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PROTON-INDUCED X-RAY EMISSION ANALYSIS - A NEW TOOL IN QUANTITATIVE DERMATOLOGY

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Abstract

Proton-Induced X-ray Emission analysis (PIXE) constitutes a method for trace element analysis characterized by multielemental capability, detection limits in the low ppm-range and size resolution down towards a micrometre. In applications where the sensitivity of the Electron-Induced X-ray Emission (EIXE) analysis is not sufficient and where a spatial resolution not better than a few micrometres is required, the PIXE technique provides a powerful tool. In this paper properties of the PIXE method are demonstrated by quantitative results from three different samples of dermatological interest. Firstly, mercury results from a longitudinal scan of a single hair strand from a mercury poisoned person are shown. With a spatial resolution of one or a few millimetres very fast scans may be performed on hair strands giving information on time and magnitude of intoxication or other exposures, as well as deficiencies. Secondly, results are given from a radial scan with a beam width of 4 μm on hair from a person exposed to high amounts of iron. The calcium, iron and zinc distributions but not the sulphur and potassium distributions show narrow peaks of concentration (less than 4 μm) about 15 μm from the surface of the hair. Further investigations have to be performed in order to interpret these data. Thirdly, the depth profiles in skin of some elements were measured with a beam width of 10 μm. The results show significant increases in sulphur, calcium and zinc concentrations and significant decreases in phosphorous and potassium concentrations at the skin surface, i.e. in the stratum corneum.

KEY WORDS: Proton microprobe, proton-induced X-ray emission (PIXE), external beam, energy-dispersive, electron microprobe, dermatology, hair, skin, cryosectioning

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Introduction

Although it has been possible to collect data from skin and its appendages through biochemical and biophysical methods as well as through light and electron microscopy, information on the elemental content in situ has only recently been available (27). Energy-dispersive X-ray microanalysis with an electron microprobe (EMP) provides such data collection on frozen hydrated or freeze-dried sections obtained after freeze-quenching of the specimens. However, the EMP is not a trace element method and important electrolytes such as calcium and magnesium are barely detectable above the significant background obtained by analysis. In the search for methods allowing the detection of elements at concentrations close to trace element levels, i.e. 10-500 ppm, we have investigated the analytical potential of PIXE (proton induced X-ray emission analysis). The high sensitivity and rapidness of PIXE makes it an attractive tool in many applications of trace element analysis. For detailed descriptions and reviews see Johansson and Johansson (14) and Khan and Crumpton (16).

Longitudinal hair scan

The technique of longitudinal scanning of hair in a helium atmosphere with the PIXE technique provides an accurate, non-destructive and rapid method for detecting enhanced concentrations of toxic elements, which may be due to acute poisoning, with applications in, for example, forensic science (11). Furthermore, it may be of great use in analysing hair samples for extensive epidemiological studies when occupational or environmental pollution is suspected.

Inherent in the PIXE technique is the capability of estimating surface to bulk distributions of elements in thick samples (1). In the case of hair a repeated analysis at 2 or 3 different proton energies have been used to roughly calculate the surface to bulk ratios of several elements (21). This technique may be of use for separating any possible exogenic contamination from endogenic components.

Several investigations of the spatial distribution of elements in hair have been performed using PIXE analysis (15,25). Another suitable technique is the use of X-ray fluorescence analysis. Toribara et al (23) used molybdenum-

filtered radiation from a molybdenum X-ray tube and a spatial resolution of a few mm in studies of trace elements in hair.

A similar technique to obtain time records of trace element concentrations would be to use another appendage of skin, i.e. nails. Samples can easily be collected from, e.g. occupationally exposed workers and the elemental distributions along the nail may be determined by direct PIXE analysis. This technique has been used with neutron activation analysis in a case of repeated arsenic poisoning (10). Preliminary attempts have been made at our laboratory to sample thin strips from nails and to scan the strips longitudinally. Sampling on workers exposed to arsenic is at present being planned.

Microprobe analysis

In common with the electron beam in an electron microscope the proton beam may be finely focused by magnetic or electrostatic means, allowing elemental analysis with high spatial resolution. A detailed review of the properties and possibilities of proton microprobes has recently been given by Martin (22).

There is a strong trend towards an increasing number of laboratories developing microbeams for applications in different fields. In a recent conference in Namur, Belgium, many laboratories presented their progress (8). Cookson and Pilling (6) and Bos et al. (3) have reported interesting results from cross sectional scans of hairs performed on thick slices of hair strands. Bischof et al. (2) have reported depth profiles of some elements in skin showing strong variations in concentration introduced by the presence of a hair root. In the present communication we report the first quantitative results from a freeze-quenched, freeze-sectioned and freeze-dried thin section of hair obtained with a finely focused microbeam of protons as well as quantitative elemental profiles on normal human skin provided under the same conditions. Furthermore, PIXE data on single hair strands obtained with a macrobeam will be discussed in this context.

Materials

Hair

One hair sample was obtained from a woman aged 36, who had been exposed to very high amounts of iron in domestic water supply (13 mg/l). Hair fibres were plucked ensuring that anagen fibres were obtained and sent by post without any further preparation. Short pieces of hair (2-4 mm) were cut from the first 1.5 cm (root end) of the fibre and placed in carboxymethyl cellulose, swelled in doubly distilled water and immediately quench-frozen in liquid nitrogen. Thin sections (< 5 µm) were obtained with a specimen knife of glass at a temperature of -100 degrees C by manual sectioning on a Reicher OMU3 FC2 cryoultramicrotome. The sections were collected on a 2 µm thick sheet (Kimfol®) and fixed to it by thawing and subsequent air drying. The plastic sheet carrying the sections were then transferred to circular specimen holders allowing free passage of the protons after interaction with the specimen.

A second hair sample for analysis with a macrobeam was collected from a victim of mercury poisoning. The hair was stretched and glued, self-supporting, without any pre-treatment onto a plastic frame (20 x 200 mm^2). The length of the hair to be analysed was 150 mm.

Skin

Skin samples were taken by punch biopsy from the lower arm of a healthy volunteer without any previous local anesthesia. Semi-thick sections (10 - 30 µm) were cut at -25 degrees C on a Leitz cryostat and the sections were subsequently freeze-dried in the cryostat at -25 degrees C for 6 hours. Each freeze-dried section was then mounted on a 2 µm thick sheet (Kimfol®) in the circular specimen holder designed for our instrument. To prevent the uptake of water before analysis the specimens were stored over Drierite®.

Methods

For the PIXE analysis protons with an energy of 2.5 MeV were delivered by an electrostatic accelerator (Pelletron 3UDH). The characteristic X-rays produced on impact by the protons on a sample are detected by an 80 mm^2 Si(Li) detector (Kevex; nominal resolution 158 eV at 5.9 keV) placed at an angle of 135 degrees relative to the beam. The presence of all elements heavier than sodium can be determined simultaneously by storing the X-ray events in a multichannel analyzer (Nuclear Data 6620) and transferring the X-ray spectra to magnetic tape for subsequent evaluation. For routine analysis a beam diameter of 1 to 10 mm is used. Precision and accuracy are normally better than 5 to 10 % (13,5).

External beam PIXE

For macrobeam analysis of hair a tantalum beam collimator of 2x4 mm^2 was used with the long side perpendicular to the hair. The proton current was kept below 40 nA. To reduce heating and charge build-up during irradiation the hair was placed in an atmosphere of helium (pressure 50 kPa). The proton beam was extracted from vacuum through a thin polyimide foil (Kapton®, 7.5 µm). After passing through the hair the proton beam leaves the target chamber through another foil to a high-vacuum Faraday cup where the current is measured. Detection of X-rays is performed as described above.

The plastic frame with the hair was placed in a tray drawn by a linear stepping motor (Airpax L92121-P2) allowing a spatial resolution of 50 µm. After complete analysis of one section of the hair the next section is moved into the beam by remote control (computer controlled).

The hair strands represent samples of non-negligible thickness (diam: 40 - 110 µm) and to be able to calculate accurate elemental concentrations a surface barrier detector is used to measure the energy distribution of scattered protons. From this particle energy spectrum it is possible by a special algorithm, to determine the hair diameter (19,20).

The proton microprobe (PMP)

An adjustable collimator is situated after the accelerator at a distance of 4 metres from the sample chamber. With the aid of micrometre screws the opening of this object collimator can be set separately from 10 to 1500 µm in two perpendicular directions. With 4 quadrupole magnets positioned

about 0.3 m in front of the sample, beam dimensions down to a few micrometres may be attained by focusing. In these experiments only two magnets were used. The experimental demagnification factors with this doublet configuration were 8 and 38 in the horizontal and vertical planes respectively.

The sample holder can be moved by remote control in steps of 1 μm or more in both horizontal and vertical directions. To identify and position the samples they are illuminated by an external light source of high intensity through a system of mirrors and windows. Viewing is carried out using a stereo microscope (Nissho ZN 240 with a zoom lens and a maximum magnification of 180x).

The chamber vacuum was about 10^{-5} torr and the highest obtained proton current was approximately 3 pA/μm^2. With a pair of extra quadrupole magnets situated in front of the object collimators the proton density can be strongly increased although the divergence of the beam and thus the aberration in the lenses will increase and only for larger beam sizes this will be an advantage.

For the skin sample the beam was focused in a spot of 10x100 μm^2 oriented with the long side parallel to the skin surface and the irradiation was started about 35 μm into dermis. The sample could be moved perpendicular to the beam (in steps of 8 μm) to analyse the various epidermal strata. Each spot was irradiated for 300 to 500 s.

For the elliptical hair section (see upper left part of fig.3) the beam was focused on an area of 4x15 μm^2. The beam profile parallel to the short side, measured by scanning the edge of a thin, self-supporting tantalum foil, is shown in fig.1.

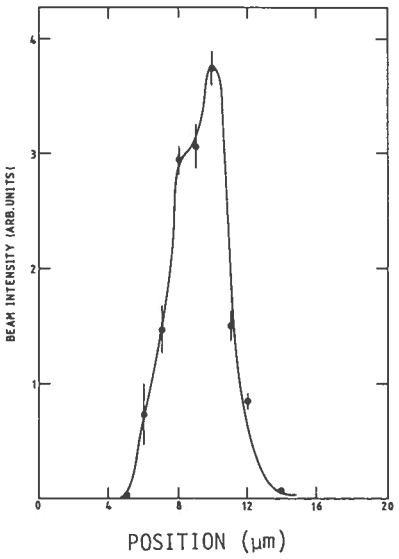

Fig.1. Vertical beam profile as determined by scanning the edge of a thin self-supporting tantalum foil. The error bars represent one S.D.

The hair sample was moved with 3 μm increments and the irradiation times were approximately 500 s per point. The zone irradiated by the proton beam formed a 15 μm wide strip passing through the centre of the hair. Unfortunately the strip at one side of the hair passed a part of the cuticula which was partly damaged during sectioning.

For both the hair and the skin sample no corrections due to proton slowing down and X-ray absorption were required for elements heavier than silicon.

Results and discussions

Longitudinal hair scan

In fig.2 the concentration of mercury along the hair strand is plotted. The concentration is calculated by assuming a constant sulphur concentration of 4.3 %. This value was obtained from PIXE-analysis of a total of 40 hair strands, which were collected from Caucasian as well as Asian subjects (20).

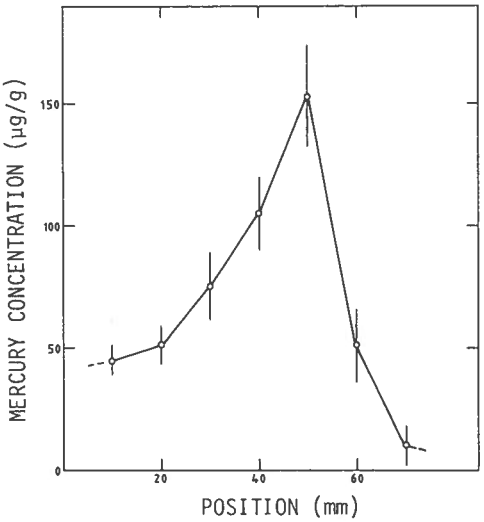

Fig.2. Mercury concentration along a single human hair strand as determined by macrobeam PIXE analysis. Origin corresponds to the root end. The donor of the hair was a victim of mercury poisoning. Irradiations were carried out in a helium atmosphere with a beam of 2.5 MeV protons with a beam collimator of 2x4 mm^2 and a current density of less than 5 nA/mm^2. The solid lines are drawn to guide the eye and the error bars show statistical uncertainties of one S.D.

Since only 2 mm sections were analysed every 10 mm along the hair the spatial resolution may easily be improved without any reduction of the sensitivity - thus facilitating determination of elemental variations with a time resolution of less than one week. The detection limit for mercury in this set-up is around 8 μg/g using 100 s irradiation time.

Cross sectional hair scan

The results from the transverse scan of the thin hair section are shown in fig 3. In calculating the elemental concentrations the sulphur concentration has been assumed to be 4.3 % as determined by Li and Akselsson (20). Part of the cuticula was damaged during sectioning (see fig.3) and results from this part have consequently been excluded in the diagram. For orientation, the centre of the elliptic hair cross section as determined by a light microscope is indicated.

In fig.3 the sulphur distribution is given in arbitrary units as an index of mass distribution, showing a rather smooth distribution over the whole hair cross section. The small variations in the sulphur distribution cannot be explained by pulse statistics but rather by the structure of the hair, i.e. a looser arrangement of the cells in the centre.

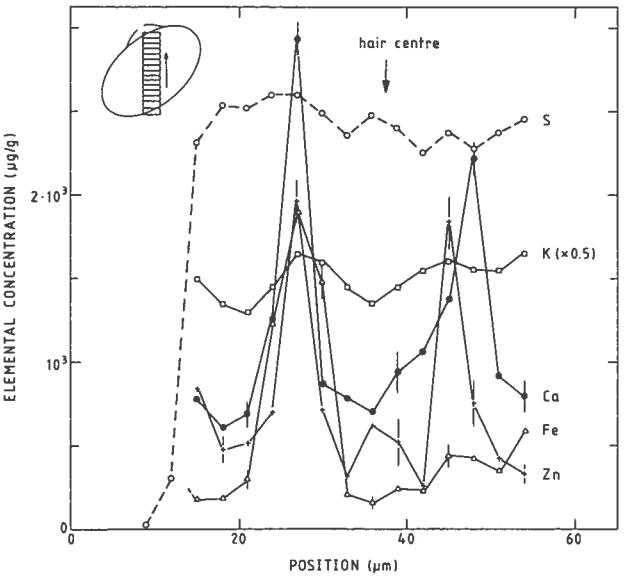

Fig.3. Elemental distributions across the thin section of a human hair. The scanning was performed according to the drawing in the upper left corner. Due to the damage caused during sectioning no values are given for the upper edge of the hair section. The analysis was performed with a proton microbeam of 4x15 μm^2 and a current of about 200 pA. The broken line shows the sulphur distribution in arbitrary absolute units and does not refer to the ordinate scale. The solid lines are drawn to guide the eye and the error bars show the uncertainty due to pulse statistics (one S.D.).

For potassium the distribution is rather constant and the concentration rather high compared with the values in the literature, normally citing potassium concentrations well below 1000 ppm (17). For the elements calcium, iron and zinc the distributions are quite different with sharp and high maxima occurring approximately symmetrically around the hair centre, about 15 μm inside the edge of the hair section. In the remaining parts the levels are rather constant with variations approximately within the statistical uncertainties. However, for iron no maximum is found in the right part of the diagram. Since the spatial resolution of the proton beam is limited (FWHM= 4 μm) the maxima may appear wider than their actual widths. Thus if the widths of the maxima are considerably less than 3 μm the peak concentrations are considerably higher than indicated in fig.3.

In a few other investigations, a PMP has been used for cross sectional scans of hairs (6,12). In their works they used a beam twice as broad as the one in the present work and the hair sections analysed were infinitely thick for the protons. In their investigations maxima were found for several elements but normally they occur at the hair surface (the edge of the section) and are probably caused by exogenic contamination. However, in the report by Houtman et al. (12) calcium and zinc distributions for a few hairs were found to peak somewhat inside the hair similarly to that found in the present work. They explain this by exogenic contamination and diffusion followed by a superficial extraction. The smoothened peaks due to their limited spatial resolution make this a plausible explanation. In our case the very sharp maxima can hardly be explained in this way. The possibility of a contamination transferred to our sample during processing cannot be totally excluded but the probability is very small that the contamination would occur in such a symmetrical fashion as found in fig.3. Since the donor of the hair is suspected to suffer from a too heavy exposure to iron from domestic water supply the elevated levels of iron and several other elements might be explained by a concomitant endogen uptake as well as contaminant cosmetic care.

Apart from the elements given in fig.3 chlorine and copper were also found to be present. In fig.4 is shown a PIXE spectrum formed by summing the spectra obtained in the transversal hair scan.

The detection limits attainable in PIXE analysis depend on the beam intensity and the solid angle of the X-ray detector. However, an upper limit for the beam intensity is set by the heating induced in the sample. By using a PMP in beam scanning mode analysis (7,18) the average irradiation intensity will be much lower and the beam current can thus be considerably increased without any excessive heating of the sample. Also the sample thickness is of importance in regard to the detection limits. A thick sample (> 12 mg/cm^2 for 2.5 MeV protons) gives a higher yield, but also absorbs significantly more of the beam energy. To perform accurate matrix corrections the composition must be known. In thin samples no, or at least very small, corrections for X-ray self-absorption are required, but when calculating the elemental concentrations in a thin sample the sample thickness (mg/cm^2) must be known.

The proton microprobes, when used with small beam areas, can usually deliver only a modest beam intensity and if this is a limiting factor the thick sample with its higher yields will give the lowest detection limits. In the case of samples of dermatological material the optimal thickness is

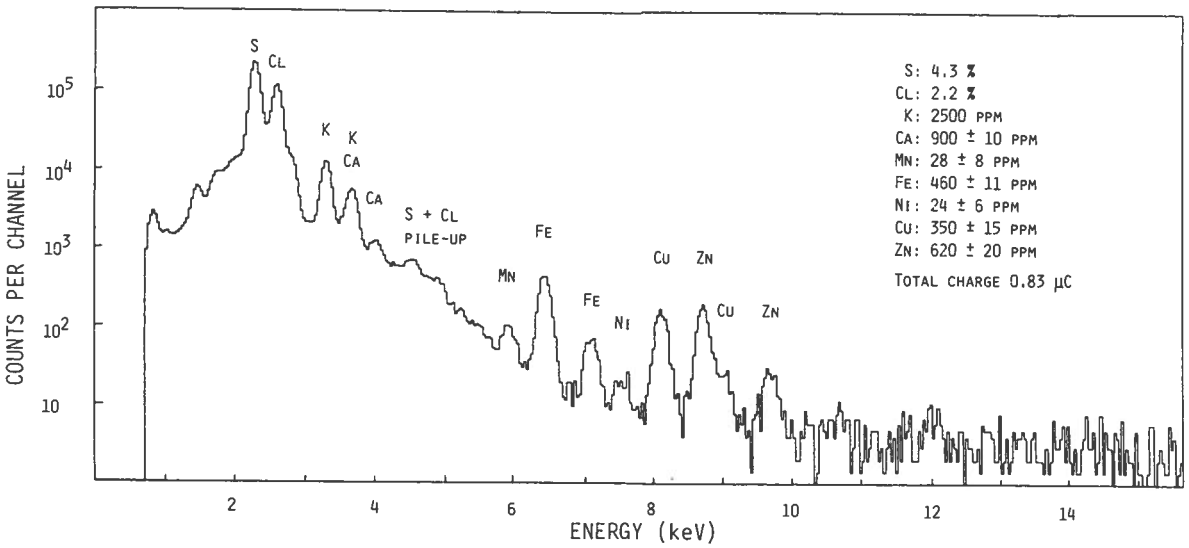

Fig.4. PIXE spectrum formed by summing the individual spectra obtained when scanning a thin human hair section. The total accumulated charge of protons was 0.83 μC. The elemental concentrations and uncertainties (one S.D.) are determined from this spectrum.

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estimated to be about 3 mg/cm^2. With a sample thickness of 3 mg/cm^2 only 18 % of the beam energy is absorbed by the sample, but the yield will be more then 55 % of that of a thick sample. With this sample thickness a surface barrier detector can be used to measure the energy of scattered protons, from which the sample thickness can be calculated. Since the yield of the $^{27}Al(p,p'\gamma)^{27}Al$ reaction is very sensitive to the proton energy, it is also possible to use the intensity of induced gamma rays from a beam stop of aluminium to determine the proton energy loss in the sample and thus monitor the sample thickness (4).

If a sample contains very high concentrations of elements with atomic numbers below 28 an X-ray filter with a hole can be used to decrease the solid angle of low-energy X-rays for the detector rather than decreasing the beam current. In this way a high sensitivity is maintained for heavier elements, while keeping the detector count rate at a suitable level.

Microprobe scan of human skin

In figs. 5a and b are shown the elemental depth distributions in the human skin sample.

The sample thickness (mg/cm^2), which is needed when calculating the elemental concentrations has been determined by comparison of the intensity of the bremsstrahlung continuum for different positions with that of the supporting foil, which has a known thickness (0.18 mg/cm^2). This continuum is proportional to the thickness of the irradiated material (24). The broken line in the figure shows the determined mass distribution with its maximum value in the dense stratum corneum. The zones of epidermis and dermis as determined visually in a light microscope are indicated in the figures. As can be seen in the diagrams the statistical uncertainties are very small for the elements phosphorous, sulphur, chlorine and potassium which occur in high concentrations in skin. For calcium the uncertainties are more important due to overlapping between the large potassium K-beta peak and the calcium K-alpha peak. The trace elements iron and zinc are plotted in fig.5b.

The phosphorous concentration, which is probably correlated to the phospholipids of the living cells, is low in dermis and has a maximum in the lowest part of the epidermis, stratum germinativum, where the cells are formed. The phosphorous concentration further out decreases slowly to show very low values in the stratum corneum where the phospholipids in the living cells are replaced by ceramides in order to form the barrier of the horny layer (28). The sulphur distribution shows only smooth variation with a slight maximum in the border region between dermis and epidermis but with a significant increase in the stratum corneum.

The potassium concentration is rather constant in the scanned section except in the outer part where it decreases in a similar fashion to phosphorous showing very low values in the stratum corneum. Chlorine is high in the dermis and decreases to a level which is constant through the whole of the epidermis.

Calcium is below the detection limit in the inner half of the epidermis, but the calcium distribution shows a steep increase in the outer part with a maximum at the surface of the stratum corneum. This may be due to an endogenic increase in the horny layer but the shape of the distribution, reminiscent of a diffusion gradient, may also be explained by external contamination from, e.g. domestic water supply with a high calcium content.

In fig.5b it is clear that lower detection limits would be required to reach any decisive conclusions regarding the distribution of the trace elements. However, both zinc and possibly iron seem to increase in a fashion similar to

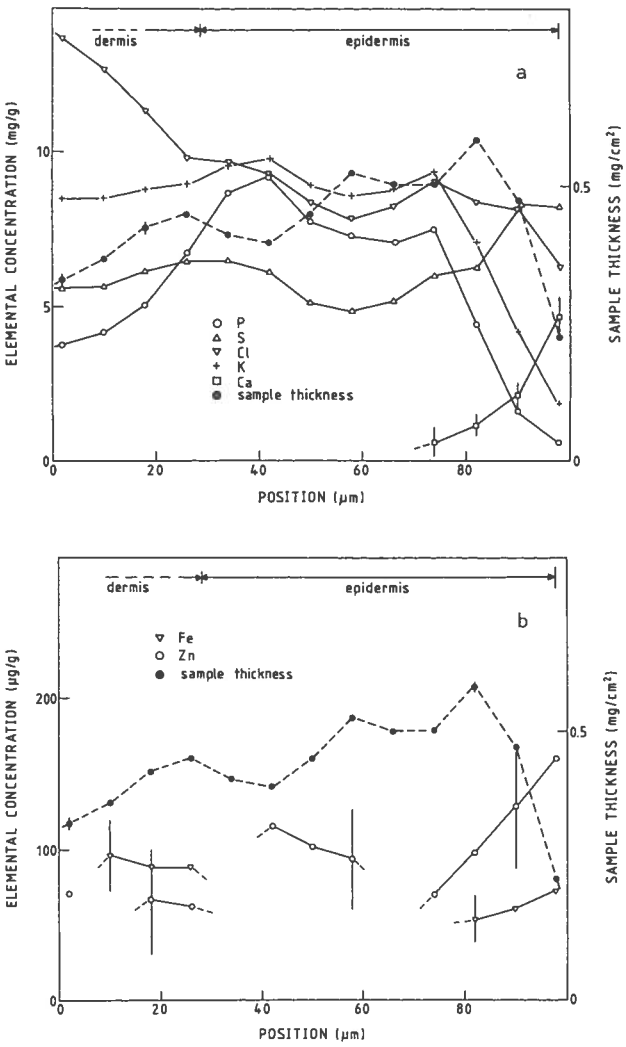

Fig.5 a/ Elemental distributions in normal human skin as determined by a proton microprobe with a beam area of 10x100 μm^2 having the long side parallel to the skin surface. The broken line shows the sample thickness (right scale) as determined from the background in the PIXE spectra. The solid lines are drawn to guide the eye and the error bars represent the uncertainties due to pulse statistics (one S.D.).
b/ As fig.5a except that the trace elements Fe and Zn are sometimes below the detection limits and are then not marked. Detection limits for the two elements are between 30 and 50 ppm. Error bars showing one S.D. are occasionally given to show the magnitude of the uncertainty.

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calcium in the stratum corneum, indicating possible exogenic contamination and diffusion. By improving the detection limits by a factor of five (to about 5 to 10 ppm), which may be achieved by increasing the beam intensity, irradiation time and the solid angle of the X-ray detector (see also the section "Cross sectional hair scan"), the distribution of several metals, such as chromium, manganese, iron, nickel and copper, may be determined. This is of interest in, e.g. occupational dermatology, in which mechanisms of uptake of metals through skin is very important.

The results from this study show agreement with those of e.g. Forslind et al (9) who studied the elemental distribution of several elements in guinea pig epidermis by the EMP. The distributions of the elements phosphorous, sulphur and potassium behave similarly to ours, while some elements, sodium and magnesium, were not detected in our study. In their investigation Bischof et al. (2) studied the whole skin (0.5 - 1 mm thick) with a rather limited spatial resolution. Interesting results, however, were obtained in this scan when passing a hair follicle deep in dermis. It was shown that sulphur, chlorine and zinc concentrations were high in the hair cortex, while the phosphorous concentration was high in the follicle region surrounding the hair strand, where cell formation takes place.

As far as detection of the lightest elements are concerned there exist no other restrictions in PIXE-analysis than in the other X-ray techniques. Except for a thin detector window it is also required to keep the sample very thin to reduce self-absorption of the very soft X-rays from elements like sodium and magnesium. However, for high concentrations of sodium and rather thick samples it is possible to use a complementary technique simultaneously with PIXE-analysis. By using 2.5 MeV protons and detecting gamma rays from the inelastic reaction $^{23}Na(p,p'\gamma)^{23}Na$ (E = 439 keV) in a Ge(Li) detector and calibrating with a sample of known composition it is possible to detect less than 50 ppm sodium in short macrobeam irradiations (5). For the microbeam irradiations the total accumulated proton charge is 50 to 100 times less than for macrobeam analysis and consequently the estimated detection limits will be approximately 300 to 500 ppm. Since this technique is not sensitive to attenuation it is quite promising for use in combination with the PMP in the analysis of skin. Further experiments in this field will be carried out in the near future.

For magnesium, the second light element of relevance for skin characterization, our present microbeam detection limit in thin samples is around 800 ppm. This value may be improved by using a detector with a thinner window inside the chamber (or a windowless detector).

PIXE versus EIXE

EIXE and PIXE are closely related techniques. However there are important differences between the two methods.

Although the EMP can be focused down to a diameter well below 0.1 μm the effectively excited volume has a considerably larger extension due to the pear-shaped form of the excitation volume.

However, in thin sections localization of the electron probe within a subcellular compartment such as a mitochondrion is feasible and makes it possible to detect gradients of elements within the cell envelope. Whereas the EMP has the advantage of a precise localization even within subcellular compartments the PMP can only be focused down to a width of a few micrometres. In some microprobes (22,26) it is possible to work with a resolution of 1 - 2 μm, but with a further limited beam current. For samples not extremely thin, such dimensions are rather competitive to the electron probes since for protons no significant degradation of resolution occurs due to scattering within the sample.

The sensitivity of the EMP system is such that trace elements cannot be determined without certain preparatory measures such as micro-incineration. In applications to biological material the method has an uncertainty in the determinations at a 10 % level. The minimal amount of an element that can be detected is at optimal conditions of the order of 10^{-18} g, the minimal concentration detectable lies in the range 0.02 - 0.2 % depending on the particular element and the type of specimen under analysis. The proton microprobe, on the other hand, has minimal detectable concentrations of the order of 1 - 100 ppm. The quantification technique is absolute and no standards are required, since the number of protons hitting the sample can usually be measured. The uncertainties are normally below the 10 % level. With high beam densities and very small beams (1 μm) the minimum detectable absolute amount is of the order of 10^{-16} g. When selecting the proper technique great care has to be taken when considering whether very high spatial resolution is required or if a less resolved beam but more sensitive analysis is suitable. Comparative analysis on the same targets from skin and its appendages with both techniques are under way at our laboratories.

Conclusions

Using a proton beam of millimetre dimensions the longitudinal distributions of many elements in human hair can be determined non-destructively. Thus measures of exposures and deficiencies with a time resolution of less than one week are offered. Such information may also be of interest for forensic investigations and in environmental and occupational health research. In contrast to the electron microprobe which can be used well below 0.1 μm resolution it is with the proton microprobe only possible to attain a spatial resolution of about a few micrometres. This implies a restriction when using a PMP in sub-cellular compartments. However, due to its significantly higher relative sensitivity the PMP may be used instead of or complementary to the EMP in many applications in dermatology.

In this work a radial scan of a thin hair section from a subject with high iron concentration in the domestic water supply has been performed using a proton microprobe. Several interesting results were found regarding the distribution of elements such as calcium, iron and zinc. A comparison with some other investigations made in thick hair sections shows similar distributions except for the very sharp peak concentrations found in this study.

In a scan of a thin section of normal human skin the elemental depth distributions were in good agreement with those from EMP studies on guinea pig skin. With the PMP method elements like calcium, iron and zinc were detected. With minor improvements of the analytical procedure we estimate that there is a substantial probability of detecting other trace metals at the 5 to 10 ppm level.

Although the material reported in this work is rather limited, it is together with results from other studies discussed possible to establish that the high sensitivity of PIXE analysis makes it a valuable tool in quantitative analysis of dermatological materials.

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Discussion with Reviewers

B. Gonsior: In all interdisciplinary work there is the problem of correlating the possibilities of the method and the way of thinking of one discipline with the philosophy of research in the other discipline. Could the authors comment on research lines they plan to follow in dermatology beyond data collecting and comparison with the body of knowledge in this field? Using this as a starting point is quite all right.

Authors: The physiological regulation of cell metabolism in single strata of the stratified epidermis is of importance for understanding the reactions leading to skin changes in contact skin reactions. No physiological probe allows detection of changes in, e.g. calcium levels which means that elemental analysis in quench-frozen sections is at present the only acceptable means of assessing elemental redistribution in a single cellular stratum. The electron microprobe is not sensitive enough to allow assessment of changes in calcium and magnesium levels at statistically significant levels.

Consequently, PIXE has been the method of choice in the search for methods allowing such determinations in normal and diseased skin. We expect to be more successful in this respect in the future when we have accumulated more experience in the analysis of skin.

Concerning trace element analysis studies on the distribution of elements in normal hair and in hair from patients with systemic (as well as systemic genetic) disease(s) we are investigating the possibilities of elemental analysis as a resource for precise clinical diagnosis. Studies of hair cross sections will lead to an understanding of the effects of external contamination as contrasted to endogeneous up-take.

B. Gonsior: What are the detection limits obtained by the authors, (a) with normal beam, (b) with beam in helium, (c) with microbeam?

Authors: For the single hair strand irradiated in helium gas (b) the detection limit (3 S.D.) for mercury was 8 μg/g (peak area: 5 pulses; proton current: 4 nA/mm^2; hair section length: 2 mm; analysis time: 100 seconds). By scanning the hair back and forth with a speed of 1 mm/second the time can be reduced by a factor of 2 - 3. With vacuum analysis (a) approximately 4 times longer

time is required to obtain the same spectrum (the helium gas has negligible influence on the spectrum shape).

The detection limits for one of the points in the microbeam hair scan (c) are given in figure 6.

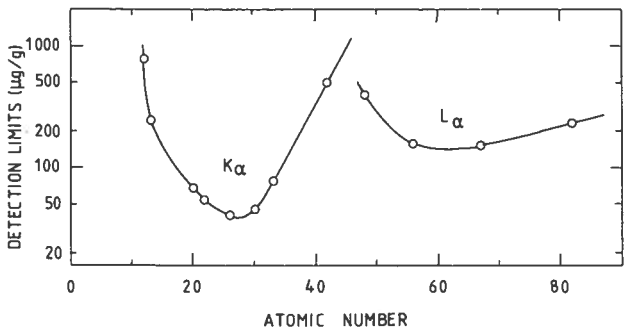

Fig.6. The detection limits for our present microprobe system. The analysis parameters were: time 250 s; current 0.2 nA; count rate 400 pulses/s; detector solid angle 0.0179 sr; sample thickness 0.5 mg/cm^2. The detection limits are defined as three times the uncertainty of the background in a 2 FWHM region under the peak of interest. The gaussian components of the characteristic peaks are not included in the background.

For a thick sample and equivalent conditions the detection limit for heavier elements (Z>22) would be 10 times lower, however, the energy deposition in the sample would increase by a factor of about 50.

J.A. Swift: It seems remarkable for so many elements in PIXE analysis of hair cross sections (both your work and that mentioned under reference 6) that there is apparently an annulus about 10 µm below the hair surface containing the element at much higher concentration than in the rest of the section. In your own work for example the zinc concentration is nearly one order of magnitude greater in this annulus than at the centre of the hair section. Can this be explained in biosynthetic or structural terms or is it possible that this annulus is the artifact caused by an 'edge' effect between the hair surface and the supporting medium?

Authors: This is an interesting question which obviously deserves attention. Several explanations for this non-uniform distribution may be offered. The cortex cells of the outer annular zone are derived from matrix cells low down on the papilla of the root whereas those of the centre of the cortex derive from areas close to or at the tip of the dermal papilla. The different areas of the matrix cells on the papilla may, due to differences in diffusion path distances from the capillary bed of the papilla, have different chances of elemental uptake.

One of the obvious reasons for an uneven distribution may be related to an uneven distribution of the mass of the keratin matrix which is probably the supporting structure for the incorporated trace elements (metals). As yet we have not correlated the assessed amount of trace elements to the corresponding mass distribution as measured by stereological methods. In our experience we have found that in general the centre of a hair fibre has a much more loose arrangement of the cortex cells than is seen at the fibre periphery. Open intercellular spaces may thus be observed in the centre of the hair fibre whereas the cells of the periphery are closely adjoined leaving no open intercellular voids.

The external contamination is an interesting problem. To the best of our knowledge, so far no systematic study of the barrier offered by the cuticular cells to metal ions has been performed. Such a study also involves the problem of possible cuticular damage due to weathering which may drastically change the barrier capacity of the cuticle. The cuticular cells are definitely permeable to amino-acids such as cystine as previously shown (cf Forslind B. (1971). Electron microscopic and autoradiographic study of s-35-L-cystine incorporation in mouse hair follicles. Acta Dermatovener 51, 9-15 and Forslind B, Lindström B, and Swanbeck G. (1971). Microradiographic and autoradiographic studies of keratin formation in human hair. Acta Dermatovener 51, 81-88). We do not know if the cuticular cells offer any permeability resistance to metal ions .

The uneven distribution is not an 'edge' effect since the supporting medium contained no recorded traces of the elements determined within the hair cross sections. It would, however, be possible to get unreasonably high values of concentrations exactly at the surface of the hair since a high concentration of an element situated at the surface of the hair cannot be normalised to the matrix in a meaningful way.

J.A. Swift: I am concerned about the very considerable dwell times of the proton beam on the hair during studies of linear distributions of elements (200-500 seconds). Horowitz et al (reference 11) used a method of rapidly scanning the hair about the beam to do this job and to dissipate beam power over a wider area without compromising either spatial or elemental resolution. Have you examined your hairs in the SEM to see whether there is any damage inflicted by the proton beam?

Authors: When PIXE analysis is applied to single hair strands as described in the text the problem of beam damage is highly significant. Precautions are taken in using helium gas, an excellent cooling medium, in the irradiation chamber during bombardment. We have also used a SEM to examine hair strands irradiated under different conditions. As seen from fig. 7 we observe phenomena like 'swelling' or 'bursting' of the irradiated part when too high current densities are used. It is likely that some elemental losses occur at excessive current densities particularly in vacuum.

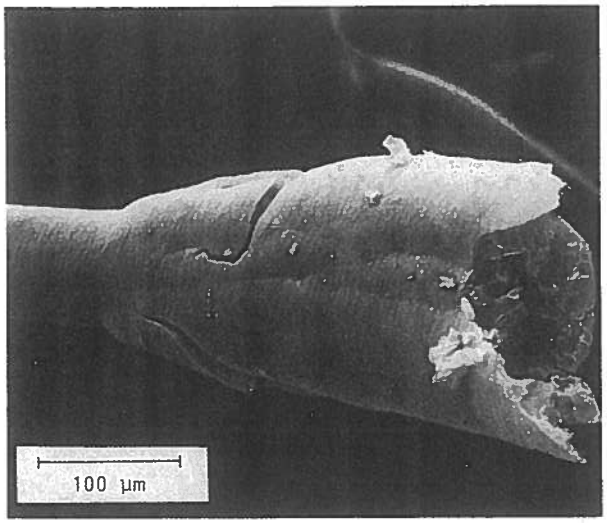

Fig.7. SEM picture of a hair bombarded with 2.5 MeV protons in vacuum; intensity: 2-3 nA/mm^2. The hair was not broken until it was mounted for the SEM.

For acceptable current densities no changes on the hair was seen in the SEM. The method cited in reference 11 has later been introduced into our irradiation facility and the estimated increase of the maximum current density is about a factor of 3. (cf Li HK, Malmqvist KG, Carlsson L-E and Akselsson KR. (1983). A PIXE system for routine longitudinal scanning of single hair strands. In: Proceedings of Third Int Conf on Particle Induced X-ray Emission and its Anal. Applic, will be published as a volume of Nucl. Instr. and Meth. in Phys. Res. in 1984.)

J.A. Swift: Some of the references you quote (particularly numbers 19,20 and 21) are not generally available outside Sweden. Since the information in them is the key to your work and of general interest can you give a brief synopsis of them? This particularly concerns the special algorithm for determining hair diameter from the particle energy spectra and the principles for calculating surface-to-bulk ratios of elements.
Authors: The references quoted will be submitted shortly (in 1983) for publication in Nucl. Instr. and Meth. in Phys. Res. In the present work these references are mainly relevant for the minor part concerning longitudinal scans of single hair strands.

In reference 19 it is shown, theoretically as well as experimentally, that the intensity of protons back-scattered from a thin surface layer of the hair, which corresponds to the number of counts between two chosen channels in the observed proton back-scattering spectrum, is linearly proportional to the hair diameter.

In reference 20 the significance of the geometrical hair diameter for calculating correction factors for proton slowing down and X-ray attenuation is shown. In reference 21 a semi-quantitative method for determining surface-to-bulk ratios of elements is discussed. By analysing with 2 or more different proton energies and comparing the yields of characteristic X-rays from an element at the different irradiation energies it is possible to reveal whether an element is homogeneously distributed in the hair matrix (bulk) or mainly situated at the surface.

J.A. Swift: What is the mean free path of 2.5 MeV protons into hair and skin?
Authors: The total range of 2.5 MeV protons into hair is about 10.8 mg/cm^2 and in skin about 10.2 mg/cm^2. The difference is mostly due to the different hydrogen contents. In the 1 mg/cm^2 samples in microbeam applications the protons lose approximately 5.5 % of their initial energy and this causes the X-ray production to decrease by 2 to 4 % from the front to the back surface.

G.J.F. Legge: In elemental microanalysis of biological tissue, extreme precautions must usually be taken to preserve not only the morphology but more importantly the elemental integrity of the sample. Although hair was quench frozen and cryosectioned, it was first swelled in distilled water and later thawed and air dried - all processes of some risk with most biological tissues. What were the justifications for these treatments of this tissue?
Authors: The cross sections of the hair studied were put dry into the embedding medium immediately before the freeze-quenching and subsequently freeze-sectioned (We have unfortunately expressed ourselves vaguely - it is the carboxy methyl cellulose which is swelled in distilled water). Effectively the sections were freeze-dried in the cryo-chamber for approximately half an hour before they were fixed to the Kimfol foil at ambient temperature. Seen in the preparative microscope the thawing involved no detectable melting of ice. We are therefore confident that thawing has little effect on the elemental distribution in hair cross sections. However, it must not be inferred that this particular preparation method can be applied to other tissues which are not dehydrated in the natural state as are hair fibres.

G.J.F. Legge: Mention was made of reducing detection limits partly by increasing current density. The problem of beam heating was also mentioned. What evidence is there concerning the ability of hair, in vacuum or in helium atmosphere, to withstand bombardment by high density proton beam, particularly in spot (unscanned) mode? Is there any evidence on possible losses of elements or mass from the samples during bombardment? How repeatable were the small variations in sulphur distribution mentioned for the cross sectional hair scan?
Authors: In order to increase current density in longitudinal hair scans it is necessary to perform analysis in rapid scanning mode. Such a system is at present being installed in our irradiation facility. According to preliminary tests the current density can be raised by a factor of 3 in scanning mode relative to the stationary mode (Li HK, Malmqvist KG, Carlsson L-E and Akselsson KR.

(1983). A PIXE system for routine longitudinal scanning of single hair strands. In: Proceedings of Third Int Conf on Particle Induced X-ray Emission and its Anal. Applic, to be published as a volume of Nucl. Instr. and Meth. in Phys. Res. in 1984).

Since our microbeam facility is not equipped with a beam scanning system we have deliberately been using moderate current densities (normally 0.15 nA on a 4x15 μm^2 area). We have not observed any signs of long-term losses of mass or elements. Systematic tests of possible elemental losses have been carried out by Legge (Legge GJF, In: The Scanning Proton Microprobe. Microbeam Analysis - 1980, San Fransisco Press Inc, 1980). He used an extreme current density (10 nA on a circular beam area with a 12 μm diameter) on a 5 μm thick section of biological matrix in an unscanned mode. The results show no significant losses of mass but very rapid (within a few seconds) losses of some elements, e.g. chlorine. Another systematic long-term test of beam effects on hair sections was carried out by Bos et al (Bos AJJ, van der Stap CCAH, Valcovic V, Vis RD and Verheul H. On the incorporation of trace elements into human hair measured with micro-PIXE. In: Proceedings of Third Int. Conf. on Particle Induced X-ray Emission and its Anal. Applic., to be published as a volume of Nucl. Instr. and Meth. in Phys. Res. in 1984). With a one-hour irradiation in unscanned mode at 0.5 pA/ μm^2 (beam area 5x12 μm^2) on a 30 μm thick section of hair no significant losses of the elements S, Cl, Cu and Zn were detected.