31, 32). Epidemiological studies have not yet revealed an unequivocal link between the use of antiestrogenic tamoxifen therapy and colon cancers. While the original investigation of Rutquist *et al.* (5) showed an excess of gastrointestinal cancers associated with tamoxifen, the larger NSABP trial found no significant difference between the treatment arms in secondary colon cancers (1). The present study indicates a mechanistic route that could result in colon cancers but does not directly involve estrogen agonist or antagonist action.

In women treated chronically, tamoxifen is extensively distributed and steady state tissue concentrations are typically an order of magnitude higher than plasma levels (33). The plasma concentrations of tamoxifen equivalents in this study were consistent with published reports following a single dose (34, 27) and ~16-fold lower than tissue concentrations. In women undergoing hysterectomy, uterine concentrations after one 20 mg capsule of [^{14}C]-tamoxifen were approximately 3-fold lower than that attained in colon tissue (27). The relatively high tissue concentrations in the present study may be attributed to the fact the capsules were given orally, delivering the contents directly to the gastrointestinal mucosa. It is also possible that

although healthy tissue was taken, the presence of inflammatory disease in adjacent tissue could influence tamoxifen absorption.

Tamoxifen is metabolically activated via α-hydroxylation of the parent compound and other Phase I metabolites; a reaction catalysed by CYP3A4 in human tissues (13). Although in rat liver the proximate reactive species is considered to arise as a consequence of further sulfation, the alcohol itself shows intrinsic activity towards DNA albeit ~1600-fold weaker than the sulfate ester (16). The presence of detectable CYP3A4 protein in colon tissue of all 10 patients therefore indicates that α-hydroxytamoxifen may be formed *in situ* as well as occurring in the systemic circulation (35). The high levels of DNA binding in rat liver is due to sulfation of α-hydroxytamoxifen by an isoform of hydroxysteroid sulfotransferase, ST2A2 (36). Evidence suggests that α-hydroxytamoxifen is a poor substrate for the human form of the enzyme (SULT2A1) that produces in the region of 3-fold lower to undetectable levels of α-sulfate tamoxifen compared to rat hydroxysteroid sulfotransferase (37, 38). Furthermore, SULT2A1 activity in human colon, measured using dehydroepiandrosterone, is almost non existent relative to liver tissue (39). We therefore propose that the low levels of binding detected in colon tissues by AMS is primarily due to the direct interaction of α-hydroxytamoxifen with DNA, a reaction that may be enhanced by the slightly acidic environment in the colon (15). It follows that the level of adducts formed will be dictated by the concentration of tamoxifen and α-hydroxytamoxifen in the tissue, which will be influenced by tissue distribution and local CYP3A4 expression, balanced against the activity of detoxification pathways. Consistent with this idea is the strong positive correlation between total DNA binding and [^{14}C]-tamoxifen equivalents in the colon, with those patients having the greatest

tissue concentrations (1, 9 and 10) clearly exhibiting the highest levels of DNA binding.

Rats given α-hydroxytamoxifen orally for four days (47 mg/kg per day) show hepatic DNA damage but surprisingly, none was detected in the colon (40). Failure to detect DNA adducts may be partly associated with the relatively lower sensitivity of ^{32}P-postlabeling methods used. In contrast, tamoxifen adducts have been detected in multiple organs (brain, liver, uterus, ovary and kidney) of Cynomolgus monkeys after daily tamoxifen dosing (2 mg/kg) for a month (41, 42). This supports the potential for tamoxifen to act as a genotoxin in the colon of treated women.

HPLC-AMS analysis of digested DNA samples provides evidence indicating the [^{14}C]-radiolabel in intact DNA was due to the presence of covalently bound tamoxifen. Under steady state conditions tamoxifen is >98% reversibly bound to plasma proteins (33) and reactive metabolites of tamoxifen are also capable of binding covalently to proteins. Minor amounts of protein contamination (~2%) were detected in extracted non-digested DNA samples. This suggests that binding of [^{14}C]-tamoxifen to protein could conceivably contribute to the excess ^{14}C measured above background levels by AMS. However, for it to entirely account for the ^{14}C content, the extent of protein adduct formation would have to be at least ~ 44 to 50-fold higher than DNA adduct formation. This is unlikely considering previous data comparing [^{14}C]-tamoxifen binding in uterine tissue, in which total protein binding was only ~14 times higher than DNA binding (27). Furthermore, after DNA samples were digested to nucleosides, they were filtered to remove enzymes and any partially hydrolysed DNA or protein contaminants prior to injection on the HPLC system. Whilst it is

feasible that very small molecular weight adducted peptides, < 20 amino acids in length, may pass through the filter, if such species were present they would be expected to be as a mixture of peptide adducts rather than a single entity and therefore produce a broad HPLC peak. It is therefore extremely unlikely that tamoxifen adducted peptides would account for a sharp, discrete [^{14}C]-radiolabeled peak as seen in some of the HPLC-AMS runs. Another possible source of non-covalently bound radiolabel in intact DNA might be free tamoxifen or its metabolites. However, AMS analysis of the entire HPLC run of digested DNA from patient number 1 confirmed that all non-bound tamoxifen and its metabolites were efficiently extracted during isolation and therefore do not contribute to the [^{14}C]-radiolabel measured. After a single dose, [^{14}C]-tamoxifen would be expected to be the predominant circulating species in plasma (34) and account for the majority of [^{14}C]-radiolabel measured in whole tissue and plasma. All known tamoxifen metabolites are more polar than the parent drug and should therefore be more readily removed by the aqueous extraction methods employed. The absence of a peak corresponding in retention time to tamoxifen in the chromatogram, particularly in this patient, who had one of the highest concentrations of [^{14}C]-tamoxifen equivalents in the colon, is important as it suggests that none of the peaks detected in other patients are due to non-bound derivatives. Two peaks of radioactivity were evident in the radiochromatogram for patient 1, the later eluting peak corresponding in retention time to the dG-N^2-tamoxifen adduct standard and equivalent to a damage level of 1 adduct/10^9 nucleotides. This peak was also detected in DNA digests from patients 4 and 8 at a level of approximately 7 and 4 dG-N^2-tamoxifen adducts/10^9 nucleotides respectively. However, the dG-N^2-tamoxifen adduct, if present, was below the limit of detection (~ 0.4 adducts/10^9 nucleotides) in the other four patients. The limit of detection for dG-

N^2-tamoxifen adducts by conventional LC-MS/MS analysis is 0.2 adducts/10^8 nucleotides, 10-fold higher than our HPLC-AMS assay (43). Given the relatively high adduct levels detected in two of the patients, colon DNA binding may have also been detectable using LC-MS/MS. Inter-individual variability in the extent of α-hydroxytamoxifen DNA binding and/or the efficiency of adduct removal is consistent with data from Shibutani *et al*. and Umemoto *et al*.. These groups reported tamoxifen adducts in endometrial DNA from only 8 out of 16 women and adducts in leukocytes from 6 out of 47 breast cancer patients (21, 22).

The identities of the other peaks visible in the reconstructed radiochromatograms from patients 1 and 8 are not currently known. As discussed above, it is reasonable to rule out both Phase I and II tamoxifen metabolites together with adducted protein and large peptides. Remaining possibilities include nucleoside DNA adducts of a different structure, such as α-(N^6-deoxyadenosinyl)-tamoxifen (44) or dG-N^2-*N*-desmethyltamoxifen, a di/trinucleotide adduct resistant to hydrolysis or unknown degradation products.

Interestingly, the urinary metabolite profile of the dietary carcinogen 2-amino-1-methyl-6-phenyimidazo[4,5-*b*]pyridine (PhIP) has recently been described as a possible predictor of DNA adduct levels in colon tissue of humans administered a dietary relevant dose of [2-^{14}C]-PhIP (45). PhIP is bioactivated by CYP1A2 to 2-*N*-hydroxy-PhIP, which is subsequently esterified, producing the ultimate DNA reactive species, *O*-sulfonyl or *O*-acetyl esters. As with [^{14}C]-tamoxifen binding observed in the present study, the levels of PhIP DNA adducts in the colon displayed a degree of variability but more importantly, they were found to negatively correlate with the

urinary concentration of 2-*N*-hydroxy-PhIP-N^2-glucuronide. This suggests that glucuronylation is a significant detoxification pathway for *N*-hydroxy-PhIP in humans. *O*-Glucuronylation of α-hydroxytamoxifen also serves as a species-specific protective mechanism in humans, as illustrated by the fact that human liver microsomes catalyse this deactivation reaction at a rate >50 times more rapid than rat microsomes (46). Differences in the expression and activity of glucuronyltransferases either in the liver or locally in colon tissue, may therefore contribute to the different tamoxifen adduct levels detected in the ten patients.

The significance of potentially mutagenic tamoxifen-induced DNA damage in the development of colon cancer is likely to be dependent on numerous factors including DNA sequence context, efficiency of adduct repair and rates of cell proliferation. However, in considering the level of damage, it is worthwhile noting that in the present study, where dG-N^2-tamoxifen adducts were detected, binding was in the order of ~10-100 times higher than that reported for other suspect human colon carcinogens, MeIQx, PhIP and benzo[*a*]pyrene (47-49). Results suggest further epidemiological studies on the association between tamoxifen and colon cancer are warranted.

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Figure Legends

Figure 1. Concentrations of total [^{14}C]-tamoxifen equivalents in colon tissue of patients given 20 mg (1.85 MBq) [^{14}C]-tamoxifen prior to surgery (100 mg tissue). Radioactivity was measured by liquid scintillation counting and results presented are from a single analysis.

Figure 2. Western blot showing expression of CYP3A4 protein in colon tissue of [^{14}C]-tamoxifen treated women. Equal amounts of protein (50 μg) were separated on SDS 7.5% polyacrylamide gels together with recombinant human CYP3A4 standards. After electrophoresis, proteins were transferred onto nitrocellulose filters and incubated with anti-human CYP3A4 antibody. Immunoreactive proteins were detected by an anti-mouse peroxidase-coupled secondary antibody. Chemiluminescence was quantitated using Syngene image analyser. Results presented are from a single analysis. (**A**) Lanes 1 to 10 show the presence of CYP 3A4 protein in colon extracts of all 10 patients. Std represents human recombinant CYP3A4 (0.1 pmole). (**B**) Semi-quantitation of the chemiluminescence intensity of bands shown in A, relative to CYP3A4 standards.

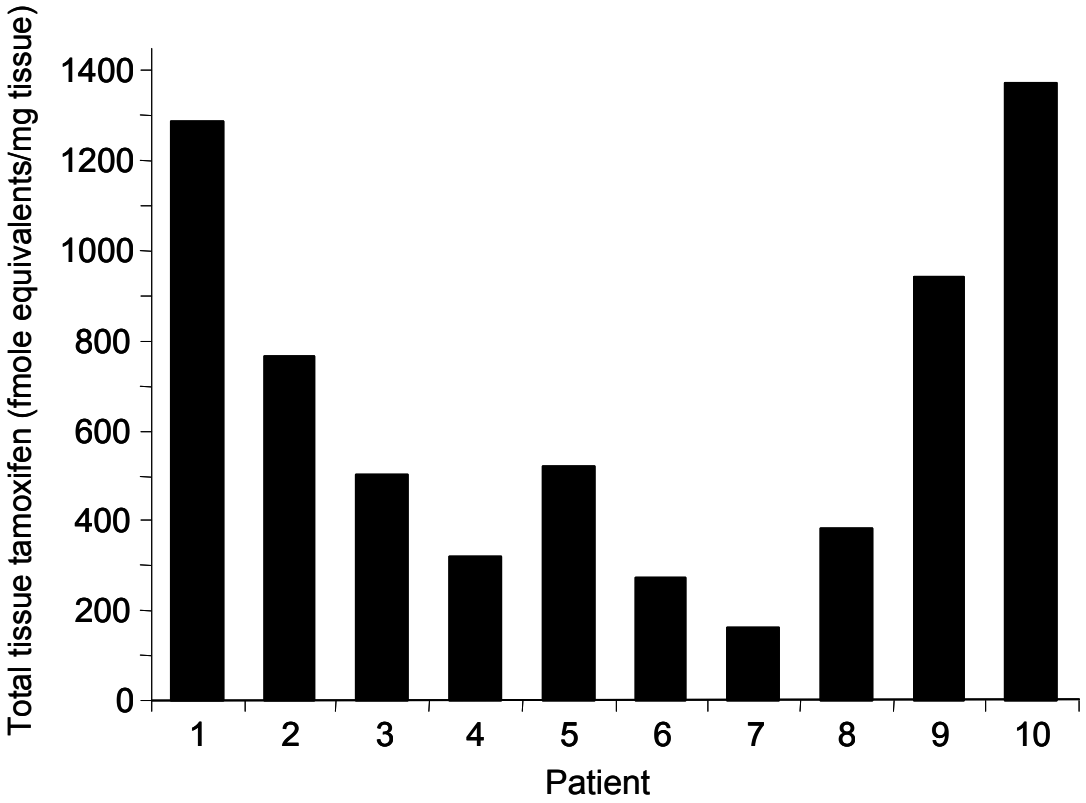
Figure 3. Radiocarbon content of colon DNA samples from women who received a single 20 mg (1.85 MBq) dose of [^{14}C]-tamoxifen prior to surgery. The natural background level of ^{14}C in DNA from a control, untreated patient is indicated by the dashed line (46.3 attomoles/mg DNA). The highest radiocarbon content was found in DNA from patient 10 (834 attomoles/mg DNA, which translates to an adduct level of 12185 adducts/10^{12} nucleotides), whilst the lowest concentration was measured in patient 8 (169 attomoles/mg; equivalent to 2472 adducts/10^{12} nucleotides). Each DNA

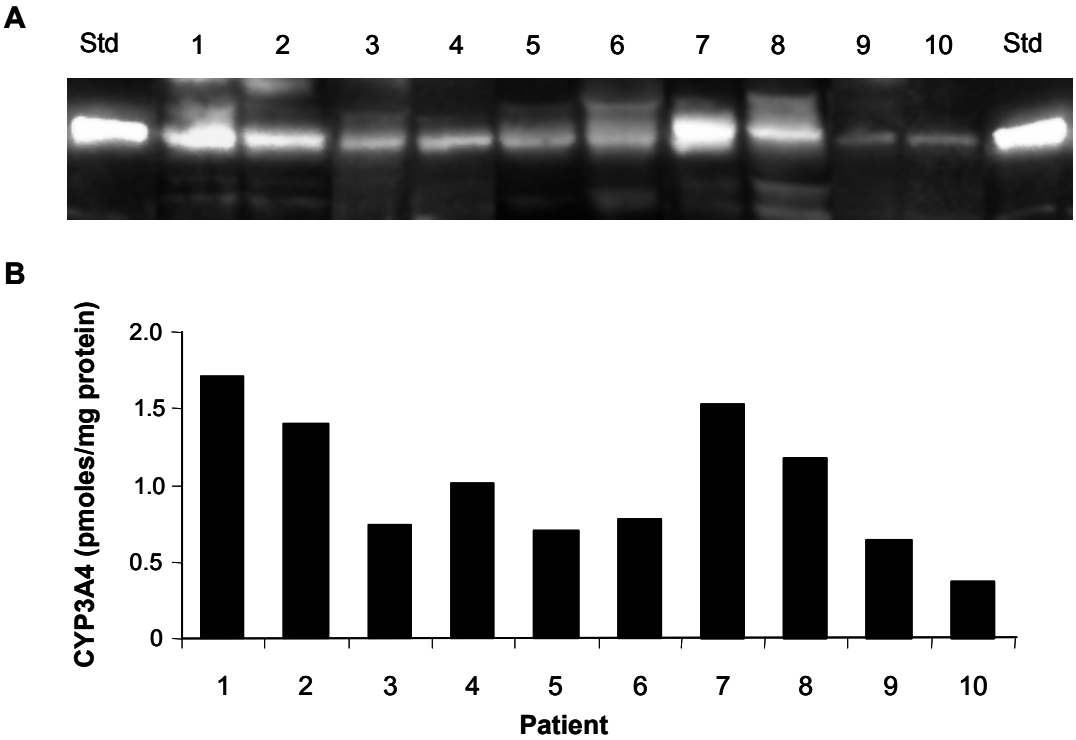
sample (500 μg) was analysed on a single occasion by AMS after conversion to graphite. The error bars represent the AMS measurement error, since each graphite sample is analysed up to seven times or until the measurement variation was within ± 5%. All values are significantly above the background ^{14}C content in control DNA ($p < 0.005$).

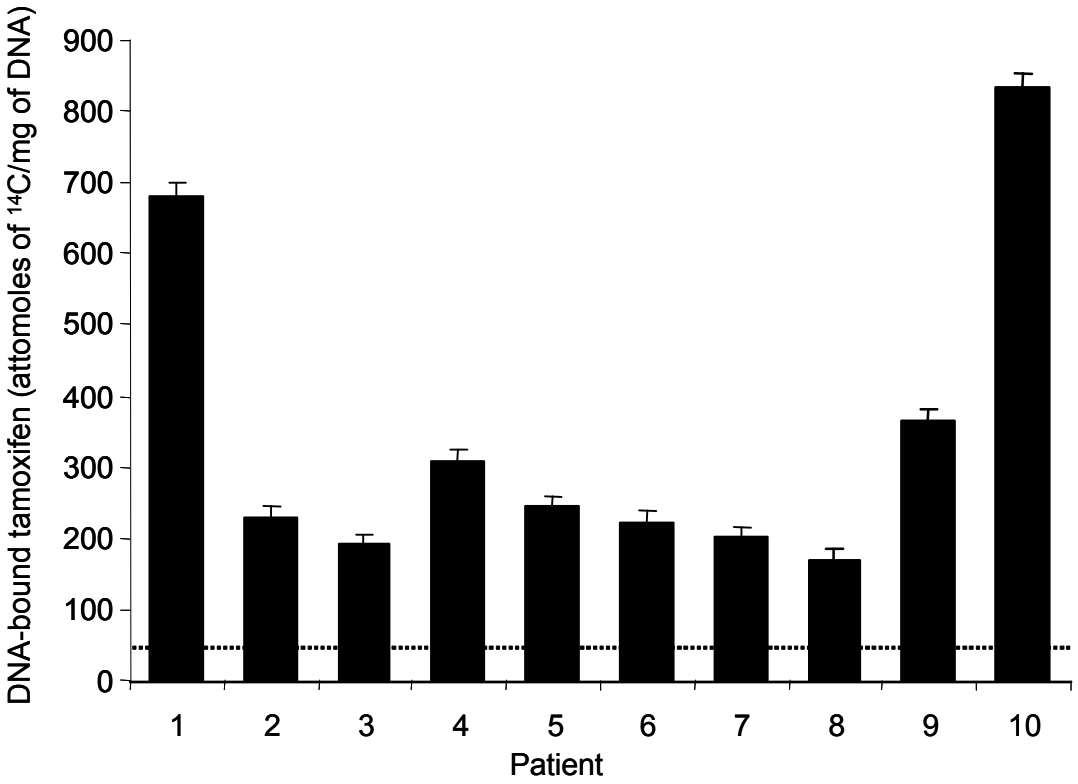
Figure 4. HPLC-AMS analysis of digested colon DNA. Panels corresponds to: (**A**) Reconstructed chromatogram of digested DNA (200 μg) from [^{14}C]-tamoxifen treated Patient 1 and (**B**) digested DNA (200 μg) from a control, unexposed patient. In each case fractions were collected at 30 s intervals throughout the run and the entire HPLC run (80 fractions) was submitted for analysis. Half of each HPLC fraction (250 μL) was converted to graphite and analysed for radiocarbon content by AMS. The contribution of tributyrin to the total measured ^{14}C was subtracted and mean radiocarbon content expressed as attomoles of ^{14}C per HPLC fraction. (**C**) Shows a dG-N^2-tamoxifen adduct standard. The inset panel is a product ion scan of the $[M+H]^+$ molecular ion at m/z 637 analysed by ESI-MS/MS, which confirms the adduct structure. (**D**) Shows the separation of tamoxifen and its major metabolites (30 μg of each) together with 2'-deoxynucleosides liberated from digested control DNA (50 μg). All standards were analysed immediately after the digested colon DNA samples were run.

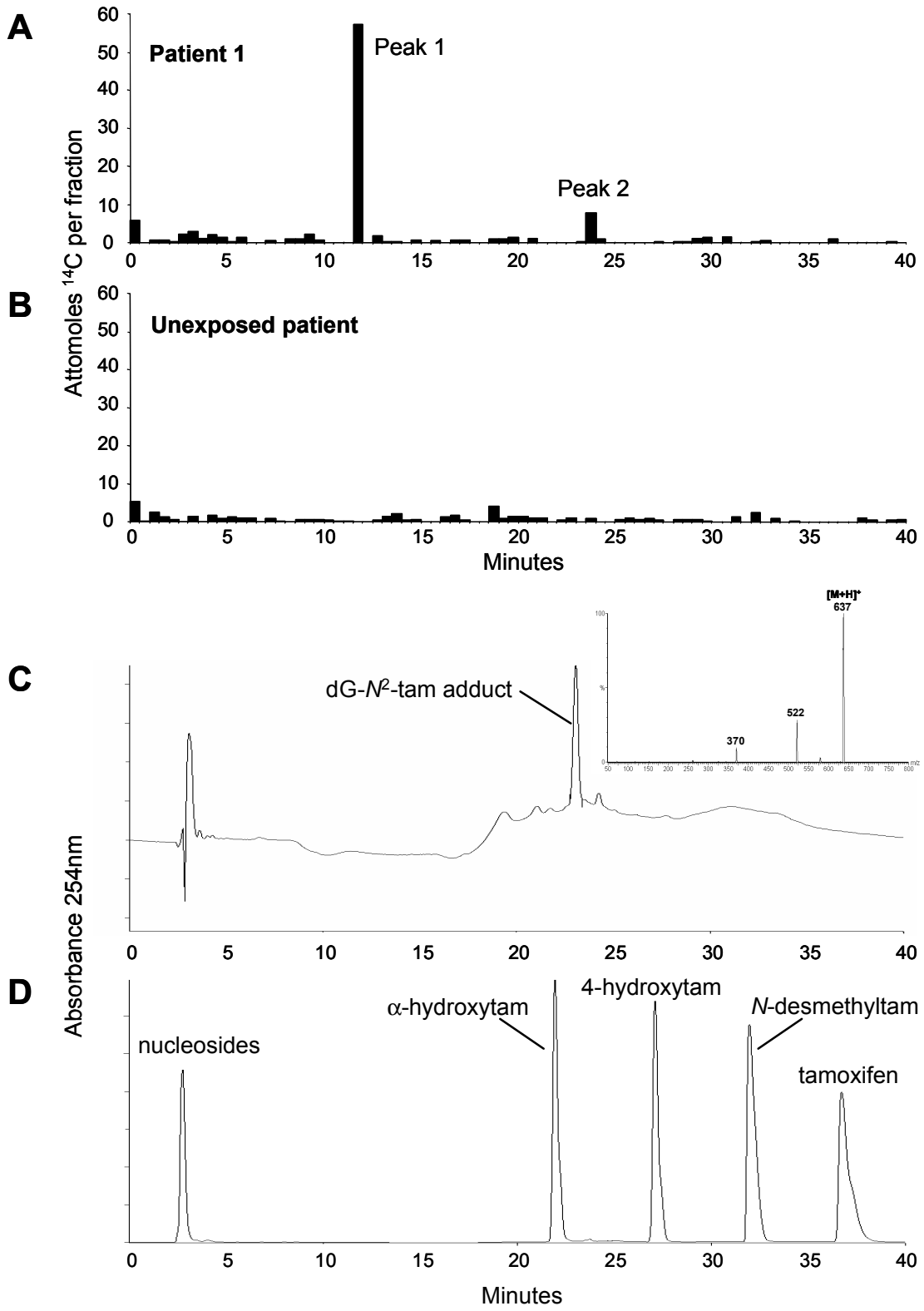
Figure 5. Partial reconstructed chromatograms of DNA digests from [^{14}C]-tamoxifen treated patients (3, 4, 5, 6, 7, and 8) separated by HPLC and analysed by AMS. DNA (150-200 μg) was digested and injected onto the HPLC. Fractions were collected at 30 second intervals throughout the run and selected samples were submitted for

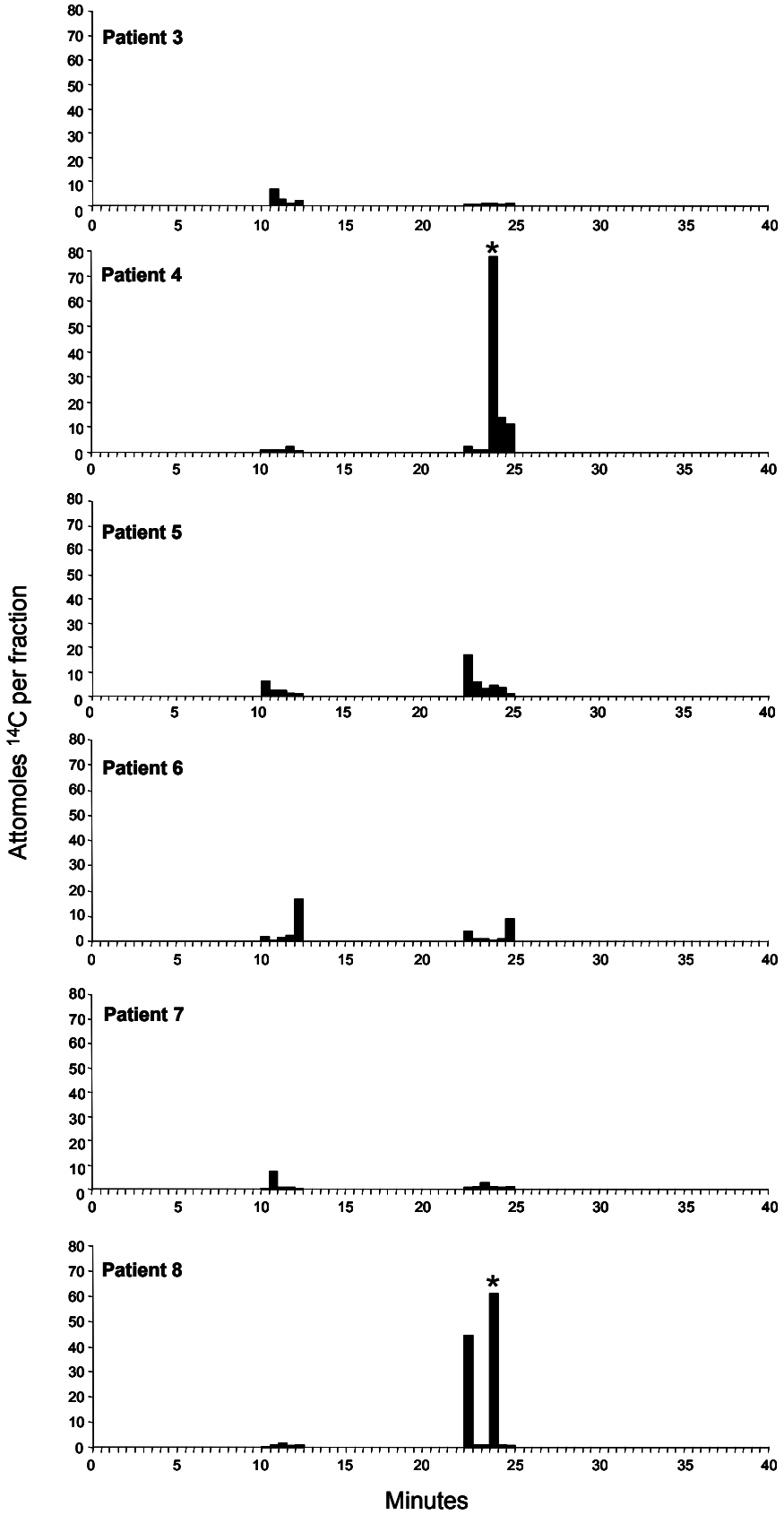
analysis (10-12.5 min, 5 fractions and 22-25 min, 6 fractions). Each HPLC fraction (500 μL) was converted to graphite and analysed for radiocarbon content by AMS. After subtraction of the contribution of tributyrin carrier to the total measured ^{14}C the mean radiocarbon content is expressed as attomoles of ^{14}C per HPLC fraction. Peaks eluting at a time consistent with the dG-N^2-tamoxifen adduct standard are indicated by an asterisk (*).


Total tissue tamoxifen (fmole equivalents/mg tissue)
1400
1200
1000
800
600
400
200
0
1
2
3
4
5
6
7
8
9
10
Patient


A
Std
1
2
3
4
5
6
7
8
9
10
Std
B
CYP3A4 (pmoles/mg protein)
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
7
8
9
10
Patient


DNA-bound tamoxifen (attomoles of 14C/mg of DNA)
900
800
700
600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10
Patient


A
Patient 1
Peak 1
Peak 2
B
Unexposed patient
Attomoles 14C per fraction
Minutes
C
dG-N2-tam adduct
[M+H]+
637
522
370
Absorbance 254nm
D
nucleosides
α-hydroxytam
4-hydroxytam
N-desmethyltam
tamoxifen
Minutes


Patient 3
Patient 4
Patient 5
Patient 6
Patient 7
Patient 8
*
*
Attomoles 14C per fraction
Minutes
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
80