Application of HPLC to the Isolation of Molecular Targets in Dosimetry Studies

by Rod Balhorn,* J. A. Mazrimas,* and Michele Corzett*

**Introduction**

Exposure to genotoxic agents may be detected by a variety of somatic and germinal assays in animals and humans, but the biological significance of the observed changes, as measured in vivo or in vitro, often remains uncertain. One reason for this is that we cannot always define the actual dose of chemical received by specific target molecules. It is essential, therefore, that we develop and routinely apply dosimetric techniques together with these bioassays. This will permit us to define both the extent of damage sustained by specific target molecules and the rate and efficiency of damage repair. By employing dosimetric techniques that can be applied to any tissue, comparisons may be made relating dose to different biological endpoints. Such studies would also provide the common denominator (target dose) required for comparisons among species exposed to similar substances.

One reason that dosimetry measurements are not made an integral part of most exposure/endpoint studies is that target isolations and binding measurements are often labor-intensive. Conventional methods for analyses of DNA adduct formation, for example, require numerous steps to isolate and purify DNA prior to the analysis of bound DNA adducts. Another reason is that few target molecules other than DNA have been extensively characterized with regard to their dose response in exposed animals.

Recent improvements in high-performance liquid chromatography (HPLC) and column packagings make it possible to rapidly separate a variety of macromolecules from exposed animals. We have used HPLC to facilitate the isolation of selected target macromolecules from the tissues of mice exposed to radiolabeled chemical mutagens and quantify the actual dose of chemical that interacts with these molecules. While the work that is described here employs radiolabeled mutagens and scintillation counting to quantify adduct formation, the DNA and proteins provided by these separations may also be analyzed by other techniques that permit direct analyses of adducts and do not require exposures to radioactive compounds.

**Isolation of Molecular Targets**

The techniques described here emphasize the isolation of macromolecules from two types of targets: molecules that compose or are closely associated with the genome (DNA, histone) and proteins in the blood (hemoglobin, albumin) that can be obtained easily and ana-
lyzed as dosimeters of recent chemical exposure.

While the ultimate target of mutagens and carcinogens is generally accepted to be DNA, other macromolecules closely associated with DNA may also sustain damage that alters the accessibility or genetic activity of specific chromosomal domains. Chromosomal proteins such as the histones and high mobility group (HMG) proteins are intimately associated with DNA and appear to modulate the packaging of the genome and its accessibility for transcription (1–3). The histones are synthesized coincident with DNA replication and are as stable, biochemically, as DNA itself (4–6). While the HMG proteins, in contrast, do turn over (7,8), two of these proteins appear to maintain genetically active regions of the chromosome in an open, accessible conformation (2,8).

Other targets of potential interest are hemoglobin and serum albumin. Adduct formation to hemoglobin has been considered for use in dosimetry studies as a means of monitoring exposure dose (9–11). Albumin, a natural scavenger and carrier of metals, ions, proteins, fatty acids, antibiotics, sugars, lipids, hormones and a variety of other molecules in serum (12–14) should also be considered. These proteins are excellent nucleophiles, and both may be isolated from blood in reasonable quantities.

**DNA Isolation by Gel Permeation Chromatography**

DNA and a number of chromosomal proteins may be isolated from tissue or cultured cells simultaneously. The entire procedure requires approximately an hour to perform and permits the isolation of from a few micrograms to over a milligram of DNA per sample. If both DNA and chromosomal proteins are to be isolated from the same sample, a brief nuclear isolation step must be performed to eliminate contaminating ribosomal and cytoplasmic proteins. Tissues (or cultured cells) are Virtis-homogenized in a medium (grinding medium: 0.25 M sucrose, 10 mM Tris, pH 8, 2.5 mM magnesium chloride) which stabilizes the nuclear membrane, and the nuclei are purified by centrifugation (10 min) through 1 M sucrose containing the detergent Triton X-100. After rinsing in grinding medium, the nuclear pellet is dissolved in 3 M guanidine hydrochloride, sonicated (to reduce DNA viscosity), and centrifuged to remove insoluble material. If only DNA is to be isolated, the nuclear isolation step may be eliminated and the cells or tissues dissolved directly in 3 M guanidine hydrochloride.

DNA and chromosomal protein are then separated by chromatography of the dissociated nuclei on a TSK-3000 SW column (three 7.5 mm × 300 mm columns connected in tandem) in 1 M guanidine hydrochloride, 50 mM phosphate buffer pH 6.5. At a flow rate of 1.0 mL/min, separation of the DNA and chromosomal protein peaks (Fig. 1) is complete in less than 40 min. RNA, a minor contaminant in these preparations (0.35%), elutes midway between the DNA and protein peaks. By judi-

![Figure 1](image1.png)

**FIGURE 1.** Gel-permeation chromatography of dissociated nuclei. HPLC of dissociated mouse liver nuclei was performed on a TSK-3000SW column in 1 M guanidine hydrochloride as described in the text.

![Figure 2](image2.png)

**FIGURE 2.** Reverse-phase separation of histones and HMG proteins. The lyophilized protein peak obtained following GPC chromatography of dissociated nuclei was dissolved in 0.1% trifluoroacetic acid and rechromatographed on a reverse-phase PRP-1 column. Separation of the HMG proteins and individual histones was accomplished using the following multistep acetonitrile gradient: 35–68% buffer B, 8 min; 68–75% buffer B, 35 min; 75–100% buffer B, 4 min; 100% buffer B, 6 min; 100–35% buffer B, 1 min. Buffer A: 0.1% trifluoroacetic acid. Buffer B: 60% acetonitrile in 0.1% trifluoroacetic acid.

The lyophilized protein peak obtained following GPC chromatography of dissociated nuclei was dissolved in 0.1% trifluoroacetic acid and rechromatographed on a reverse-phase PRP-1 column. Separation of the HMG proteins and individual histones was accomplished using the following multistep acetonitrile gradient: 35–68% buffer B, 8 min; 68–75% buffer B, 35 min; 75–100% buffer B, 4 min; 100% buffer B, 6 min; 100–35% buffer B, 1 min. Buffer A: 0.1% trifluoroacetic acid. Buffer B: 60% acetonitrile in 0.1% trifluoroacetic acid.

![Figure 3](image3.png)

**FIGURE 3.** Reverse-phase separation of histones and HMG proteins. The lyophilized protein peak obtained following GPC chromatography of dissociated nuclei was dissolved in 0.1% trifluoroacetic acid and rechromatographed on a reverse-phase PRP-1 column. Separation of the HMG proteins and individual histones was accomplished using the following multistep acetonitrile gradient: 35–68% buffer B, 8 min; 68–75% buffer B, 35 min; 75–100% buffer B, 4 min; 100% buffer B, 6 min; 100–35% buffer B, 1 min. Buffer A: 0.1% trifluoroacetic acid. Buffer B: 60% acetonitrile in 0.1% trifluoroacetic acid.

![Figure 4](image4.png)

**FIGURE 4.** Reverse-phase separation of histones and HMG proteins. The lyophilized protein peak obtained following GPC chromatography of dissociated nuclei was dissolved in 0.1% trifluoroacetic acid and rechromatographed on a reverse-phase PRP-1 column. Separation of the HMG proteins and individual histones was accomplished using the following multistep acetonitrile gradient: 35–68% buffer B, 8 min; 68–75% buffer B, 35 min; 75–100% buffer B, 4 min; 100% buffer B, 6 min; 100–35% buffer B, 1 min. Buffer A: 0.1% trifluoroacetic acid. Buffer B: 60% acetonitrile in 0.1% trifluoroacetic acid.
inates. The DNA peak is processed directly, while the protein peak must be dialyzed against water, lyophilized and rechromatographed to separate specific proteins.

DNA may be isolated from any cell or tissue by this method. Recoveries of DNA and histone from the column typically exceed 95%. Only mammalian sperm require special treatment. The disulfide bridges in the chromosomal proteins associated with the DNA in this cell (protamine) must first be reduced with dithiothreitol or mercaptoethanol and the chromatography performed in 2 M guanidine hydrochloride. The protamines cannot be dissociated from DNA without this reduction and additional guanidine hydrochloride is required to keep the protamine and DNA dissociated and soluble.

Reverse-Phase Separation of Chromosomal Proteins

Further separation and isolation of specific histones and nonhistone chromosomal proteins requires rechromatography of the protein peak on a reversed-phase column. The lyophilized protein is dissolved in aqueous trifluoroacetic acid (TFA, 0.1%) and injected into a 10 μm PRP-1 column (two 7.5 mm × 300 mm columns connected in tandem; Hamilton Co., Reno, NE). Separation of the individual histones is accomplished by using a multistep acetonitrile gradient (Fig. 2). The HMG proteins elute early, just prior to H1 histone, while the core mouse histones elute in the order H2B, H2A, H4, and H3. Only the histones and HMG proteins are present in sufficient quantities to allow analyses of mutagen binding at exposure levels which result in the formation of less than one DNA adduct per 10^9 nucleotides.

Isolation of Hemoglobin and Albumin

Mouse hemoglobin and albumin are obtained by HPLC of whole blood lysates. Aliquots of mouse blood (100 μL) are added to 1.0 mL sodium citrate (1%) and promptly frozen at -20°C. After thawing and centrifuging, 0.5 mL samples are chromatographed on a 7.5 mm × 300 mm Poly Cat A cation exchange column (Custom LC, Inc, Houston, TX) by using a multistep sodium chloride gradient (Fig. 3). Under these conditions, albumin and hemoglobin separate as individual peaks in less than 20 min. The hemoglobin peak is a composite of at least four peaks; these represent sequence variants of hemoglobin that readily separate on this column.

Sample Processing

Following their isolation, the concentration of chromosomal protein or DNA is determined from the absorbance of the sample at 230 nm or 260 nm, respectively. Albumin and hemoglobin concentrations are determined by using their absorbance at 280 nm. Absorbance measurements are made directly on the DNA fractions or blood proteins, while the chromosomal proteins are lyophilized and redissolved in water. Each sample is then extracted with ethyl acetate or water-saturated butanol to remove noncovalently bound mutagens. This step is essential if adduct formation is to be quantified using radiolabeled compounds and scintillation counting or other techniques that do not differentiate hydrophobic or electrostatic binding and the formation of covalent adducts. Experiments with several different radiolabeled mutagens have revealed that a substantial fraction of the mutagen that coisolates with DNA is not covalently bound to it (Table 1). This extractable mutagen is tightly bound; extensive dialysis does not significantly diminish the level of its binding.

| Table 1. Mutagen binding in untreated and water-saturated butanol-extracted DNA and albumin samples. |
|--------------------------------------------------|
| **DNA (adducts/nucleotide)** | **Albumin (adducts/amino acid)** |
| Unextracted | Extracted | Unextracted | Extracted |
|---|---|---|---|
| 7-BMBA | 188/10^7 | 92/10^7 | 19.8/10^9 | 13.7/10^9 |
| Benzo(a)pyrene | 16.5/10^6 | 15.1/10^6 | 25.1/10^9 | 17.5/10^9 |
| 7,12-DMBA | 33.1/10^6 | 34.4/10^6 | | |
| TRP-P-2 | 126/10^7 | 17.5/10^7 | | |
Applications

Because numerous macromolecules (DNA and a variety of proteins) may be isolated from the same cell or tissue sample and separated by HPLC, this method is particularly well suited for application in studies comparing the kinetics of adduct formation (and repair) to multiple target molecules. Analyses of 7-bromomethylbenzanthracene (7-BMBA) binding to mouse DNA, histone and albumin (Fig. 4), for example, show that peak adduct formation is reached earlier in liver DNA than either protein. The majority of the DNA adducts (70%) are removed within the first 24 hr, and 10% of the adducts persist for more than 30 days. Adduct formation to histone H3 takes longer to peak, repair (or protein replacement) occurs more slowly [other studies (15) have shown that histones containing small adducts are not turned over but that the adducts are removed; the mechanisms for dealing with large adducts (16,17) have not yet been defined], and adduct removal approaches completion. As with DNA, 7-BMBA adducts to serum albumin are rapidly lost. In albumin, however, this removal reflects the normal rapid turnover of the protein (18) in mice ($t_{1/2} = 1$ day) and does not appear to involve repair.
reactive toward DNA than protein (chromosomal or blood). Adduct binding to DNA (per nucleotide) is at least 20-fold higher than to histone H3 (per amino acid) and 4-fold higher than albumin. 7-BMBA binding to hemoglobin is also observed (Fig. 6), but at a level approximately 20-fold lower than albumin. At low doses of mutagen (below 5 nmole/g body weight), dose and adduct formation in DNA, histone, albumin, and hemoglobin are linearly related. At higher doses this binding plateau in both albumin and hemoglobin and increases nonlinearly in DNA and histone. The plateau observed in albumin and hemoglobin binding appears to represent the saturation of 7-BMBA binding sites in blood. The concomitant nonlinear increase in binding to chromosomal targets may reflect the proportionately larger dose of 7-BMBA received by these targets once the bulk of the binding sites in hemoglobin and albumin become saturated.

Mice exposed to benzo(a)pyrene at doses between 1 and 100 pmole/g body weight exhibit a linear relation between exposure dose and albumin adducts (Fig. 7). The observation that 7-BMBA adducts to albumin are present in at least an order of magnitude greater concentration than hemoglobin adducts was also observed with benzo(a)pyrene; hemoglobin binding was nearly three orders of magnitude lower than albumin. The results with these two compounds suggest that albumin binding may provide an extremely sensitive method for monitoring mutagen exposure in short term experiments. While albumin cannot, obviously, be considered for monitoring exposures occurring over long periods...
Balhorn, Mazrimas, and Corzett

Is it a to IC 10,01 10 1 100 

0.01 0.1 10 Dose: Picomoles Benzo(a)pyrene per Gram Body Weight

FIGURE 7. Dose–response of benzo(a)pyrene binding to hemoglobin and albumin. Male C57Bl/6 mice were exposed to 3H-benzo(a)pyrene (200 Ci/m mole) by intraperitoneal injection of the mutagen in dimethyl sulfoxide. Hemoglobin and albumin were isolated from 50 µL of blood as described in the text: (●) albumin; (○) hemoglobin.

100

110

0.01

0.1

10

Dose: Picomoles Benzo(a)pyrene per Gram Body Weight

FIGURE 8. Dose–response of DNA-adduct formation in the liver of mice exposed to 3H-benzo(a)pyrene. Mice were exposed as described in Fig. 7. Liver DNA was isolated as described in the text and the number of DNA adducts determined by scintillation counting after extraction with equal volumes (three times) of water-saturated butanol.

of time (the half-life of the albumin molecule in mice is approximately 1 day (18) and 20 days in humans (19)), its sensitivity does offer advantages for monitoring dosimetry immediately after exposure.

The data shown in Table 2 illustrate how the quantification of DNA-adduct formation in in vivo studies is limited by the specific activity of the radiolabeled mutagen. In experiments with mutagens containing 1-25 Ci tritium/m mole (the normal specific activity for most tritium-labeled mutagens), we routinely detect the formation of several DNA adducts per 10⁷ or 10⁸ nucleotides. By increasing the specific activity of the mutagen 8-fold, as with benzo(a)pyrene (specific activity of 200 Ci/m mole), we have been able to increase the detection limit to a few adducts per cell (Fig. 8).

Conclusions

The HPLC techniques that we have described offer a number of advantages over currently used methods for target DNA and protein isolation. Using this approach, one can easily monitor and quantitate the binding of mutagens or carcinogens in vivo to multiple target molecules (DNA, histones, HMG proteins, albumin, hemoglobin) within the same individual. The ease and speed of sample processing allow the analysis of more samples than can be handled by cesium chloride gradient centrifugation or gel electrophoresis. The mild conditions employed to dissociate and separate DNA and chromosomal protein also minimize the loss of chemically unstable DNA adducts.

With this approach, analyses of DNA adduct formation require only a few tenths of a gram of tissue. If
only DNA is to be isolated, the entire cell may be dissolved in guanidine hydrochloride and the DNA separated from total protein by GPC. Using radiolabeled mutagens, the sensitivity of the assay permits adduct detection at the level of several adducts per cell. This technique may also be coupled with monoclonal antibody or mass spectrometry methods for quantifying adduct formation in vivo without the use of radioisotopes.

Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-ENG-48 and was supported by an interagency agreement (No. DW89950708-01-0) with the Environmental Protection Agency. It has not, however, been subjected to agency review and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred.

REFERENCES

1. Georgiev, G. P., Nedospasov, S. A., and Bakayev, V. V. Supranucleosomal levels of chromatin organization. In: The Cell Nucleus, Volume VI, Part C (H. Busch, Ed.), Academic Press, New York, 1978, pp. 1–34.
2. Weisbrod, S., and Weintraub, H. Isolation of a subclass of nuclear proteins responsible for conferring a DNase I-sensitive structure on globin chromatin. Proc. Natl. Acad. Sci. (U.S.) 76: 630–634 (1979).
3. Gazit, B., Panet, A., and Cedar, H. Reconstitution of a deoxyribonuclease I sensitive structure on active genes. Proc. Natl. Acad. Sci. (U.S.) 77: 1787–1790 (1980).
4. Gurley, L. A., and Hardin, J. M. The metabolism of histone fractions. II. Conservation and turnover of histone fractions in mammalian cells. Arch. Biochem. Biophys. 130: 1–6 (1969).
5. Hancock, R. Conservation of histones in chromatin during growth and mitosis in vitro. J. Mol. Biol. 40: 457–466 (1969).
6. Balhorn, R., Oliver, D., Hohmann, P., Chalkley, R., and Granner, D. Turnover of DNA, histones and lysine-rich histone phosphate in hepatoma tissue culture cells. Biochemistry 11: 3915–3921 (1972).

7. Rechsteiner, M., and Kuehl, L. Microinjection of the nonhistone chromosomal protein HMG 1 into bovine fibroblasts and HeLa cells. Cell 16: 901–908 (1979).
8. Wu, L. H., Rechsteiner, M., and Kuehl, L. Microinjection of the nonhistone chromosomal protein, HMG-2, into HeLa cells and bovine fibroblasts (abstr.). Fed. Proc. 39: 3119 (1980).
9. Pereira, M. A., Lin, L.-H. C., and Chang, L. W. Dose-dependency of 2-acetyl-aminofluorene binding to liver DNA and hemoglobin in mice and rats. Toxicol. Appl. Pharmacol. 60: 472–478 (1981).
10. Farmer, P. B., Gorf, S. M., and Bailey, E. Determination of hydroxypropyl histidine in hemoglobin as a measure of exposure to propylene oxide using high resolution gas chromatography-mass spectrometry. Biomed. Mass. Spectrom. 9: 69–78 (1982).
11. Calleman, C. J. Monitoring and risk assessment by means of alkyl groups in hemoglobin. In: The Biological Monitoring of Exposures to Industrial Chemicals. Hemisphere Press, Washington, DC, 1983, p. 331.
12. Meyer, M. C., and Guttmann, D. E. The binding of drugs by plasma proteins. J. Pharmacol. Sci. 57: 985–981 (1986).
13. Steinhardt, J., and Reynolds, J. A. In: Multiple Equilibria in Proteins. Academic Press, New York, 1969.
14. Chignell, C. F. Ligand binding to plasma albumin. In: Handbook of Biochemistry and Molecular Biology, 3rd ed., Proteins, Volume II (G. D. Fasman, Ed.), CRC Press, Cleveland, 1976, pp. 554–582.
15. Galbraith, A., and Itzhake, R. Studies on histones and non-histone proteins from rats treated with dimethylnitrosamine. Chem.-Biol. Interact. 29: 309–322 (1979).
16. Groopman, J. D., Busby, W. F., and Wogan, G. N. Nuclear distribution of aflatoxin B1 and its interactions with histones in rat liver in vivo. Cancer Res. 40: 4343–4351 (1980).
17. Kootstra, A. Effect of histone acetylation on the formation and removal of B[a]P chromatin adducts. Nucl. Acids Res. 10: 2775–2789 (1982).
18. Gitlin, D., Klinenberg, J. R., and Hughes, W. L. Site of catalysis of serum albumin. Nature 181: 1064–1065 (1958).
19. Danielesky, I. In: Biochemistry for Medical Sciences, Little, Brown and Co., Boston, 1980.