Postlabelling Methods for Detection of DNA Adducts
Ed. D.H.Phillips, M. Castegnaro & H. Bartsch
Lyon, International Agency for Research on Cancer
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An improved ^{32}P-postlabelling assay for detection and quantitation of styrene 7,8-oxide–DNA adducts

S. Cantoreggi[1], R.C. Gupta[2] and W.K. Lutz[1]

[1]*Institute of Toxicology, Swiss Federal Institute of Technology and University of Zürich, CH-8603 Schwerzenbach, Switzerland*

[2]*Preventive Medicine, and Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40506, USA*

Summary. Using DNA modified with [7-^{3}H]styrene 7,8-oxide (SO) *in vitro* we have standardized the ^{32}P-postlabelling assay for detecting SO–DNA adducts. Nuclease P1-enriched adducts were ^{32}P-labelled and purified by high-salt (4.0 M ammonium formate, pH 6.1) C_{18} reverse-phase TLC. After elution from the layer with 2-butoxyethanol:H_2O (4:6), adducts were separated by two-dimensional PEI cellulose TLC in non-urea solvents (2.0 M ammonium formate, pH 3.5, and 2.7 M sodium phosphate, pH 5.6). One major, three minor and several trace adducts were detected. The efficiency of the kinase reaction depended on the ATP concentration. Use of standard labelling conditions ([γ-^{32}P]ATP, $\leq$3000 Ci/mmol; $\leq$2 μM) resulted in poor (4–7%) adduct recovery. An ATP concentration of 40 μM, however, increased the labelling efficiency by a factor of 5–8 (35–55% based on ^{3}H-SO labelled DNA). The results indicate that the new separation technique is suitable for the relatively polar SO–DNA adducts and that high labelling efficiency can be achieved.

Introduction

Styrene 7,8-oxide (SO) is the major metabolite of styrene, a monomer widely used in the production of plastics and rubber products. It is mutagenic in a number of test systems (Barale, 1991) and carcinogenic in the forestomach of both rats and mice following oral dosing (Maltoni *et al.*, 1979; Ponomarkov *et al.*, 1984; Lijinsky, 1986). Molecular mechanisms of the genotoxicity of SO have been examined both *in vivo* and *in vitro*. DNA adduct formation *in vivo* has been investigated in forestomach DNA isolated from rats after oral administration of 3.2×10^{9} d.p.m./kg (1.7 mg/kg) tritiated SO (Cantoreggi & Lutz, 1992). In this model, no DNA-binding was detected within the limits of the assay, i.e., 4 adducts/10^{8} nucleotides. Incubation of SO with nucleotides (Savela *et al.*, 1986) or DNA (Vodicka & Hemminki, 1988), resulted in the formation of guanine adducts with N-7 as the primary site of attack. In a ^{32}P-postlabelling assay, the N^{2}- and O^{6}-guanine adducts were found to label rather poorly and the predominant N-7-guanyl adducts were barely detectable when carrier-free [γ-^{32}P]ATP was used (Vodicka & Hemminki, 1991). Bodell and co-workers also described postlabelling analysis of *in vitro* modified DNA (Liu *et al.*, 1988a). Two minor adducts were identified as O^{6}-guanine-derived, but the labelling efficiency was not determined. The same

laboratory also reported the presence of DNA adducts in the peripheral lymphocytes of one exposed individual (Liu *et al.*, 1988b). The aim of our study was to develop an efficient and reproducible postlabelling assay for the detection of SO–DNA adducts.

Material and methods

Chemicals

^{3}H-SO (103 Ci/mol) was purchased from The Radiochemical Centre, Amersham, UK. A stock solution (specific activity 1 Ci/mol) was prepared by adding unlabelled SO.

Incubation of ^{3}H-SO with DNA

Herring sperm DNA (20 mg) was dissolved in 10 ml 10 mM Tris-HCl, pH 7.4, and incubated for 20 h at 37 °C with 114 µl (1 mmol; 100 µCi) ^{3}H-SO. DNA was purified to constant specific activity by ether extraction, hydroxyapatite chromatography, dialysis and repeated ethanol precipitation.

^{32}P-Postlabelling assay

Adducts were analysed by a modified ^{32}P-postlabelling assay adapted from the procedure described by Gupta *et al.* (1991). Control and SO-treated DNA samples (20 µg) were enzymatically hydrolysed to the 3′-deoxynucleotides (enzyme : substrate, 1:2, 37°C, 3 h), and the adducts enriched using nuclease P1 (Reddy & Randerath, 1986) (enzyme : substrate, 1:3, pH 6, 37°C, 40 min). To 7.5 µl nucleotide solution (corresponding to 3 µg DNA) was added a 7.5 µl aliquot of a radioactive mix containing 2 µl buffer mix (125 mM bicine (pH 9.5), 50 mM $MgCl_2$, 50 mM dithiothreitol, 5 mM spermidine), 2.2–5.0 µl of carrier-containing [γ-^{32}P]ATP (80 µCi, final concentration: 2 µM (2700 Ci/mmol) or 60 µM (90 Ci/mmol)), 0.1 µl T4 polynucleotide kinase (30 U/µl) and 0.4–3.2 µl water. The reaction mixture was incubated for 45 min at room temperature. Complete separation of SO–DNA adducts was accomplished by a combination of C_{18} reverse-phase and PEI-cellulose TLC as described previously (Gupta *et al.*, 1991). Labelled adducts (0.5 µg) were applied to a 0.4 M ammonium formate (pH 6.2)-prewashed C_{18} thin layer (10 cm × 10 cm), and developed overnight in 4 M ammonium formate, pH 6.1, onto a Whatman No.1 paper wick protruding outside the tank. After the plate was dry, the C_{18} layer containing the adducts (1 × 4 cm) was wetted with water using a cotton swab, scraped off and extracted twice with 600 µl each of 2-butoxyethanol : water (4:6) at room temperature for 20 min with continuous agitation. The combined eluate was dried under vacuum and reconstituted in 30 to 100 µl of water, and an aliquot (10 µl) was chromatographed on a 100 mM ammonium formate (pH 3.5)-prewashed PEI-cellulose thin layer (10 × 13 cm). Development was in 2.0 M ammonium formate, pH 3.5 (D1), and 2.7 M sodium phosphate, pH 5.6, 1–2 cm onto a Whatman No. 1 wick (D2). The chromatogram was briefly washed in water (30 seconds) and dried with lukewarm air before the D2 development. Adduct spots were detected by intensifying screen-enhanced autoradiography at –80 °C and quantitated by Cerenkov counting of the excised areas of the chromatograms. The determinations were carried out in at least duplicate.

Results

The aim of this study was to standardize the ^{32}P-postlabelling assay for SO–DNA adducts. DNA was incubated with radiolabelled SO *in vitro* to obtain a substrate with

a defined adduct level so as to allow assessment of the ^{32}P-labelling efficiency. Under the conditions of the experiment, a relative adduct level of 1.0 adducts/10^3 nucleotides was achieved. Analysis of samples by C_{18} TLC in a relatively high-salt solution (4 M ammonium formate, pH 6.1) produced a "bullet"-shaped retention of adduct radioactivity (Figure 1). Lower salt concentrations resulted in significantly lower adduct recoveries.

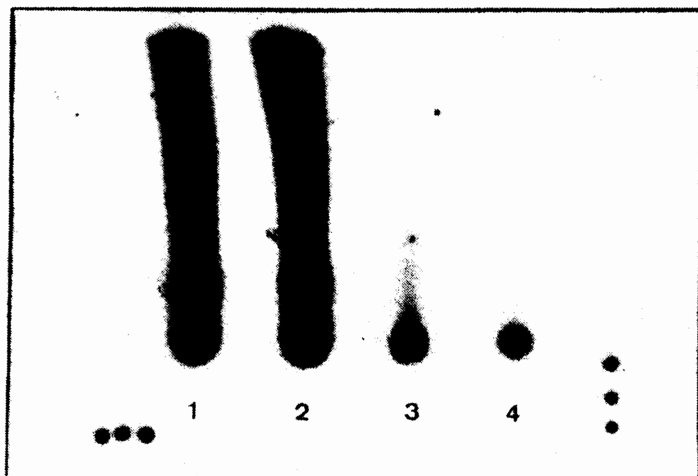

Figure 1. Ascending one-dimensional separation of ^{32}P-postlabelled adducts of DNA reacted *in vitro* with SO on a C_{18} reverse-phase plate
Lanes 1 and 2, SO–DNA; lanes 3 and 4, control DNA.

When the adducts were eluted from the C_{18} layer, usually 80–90% of the ^{32}P-radioactivity was recovered. Complete resolution of the adducts was accomplished by means of a two-directional PEI-cellulose TLC using non-urea solvents: 2 M ammonium formate, pH 3.5 (D1) and 2.7 M sodium phosphate, pH 5.6 (D2). SO–DNA showed one major (No.1), three minor (Nos 2, 3, 4) and several trace adducts (Figure 2B), as compared to control DNA (Figure 2A).

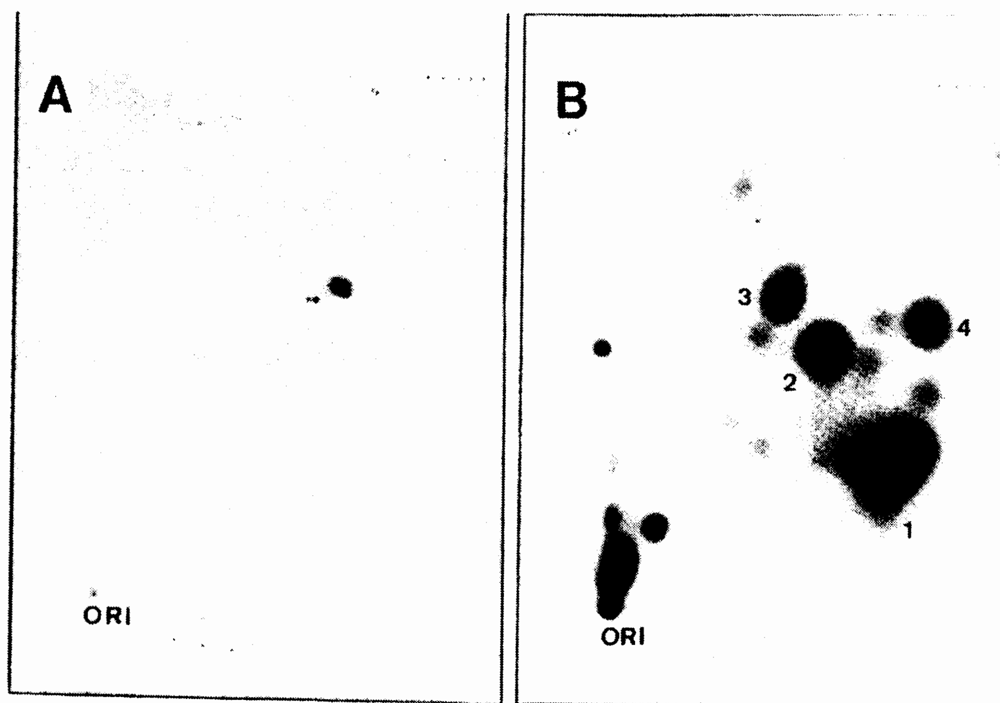

Figure 2. ^{32}P fingerprints of SO–DNA adducts
A, DNA treated with solvent only; B, SO–DNA.

Measurements of the adduct radioactivity revealed that the four major adducts comprised over 95% of the postlabelled adducts (Spot 1 = 73%; spot 2 = 14%; spot 3 = 5.5%; spot 4 = 4.1%). The adduct labelling efficiency was found to be strongly dependent on the ATP concentration. Whereas under standard conditions (2 μM ATP) the labelling efficiency was rather poor (4–7% based on ^{3}H-SO–DNA), an ATP concentration of 40 μM increased it by a factor of 5–8 (35–55%). Further increase in the ATP concentration, however, was found to be inhibitory.

Discussion

The new TLC separation technique described previously for diaziquone-DNA adducts was adaptable to SO-DNA adducts with minor modifications.

The first purification step, involving chromatography on a C_{18} layer with high ammonium formate concentration (4 M), was fully applicable to SO-adducted DNA. The subsequent separation on a PEI-cellulose layer required a combination of non-urea salt solutions (2 M ammonium formate, pH 3.5; 2.7 M sodium phosphate, pH 5.6). The buffer used in the first direction for diaziquone adducts (0.4 M ammonium formate, pH 3.3) had insufficient ionic strength to migrate SO–DNA adducts, whereas that used for the second direction (3.0 M sodium phosphate, pH 5) was almost directly applicable. The results suggest that the new methodology may be of general use for the separation of relatively polar adducts, although some adjustment to electrolyte concentration may have to be made depending upon the nature of the adducts.

The second major finding of the present investigation is that the concentration of ATP is crucial to achieve good labelling efficiency of SO-adducted DNA. This is in contrast to recent findings by Vodicka and Hemminki (1991) and is important in terms of the applicability of the assay for biomonitoring for exposure to styrene. Further development of the assay is needed to assign the spots to specific adducts and to investigate the behaviour of selected marker adducts.

Acknowledgements

We thank Karen Earley and Marietta Weber for their technical assistance.

This work was supported by BASF, Ludwigshafen, Germany (S.C. and W.K.L.) and US EPA Cooperative Agreement CR-813840 (R.C.G.).

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