Elemental Analysis of Renal Slices by Proton-induced X-ray Emission

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We optimized proton-induced X-ray emission (PIXE) for tissue analysis in a toxicity-disposition study. We used cultured rabbit renal slices as the biological system to demonstrate the use of PIXE analysis. The renal slices were exposed to HgCl₂, CdCl₂, K₂Cr₂O₇, or Na₂O₃ alone or in a mixture. The PIXE analysis provides information on concentrations of elements above atomic number 11, and it is the only analytical technique that can determine 20–30 elements nondestructively in a single, small sample (~5 mg) with detection limits of 1–5 ppm (dry weight). The renal slices are thin targets that yield X-ray emission spectra with low backgrounds and high elemental sensitivities. The nondestructive nature of PIXE and the ability to simultaneously measure uptake of multiple metals and endogenous elements are unique to this methodology. Key words: arsenic, cadmium, chromium, elemental analysis, interactive toxicity, mercury, metal analysis, nephrotoxicity, proton-induced X-ray emission (PIXE), X-ray emission. Environ Health Perspect 101:302–308 (1993)

Methods for analyzing elements in biological samples are important for studying the effects of intoxication. Since its introduction in 1970 (1), proton-induced X-ray emission spectroscopy (PIXE) has been shown to be a powerful, multielemental analysis technique with high sensitivity (2). The PIXE technique involves bombarding a sample with protons which eject inner-shell electrons of the elements in the sample; the subsequent decay of the induced electronic states results in the emission of X-rays whose energies are characteristic of the elements present in the sample and whose intensities are proportional to the concentrations of these elements. A liquid-nitrogen-cooled solid-state Si(Li) detector and a multichannel analyzer are used to determine the energies as well as the intensities of the emitted X-rays, and a computer program identifies the elements present and calculates their concentrations. Although the application of PIXE to biological samples is not new (3), and recent reviews have emphasized the usefulness of this technique (4–7), the optimization of PIXE for use in typical animal toxicity studies has not been described in the toxicology literature. Here we present the optimization of the instrumentation, sample preparation, and the application of PIXE to a disposition study of metals in renal tissue.

The implementation of the PIXE technique has been limited mostly to physics and nuclear research laboratories because no commercial instrumentation is available. The proton beams for PIXE are generated by Van de Graaff accelerators that are not in high demand for applications in physics because much higher-energy ion beams are preferred in most nuclear physics research. The availability of high-energy accelerators has therefore made PIXE an attractive alternative to other conventional, multielemental analytical methods. The simultaneous quantification of a large number of elements in a sample makes PIXE an important analytical tool for use in diverse toxicological studies.

Overview of PIXE Theory and Technique

X-rays are produced as electrons from outer shells rapidly decay to replace inner-shell electrons ejected by high-energy protons. The energies of the X-ray emissions are characteristic of the elements, and the intensities of the emissions are proportional to the concentrations of the elements in the sample. The useful range of emitted X-ray energies for PIXE is 1–30 keV; the Si(Li) detector is capable of resolving X-ray emissions as closely spaced as 150 eV, and, as a consequence, 20–30 elements can be readily identified and determined simultaneously.

An X-ray spectrum of a thin copper film deposited on a carbon substrate is shown in Figure 1. The dominant features of the spectrum are the copper Kα and Kβ X-ray emission lines resulting from the ejection of the K (innermost) shell electrons of copper. The shape of the X-ray emission peaks in the spectrum is primarily Gaussian; the width of the peak is defined by the statistical distribution of the electrical pulses generated by X-ray photons in the Si(Li) detector and noise in the pulse-processing electronics. The peak width, which is typically 120–150 eV, defines the resolution of the system. The background in PIXE spectra is primarily the result of bremsstrahlung from the deceleration of the proton beam and of secondary electrons generated in the target. Secondary electron bremsstrahlung is predominant in the spectrum at the low-energy region, and proton bremsstrahlung contributes to the background in the low-energy as well as the high-energy region of the spectrum. The use of detector filters attenuates the low-energy region of the spectrum and reduces the contribution of secondary electron bremsstrahlung and X-ray emissions from low atomic number elements such as phosphorus, sulfur, chlorine, potassium, and calcium, which are often present at high concentrations in biological samples. Attenuation of the low-energy X-rays allows more efficient detection of elements with higher atomic number, thereby reducing the analysis time. Instrumental contributions to the PIXE spectrum are 1) silicon escape peaks, which arise from the loss of a silicon K X-ray from the detector (an apparent X-ray energy signal equal to the original X-ray energy reduced by the 1.75 keV lost as a result of the emission of the silicon X-ray is recorded); 2) pile-up peaks, caused by the detection of two X-ray photons that enter the Si(Li) detector simultaneously and are interpreted by the detection system as a single X-ray photon (8). Pile-up peak rejection electronics can recognize small inconsistencies in the generated electronic pulse profile and can reject some but not all pile-up peak signals. The aluminum peak in the spectrum results from the emission of X-rays from the aluminum detector filter stimulated by higher energy X-rays.

One of the advantages of the PIXE technique is the ability to detect trace levels of elements in a sample. Typically, the PIXE system can detect approximately 1 ppm (µg/g, dry weight) for thin organic samples (8). The high sensitivity of the PIXE technique is largely due to the low

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background in the spectrum. To obtain high sensitivity, it is important to prepare the thinnest possible sample targets so that secondary electron and proton bremsstrahlung are minimized and to use a relatively low beam energy for analysis to avoid γ-ray contributions to the background. Gamma rays are produced by nuclear reactions between high-energy protons and low-atomic-number elements in the target. Scattering of the γ-rays will generate a continuum of background in the spectrum. Proton beams of 2–3 MeV are therefore best suited for PIXE (2).

Charging the sample by the proton beam sometimes results in an electrical discharge from the sample to a nearby conductor and can contribute to background in the PIXE spectrum. Sample charging can be eliminated by using a conductive coating on the target (9), using a helium atmosphere which supplies an ionized path for the dissipation of the charge, using a thin carbon foil placed in the proton beam close to the target (10), or by spraying the target with electrons to neutralize the charging effect of the proton beam (11).

It is necessary to decompose overlapped features of the X-ray spectrum and to estimate background contributions to the spectrum. Unravelling these features in the spectrum requires the use of a computer program that employs the theoretical relative line intensities (Kα, Kβ, Lα, etc.) for each element and the calculated shapes of peaks in the spectrum. The GUPiX spectrum program (12,13) models the experimental spectrum by using a nonlinear least-squares iteration of peak intensities and position parameters and constructs a model spectrum in which all characteristic X-ray peak intensities have been calculated. The degree of correspondence of the calculated spectrum to the experimental spectrum is evaluated by using a chi-square test. After the intensity of each peak in the spectrum has been calculated, the concentration of each element in the sample is determined from the peak intensity by using the theoretical X-ray fluorescence yield, ionization cross-sections for protons for each element, detector efficiency and detector filter transmission, the charge deposited on the sample (determined experimentally), and the calibration factor.

The accuracy of the quantification is improved by using a standard to provide a calibration factor that corrects for errors in the measured detector solid angle and provides an absolute correction for any systematic errors in the measurement of the charge deposited on the target. In practice, a standard is run before and after every set of samples to provide the calibration factor for each sample set, which is used to correct for any small differences in beam–sam-

**Biological Sample Preparation**

As noted earlier, the high sensitivity of the PIXE technique at thin targets, although thick samples can easily be analyzed, with concomitant increases in detection limits. Numerous methods for preparing biological material as thin targets have been developed (5,16–19), and each type of sample must be prepared differently. In some cases, the sample must be pre-concentrated to determine low concentrations of analytes. Wet or dry digestion, evaporation, extraction, and precipitation have been used successfully as preconcentration techniques in PIXE sample preparation. Thin samples have been prepared by deposition of the sample on thin, polymer-backing materials or by sandwiching a thin sample between two polymer films.

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**Figure 1.** PIXE spectrum of a thin copper film on carbon.
Thin specimens for PIXE analysis require an appropriate support. Reviews of the available backing materials are given by Campbell (13) and Russell (20). Thin plastic films, although they are electrical insulators, are desirable supports for samples because they consist of light elements and will not contribute much background to the spectrum. These films also contain only small amounts of contaminating elements, which are detected by PIXE. The thinnest backings are formed by dropping a solution of a polymer onto the surface of pure water and picking up the dry film with a frame. Thin carbon foils also have been used in PIXE. These thin films or foils have a very low bremsstrahlung background, but they are fragile and should be used when the maximum sensitivity is required.

### PIXE Instrument and Sample Chamber

Although similar to other systems, the University of Arizona PIXE instrument has several unique features (Fig. 2), the most important of which are the ability to convert rapidly between the XRF and PIXE modes and the ability to measure the total charge deposited on the sample by the use of an RBS detector. Hydrogen is ionized in the Penning source in the high-voltage terminal of the Van de Graaff generator, and the H$_2^+$ is accelerated across a 4 MV potential. The initial ionized species, which is 4 MeV H$_2^+$, is decomposed into two 2 MeV H$^+$ by passage through the Kapton exit window at the end of the beam line. The ions proceed down a beam line evacuated to $10^{-6}$ torr and are bent through 90° by a large magnet whose field is stabilized. A pair of control slits downstream from the magnet are used to monitor the beam position and vary the acceleration potential so that a beam of constant energy is sent down the beam line. A quadrupole magnet allows some degree of beam focusing or defocusing to aid in the control of the beam profile that reaches the defining-slit chamber. Two sets of four slits in the defining-slit chamber configure the beam to a 4-mm square, which passes through a 7.6-µm thick Kapton window and strikes the sample.

A custom sample holder and chamber was constructed for our PIXE system (Fig. 3). Up to 10 samples can be mounted in a vertical plane on the automatic sample changer. The beam line is isolated from the sample chamber by a 7.5-µm thick Kapton window. This permits the sample wheel to be changed without breaking the vacuum in the beam line. The beam strikes the target and generates X-rays, which are detected by the liquid-nitrogen-cooled Si(Li) detector. If the sample is thin enough to transmit the beam without significant attenuation, the charge passing through the sample can be measured by integrating the current generated when the beam is captured in the Faraday cup. Secondary electrons generated by the beam can escape from the Faraday cup and, unless suppressed, can cause inaccuracies in the measurement of charge. Thin-sample targets, however, are rarely encountered in the analysis of biological specimens such as tissue slices, and the total charge is measured using an alternative method. This alternative method is similar to that reported by Mitchell et al. (21). Protons back-scattered from the Kapton exit window are measured using an RBS detector (Fig. 3).

In our system, the Kapton window is coated with a thin layer of cobalt whose areal density (atoms per unit area) is well characterized in a separate RBS experiment. The number of protons back-scattered from the cobalt is directly proportional to the number of protons that pass through the window and can be calculated from the beam energy, RBS detector solid angle, and Rutherford cross-section for protons on cobalt. Provided that the beam is smaller in area than the sample, the total number of protons that are incident on the sample is known from the intensity of the cobalt peak in the RBS spectrum. In practice, one RBS spectrum is recorded concurrently with each PIXE spectrum, and the
charge deposited is calculated. In addition, the cobalt-treated Kapton window is sufficiently close to the sample to provide a spray of electrons that reduce the effects of sample charging by the beam (9). The 7.6-µm Kapton window is highly resistant to the effects of the beam because Kapton is a beam-resistant material, and, when coated with cobalt, it facilitates the dissipation of heat and electric charge.

The use of thin backing materials and thin specimens, optimization of detector placement with respect to the target, and reduction of instrumental contributions to the background are important factors in optimizing the PIXE instrumental technique for analyzing biological samples (22). To demonstrate the precision and detection limits obtainable with PIXE, we examined 35 control rabbit kidney slices from 9 experiments. A typical spectrum of a control rabbit kidney slice is shown in Figure 4, and the results of the analysis are shown in Table 1. The sample preparation technique is described later. A detector filter consisting of 25 µm aluminum and 44 µm mylar was used in the analysis to attenuate the intense X-rays from elements with low atomic number. In Table 1, values of nanogram per square centimeter have been converted into parts per million by using averaged measurements of the area and mass of the analyzed kidney slice. The conversion factor was 0.172 ± 0.029 cm²/mg and was determined by measuring approximately 20 prepared kidney slice specimens with a micrometer and weighing the unmounted, desiccated slice on an electronic balance. The reported elemental concentrations in the blank mylar sandwiches are from separately analyzed sandwich assemblies without kidney slices; the low detection limits are due to the lower background from the thinner blank targets.

Uncertainties in the thickness measurement of the detector filter are magnified for low-energy X-rays that have a low percent transmission through the filter. We calculated the uncertainty in concentration for each element from the average of the standard deviation of four replicate samples, which were prepared from adjacent kidney slices from a single core sample, averaged over the 9 experiments. The GUPIX spectrum processing program calculated the detection limits by measurement of the background in the region of the spectrum under the analytical peak. The statistical errors were calculated from the counting statistics by the GUPIX computer program, which approximated the error by calculating the inverse of the square root of the number of counts in the spectral peak; included in the calculation of the statistical error is an estimated uncertainty from errors in detector filter

| Element | ng/cm² (±SD) | ppm (avg) | SD (%) | SE (%) | LD (ng/cm²) | Blank (ng/cm²) |
|---------|-------------|-----------|--------|--------|-------------|----------------|
| K       | 24400 ±3120 | 4193      | 12.8   | 0.60   | 79.0        | <45            |
| Ca      | 3570 ± 480  | 614       | 13.3   | 1.57   | 133         | 1805           |
| Cr      | 12.3 ± 9.1  | 2.12      | 74.1   | 60.6   | 4.40        | <2.4           |
| Mn      | 28.1 ± 3.0  | 8.83      | 10.6   | 9.12   | 4.17        | <1.8           |
| Fe      | 462 ± 54    | 79.5      | 11.6   | 1.54   | 6.32        | 6.07           |
| Co      | 21.9 ± 2.2  | 3.77      | 10.1   | 20.9   | 9.54        | 20.5           |
| Ni      | 13.4 ± 4.9  | 2.30      | 34.5   | 17.4   | 2.95        | 13.4           |
| Cu      | 42.3 ± 4.1  | 7.27      | 10.2   | 7.67   | 2.93        | <1.9           |
| Zn      | 284 ± 10    | 48.8      | 3.7    | 2.74   | 4.29        | 23.7           |
| Se      | 176 ± 4.3   | 3.04      | 27.5   | 24.9   | 6.46        | <3.1           |
| Br      | 17.2 ± 3.0  | 2.95      | 32.1   | 30.7   | 7.82        | <4.2           |

Thirty-five slices were analyzed. Al (25 µm) and Mylar (44 µm) detector filters were used. A 7–10 µC charge was deposited over 5 min analysis time. LD, limit of detection. The high counts for Ca, Fe, Ni, and Zn in the results for the blank are due to impurities commonly found in the Mylar film used for kidney slice sandwich assemblies. The blank was two sheets of Mylar without a tissue slice. The Co counts are an instrumental contribution which originates in the cobalt-coated Kapton sample chamber window.

Figure 4. PIXE spectrum of a rabbit kidney section mounted between two 3.6-µm Mylar films. The scale was magnified by a factor of 5 above 5 keV.

Figure 5. Sandwich assembly for mounting rabbit renal slices.
thickness measurement. Large statistical uncertainties are expected for small peak areas, and comparing the statistical error and the average standard deviation gives a measure of the contribution of statistical error to the overall uncertainty. The presence of the small cobalt peak in the PIXE spectrum is a result of the use of the cobalt-coated Kapton window and is due to elastic scattering of the characteristic cobalt X-rays emanating from the window.

The accuracy of the PIXE technique is usually determined by the analysis of standard materials. Elemental concentrations in NIST standards were measured, and the results showed that the uncertainties in the elemental concentrations were approximately ±10% (23).

Analysis of Rabbit Renal Tissue by PIXE
PIXE is recognized as a superior multielemental method for determining trace elements, with detection limits in the range required for the analysis of trace metals that are present in biological systems (24). PIXE is capable of assessing interelement interactions in a single biological sample of about 5 mg, which can be prepared as a thin film. One limitation of the technique is the shallow sampling depth of proton beams. The penetration of protons in the MeV range of energy is limited, typically, to depths of several tens of microns in the sample.

Sample Preparation
We prepared renal slices from male New Zealand white rabbits (1.5–2.5 kg) using a Krumdieck tissue slicer (25). Cylindrical cores (0.6 cm) were taken through each kidney along the cortico-papillary axis. The cortical areas were trimmed from the medullary regions and were subsequently cut with the slicer. The slices obtained were approximately 275 μm thick and 10 mg in wet weight.

We incubated renal slices at room temperature in an incubation vessel consisting of 20 wells, through which 95% oxygen/carbon dioxide was bubbled (25). Each well was filled with 20 ml of DME-F12 media containing 2 mM valeric acid. All buffers and media used in the studies were aerated and pH adjusted to 7.4. We placed four slices on a screen located at the base of each well. We added HgCl₂, CdCl₂, K₂Cr₂O₇, NaAsO₂ individually or in combination at concentrations from 10⁻⁶ to 10⁻³ M. Incubations were terminated at 0, 4, 8, and 12 hr for PIXE analysis.

After incubation, renal slices were blotted dry and placed on 3.5 μm Mylar film stretched across 32-mm diameter, opened XRF sample cups (Chemplex In-

Figure 6. Uptake of (A) NaAsO₂, (B) CdCl₂, (C) K₂Cr₂O₇, and (D) HgCl₂ by rabbit renal cortical slices. Values are the average of four determinations. Error bars have not been included for clarity. Standard deviations average 13%.

Figure 7. Uptake of mixtures of NaAsO₂, CdCl₂, K₂Cr₂O₇, and HgCl₂ by rabbit renal cortical slices. (A) 2.5 × 10⁻⁵ M, (B) 5 × 10⁻⁵ M, (C) 7.5 × 10⁻⁵ M, and (D) 1 × 10⁻⁴ M. Values are the average of four determinations. Error bars have not been included for clarity. Standard deviations average 13%.
dustries Inc., Tucahoe, New York) (Fig. 5). We placed another piece of film on top to create a sandwich. The snap-ring pulled the assembly taut, and two holes were punched in the sandwich to allow air to escape and the sample to dry. We placed samples in the freezer immediately after preparation and left them there until the day before the analysis, at which point we placed them in a desiccator and allowed them to thaw and dry. The dried samples were then analyzed by PIXE.

The PIXE technique was capable of detecting the uptake of the individual metals into the renal slices over a wide range of concentrations (Fig. 6). The quantity of metal accumulated in the slices was unique to the metal: mercury (HgCl₂) was taken up the most and arsenic (NaAsO₂) was taken up the least. We chose concentrations known to be cytotoxic to the kidney slices (25). Although there were instances where the metal accumulation plateaued (Fig. 6D), this was not due to exhaustion of the metal concentration in the incubation media. Saturation of uptake and binding sites within the slices is apparently occurring.

A particularly attractive feature of PIXE is that it allows one to examine the presence of more than one metal (or element) simultaneously. When four metal compounds are present at the same time, their selective uptake and interactions can be examined (Fig. 7). The ability to examine preferred uptake of one metal compound and the effects metals have on each other’s accumulation is important for interactive toxicity studies. At higher concentrations, mercury (HgCl₂) content was the greatest, as was seen when the metals were present individually (Fig. 6D). It is surprising that cadmium (CdCl₂) was the least accumulated because arsenic (NaAsO₂) had the lowest accumulation levels when present individually (Fig. 6A). This shift in accumulation patterns when the metal compounds are present together emphasizes the importance of coupling uptake analyses when performing interactive toxicity studies.

**Discussion**

Analyzing tissue in thin, desiccated sections sandwiched between two pieces of thin mylar has significant advantages. The low detection limits obtainable by analyzing a digested sample are more than offset by the time required for digestion and the possibility of contamination of the sample. Care must be taken in quantification, however, because the use of internal standards is precluded by this method of sample preparation, and the fundamental parameters of the instrument must be used. Reproducibility was aided by drying the tissue slice after sandwiching; the surface uniformity and retention of original sample area obtained by sandwiching improve reproducibility. Freezing the sandwiched tissue slice before drying appeared to improve the homogeneity of the dried sample.

Jun et al. (26) compared the preparation of tissue for PIXE analysis by 1) slicing frozen tissue, 2) slicing tissue while embedded in paraffin, and 3) preparing tissue as a homogenate. The slices or homogenate were placed on a Formvar (polystyrene) backing material and were desiccated. Jun et al. found that the frozen sections were easiest to prepare; films as thin as 100 µg/cm² could be obtained, and fewer analytes were lost than in the preparation of paraffin-embedded or homogenized specimens. In our study, the tissue slices were prepared from fresh, nonfrozen tissue for use in uptake and toxicity studies. As in the studies by Jun et al., the slices were easy to prepare for PIXE analysis with minimum loss of analytes. Precision-cut tissue slices have rejuvenated tissue slices as a tool for in vitro toxicology studies (27). However, it is important to quantify the amount of chemical to which the tissue slice, in this instance the kidney, is exposed (28,29) so extrapolations can be made to in vivo studies. Previous studies have required the use of radiolabeled metals, laborious atomic absorption methodology, sophisticated histochemical techniques, or electron probe microanalysis. The PIXE system allows for quantification of virtually any metal (elemental) toxicant above atomic number 11. The ability to simultaneously evaluate the uptake of multiple metals is extremely useful for metal–metal interaction studies (30,31). In the past, many researchers had to analyze for different metals using different techniques and often using separate samples. The PIXE technique allows the analysis to be performed on the same sample, thus minimizing intersample variability. The effects of metal–metal interactions on uptake and accumulation mechanisms should be readily addressed using the PIXE technique.

Although the application of the PIXE technique in this investigation was limited to renal slices incubated in vitro, this same methodology can be used for in vivo studi-
ies. Precision-cut slices can easily be prepared from numerous tissues from various species (27). These tissue slices from naive or exposed species can be prepared for PIXE analysis using the same techniques described here. In fact, slices can be taken from different sections of the tissue to ascertain the distribution of the metal compound through the organ.

In the PIXE analysis of organic samples, heating of the sample by the proton beam during the analysis can cause problems, but reducing the beam current or using a helium atmosphere in the sample chamber allow the sample to dissipate heat. In our work with rabbit kidney sections, beam currents of around 30 nA and beam diameters of about 6 mm were used for analyzing a dried tissue section for 10 min with no beam damage and only minor discoloration of the tissue.

PIXE analysis of biological specimens can be used to determine the concentrations of major and trace elements with a high degree of precision and accuracy. This ability to quantify a large number of elements in a single analysis of a small sample with high sensitivities is unique to the PIXE technique. PIXE is virtually the only method that is available for nondestructive, multielemental analysis of a small biological sample; none of the conventional analytical techniques can provide the same amount of information on the elemental composition of the sample, especially if the size of the sample is limited to a few milligrams. The use of PIXE has increased rapidly in the analysis of biological specimens, and while the instrumentation and spectrum processing hardware and software have developed steadily since the early days of PIXE, sample preparation is still the major limitation in obtaining reproducible results. In practice, each sample type requires a unique method of sample preparation. In studies on the uptake of toxic metals by rabbit kidney tissue, PIXE has provided results that have been pivotal in understanding interelement effects.

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