Simultaneous Determination of Trace Elements in Lavage Fluids from Human Bronchial Alveoli by Energy Dispersive X-Ray Fluorescence. 1: Technique and Determination of the Normal Reference Interval

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We have applied energy dispersive x-ray fluorescence to the analysis of broncho-alveolar lavage fluids, to determine the concentration of several elements simultaneously with manipulation of the sample minimized. A 20-μL sample of a homogenized lavage fluid, to which two internal standards (containing Y and V) have been added, is deposited on a polypropylene film, 4 μm in thickness, and analyzed by x-ray fluorescence. We report the analytical procedure, the detection limits, and the reproducibility. The normal concentration range for trace elements is reported for a selected population. The simplicity and rapidity of the method, and the possibility of automating measurements, make this procedure suitable for screening large numbers of lavage specimens and to establish the diagnosis of some pneumonioses.

Additional Keyphrases: screening • pneumoniosis • Y and V as internal standards • lung disease

Pulmonary disease related to exposure to inorganic dust has long been recognized, and in recent years it has received increasing interest. The broncho-alveolar washing technique developed some years ago to characterize certain pathological reactions, if associated with a highly sensitive analytical procedure, could be useful in establishing or confirming the medical diagnosis of pneumonioses. This procedure, a nonsurgical method, allows rapid examination of patients without risk (1), but only a small quantity of cells is collected. This accentuates the need for a sensitive and reliable procedure that requires only small samples. The energy dispersive x-ray fluorescence offers the possibility of simultaneously determining the trace-elements in a small sample size, with very low detection limits.

The apparatus developed in our laboratory and the experience we have accumulated in inorganic analysis have led us to select the technique described here and to evaluate its features. We analyzed some 80 samples to determine the normal inorganic composition of broncho-alveolar lavage fluid. The patients were selected by physicians of the pneumology departments.3,4 The analyses were performed on lavages obtained for other diagnosis purposes.

Materials and Methods

Technique of Broncho-alveolar Washing

This is a simple technique, for which there are but few contraindications, such as severe obstructive disease and severe respiratory insufficiency. Complications are unusual and the technique is harmless if the contraindications are respected (1).

The broncho-alveolar washing was performed after intramuscular injection of atropine and diazepam and local anesthesia with tetracaine solution. A B3 Olympus fibroscope was wedged into the distal branch of the lingula. Four 50-mL portions of sterile physiological salt solution, warmed to 37 °C, were introduced, and after each of them the liquid was recovered in a vacuum trap (negative pressure, 50 cm of water). When the quantity of the liquid recovered was too low (less than 50 mL), two supplementary 50-mL fractions were injected. The liquid was immediately centrifuged (600 × g, 10 min). The supernatant fluid was separated from the cell-containing pellets, which were then washed with an "albumin-Tris" mixture. This mixture consisted of 10 mL of a mixture containing, per liter, 37.5 g of Tris, 69.5 g of NaCl, and 37 g of KCl, plus 1 mL of a 33.33 mg/mL solution of albumin in physiological salt solution, plus 89 mL of distilled water. Use of this mixture avoids any alteration in the cells in the aspirate. The cells were centrifuged down (600 × g, 10 min), the supernatant fluid was aspirated, and the cells were resuspended in 2 mL of the albumin-Tris solution. We used 50 μL of the suspension immediately to make a differential cell count (macrophages, lymphocytes, erythrocytes, etc.) in a Thomas cell; 950 μL was sent to the cytology laboratory for histological control; and the last 1 mL, plus 1 mL of the Tris–albumin solution, was used in the analysis for trace elements.

This method was followed by both pneumology departments, with use of the same products. For each sample, a qualitative control of the supernate and the albumin–Tris solution was performed. The total number of cells and erythrocytes was determined. To detect any potential sample contamination, we tested the liquid after each step, and also checked all the products used to wash and sterilize the material. Results of all these tests were negative when the physicians and the analysts did not use examination gloves to minimize contamination with zinc (talcum powder used on some examination gloves contains zinc as a contaminant). On the other hand we did detect contamination in some samples, as is discussed below.

Equipment

The energy-dispersive apparatus we used was a prototype developed primarily for analysis of small samples, to attain detection limits in the microgram per liter range. It has been described previously (2). In this study, a molybdenum filter, 150 μm thick, eliminated the x-ray continuum

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Received October 29, 1984; accepted January 23, 1985.
(Bremsstrahlung). For all measurements the Mo x-ray tube was set at 40.0 kV and 20.0 mA. Results did not differ between measurements with air in the x-ray path or under reduced pressure. Accumulation of the data from an x-ray irradiation took 300 s.

Sample Preparation and Assay

The viscosity of various specimens of broncho-alveolar lavage fluid differed greatly. To avoid pipetting problems and to prepare a homogeneous suspension, we homogenized all lavages at 2500 rpm in a Teflon grinder adapted for use with centrifugation tubes (Figure 1). To an aliquot of the suspension we added an equal volume of doubly distilled water. For quantification, we diluted a 500-μL aliquot with an equal volume of doubly distilled water that contained two internal standards: yttrium 4.50 mg/L and vanadium 50 mg/L, added as NH₄VO₃ and Y(NO₃)₃ (Figure 2). Both these standards were "pro analysis" grade (Merck, Darmstadt, F.R.G.). We pipetted 20 μL of this mixture onto a 4-μm-thick polypropylene film suspended on a Teflon ring and dried this at room temperature, protecting it from contamination with dust.

Results and Discussion

Internal Standards

The simultaneous use of two internal standards such as V and Y provides a valuable control of both the calibration of the spectrum and the influence of matrix. The equivalent dilution of the sample helps minimize the background continuum and approaches "thin film" conditions (3, 4), so that the analysis can be performed on a very wide range of cell concentrations. The peak intensities are automatically normalized with respect to yttrium for the "high energy elements" (26 < Z < 53 and 70 < Z < 92) and with respect to vanadium for the "low energy elements" (20 < Z < 28 and 53 < Z < 70) by the "Super ML" program (Northern Tracer, Middleton, WI 53562). This is a "Flextran" program for a rapid on-line analysis of x-ray spectra. It makes use of a Flextran version of the NS-880 ML (multiple least squares) analysis technique to obtain accurate measures of peak intensity in instances where continuum or peaks overlap (5), and in effect it resolves interfering peaks by comparison with stored information on reference analytes. Such information was obtained by analyzing a 200 mg/L solution of the pure element (Merck's "Titrisol" or Merck's "Suprapur").

We gave special attention to the calibration of the unknown and reference spectra (± 1 eV). However, the "Super ML" program includes a "chi-squared" value, which indicates the presence of a systematic error such as peak shift, distortion, or the presence of an unreferenced peak. We quantified all elements by the method of known additions, using the same solution as for the reference spectra. In this way we determined a coefficient for each element so that the ratio of that element to yttrium or to vanadium could be expressed directly in nanograms per milliliter. Afterwards we calculated the results in nanograms per 1000 cells.

Calibration Curves

Analysis of the broncho-alveolar lavage fluid (Figure 2) shows the presence of Cl, K, Ca, Fe, Cu, Zn, and Br. But Cl, K, Ca, and Br are also present in the physiological salt solution introduced into the lungs and in the albumin-Tris buffer. So we did not quantify these elements. We detected also Ni, Pb, Rb, Mn, and Ti, but in very small amounts. The calibration curves, obtained by the method of known additions, were determined by linear regression of readings vs added concentrations, such that the sum of the deviations of the individual readings from the straight calibration line was set equal to zero while keeping the sum of the squares of the deviation at a minimum. For Fe, Cu, Zn, Ni, Pb, and As we used the same sample to establish the calibration curves. For Mn, Se, and Rb additional lavage fluids were required.

In this paper we report the results for the calibration curves of the elements present in the broncho-alveolar lavage fluids from patients who were not particularly exposed to inorganic dust, and also of Se and As. We could not prepare a calibration curve for Ti because we could find no reference solution of Ti that was chemically compatible with the lavage fluid, and Ti suspensions were not adequate for preparing a calibration curve in the concentration range of the samples. Thus the values for Ti are only semi-quantitative, derived from the Ti/V ratio of the reference. Table 1 shows the reproducibility of the calibration points, and Figure 3 shows the different calibration curves, with the correlation coefficients for the fitted values in the legend to the figure.

We compared the present technique with electrothermal
atomic absorption spectrometry for determination of trace elements in aqueous solutions (6). For purposes of comparison, we analyzed a broncho-alveolar lavage, treated with nitric acid, by graphite-furnace atomic absorption spectrophotometry. Values for Fe, Cu, Zn, and Pb as determined by the two techniques agreed within 5%. However, there is no standard sample for broncho-alveolar lavage, and the spectrophotometric analysis requires ashing, so this comparison should be regarded conservatively, although it is valid for solutions treated with nitric acid. Finally, our results agree well with those obtained from the relative x-ray excitation curve, as described previously (2).

Analytical Variables

**Precision and reproducibility.** To evaluate the precision of the apparatus we counted the same sample five times, keeping all conditions constant; to evaluate the precision of the method, we counted one sample eight times, rotating the sample horizontally each time by 45°. To assess reproducibility, we analyzed five different aliquots of the same lavage fluid. For all these investigations we used a lavage containing an average number of cells and erythrocytes (12 000 cells/mm³, 2300 erythrocytes/mm²), and with an average concentration of iron, zinc, and copper per 1000 cells.

Table 2 lists the results. Copper and zinc show the highest coefficient of variation for the precision of the apparatus. So, errors due to sample inhomogeneity are negligible. The coefficient of variation for the precision of the method is primarily ascribable to the fact that, in rotating the sample, it is removed from the exit of the collimator of the x-ray tube. The small quantities handled and the low concentrations of some of the elements increase the likelihood of errors due to contaminations. However, to detect accidental contamination by metallic particles liberated by the fibre-optic or introduced during the centrifugation process (Ni, Pb, Ti), or due to the micro-pipette tips (Zn), we analyzed each

Table 1. Precision of the Method for Mn, Se, Rb, Ni, As, Pb, Cu, Zn, and Fe

<table>
<thead>
<tr>
<th>Element</th>
<th>Precision of the apparatus</th>
<th>Precision of the method</th>
<th>Reproducibility</th>
<th>Detection limit for 20-μL sample</th>
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</thead>
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<tr>
<td>Mn</td>
<td>mg/L CV, %</td>
<td>mg/L CV, %</td>
<td>mg/L CV, %</td>
<td>mg/L CV, %</td>
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<tr>
<td>Fe</td>
<td>72.85 1.3</td>
<td>70.15 3.6</td>
<td>69.00 3.3</td>
<td>0.140</td>
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<tr>
<td>Zn</td>
<td>0.75 0.5</td>
<td>0.73 10.8</td>
<td>0.74 5.6</td>
<td>0.045</td>
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<tr>
<td>Cu</td>
<td>0.15 27.9</td>
<td>0.16 23.3</td>
<td>0.17 9.1</td>
<td>0.060</td>
</tr>
<tr>
<td>Pb</td>
<td>0.17 34.7</td>
<td>0.15 16.9</td>
<td>0.17 38.1</td>
<td>0.080</td>
</tr>
<tr>
<td>Mn/V</td>
<td>0.190</td>
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<td>Ni/V</td>
<td>0.075</td>
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<tr>
<td>Cu/Y</td>
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<tr>
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<tr>
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<tr>
<td>Ni/ne/V</td>
<td>0.023</td>
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<td></td>
<td></td>
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</tbody>
</table>

*Mean of five analysis of the same sample, with conditions kept constant.

**Mean of analyzing one sample eight times, rotating the sample horizontally each time by 1/4 × 360°.

*Detection limit for 20-μL sample.

Table 2. Precision, Reproducibility, and Detection Limits

**Fig. 3. Calibration curves for Fe, Cu, Zn, Ni, As, Pb, Mn, Se, and Rb, prepared by the method of known additions.**

Curves for Fe, Cu, Zn, Ni, As, and Pb were prepared by use of the same lavage; for Se, Rb, and Mn on two different lavages. Correlation coefficients for the fitted values: Se: 0.9996, Rb: 0.99999, Zn: 0.9994, Fe: 0.9990, Cu: 0.99991, Mn: 0.9996, Pb: 0.991, As: 0.9991, Ni: 0.9996. Variance for the fitted values: Rb: 0.0012, Zn: 0.0045, Se: 0.0025, Fe: 0.327, Cu: 0.0001, Mn: 0.0005, Pb: 0.0003, As: 0.0002, and Ni: 0.0001
sample four times. To minimize the risks of overestimates caused by undetected contaminations, we discarded the first drop pipetted. We also did not take into account, in our statistical treatment, those samples showing very large coefficients of variation for some of the elements, mostly Zn, Ni, Pb, or Ti. With these precautions, the present technique performs well for measurement of trace elements in broncho-alveolar lavage fluid.

Detection limits. Table 2 also shows the detection limits we obtained for the broncho-alveolar lavage fluids, calculated for the respective elements as described previously for human serum (2).

Distribution of the Concentration

With the appropriate coefficient, the "Super ML" program gives the results directly in nanograms per milliliter. Afterwards we related these amounts in nanograms per 1000 cells. Figure 4 shows the distribution of the concentrations for Fe, Cu, Zn, Ni, Mn, Rb, Pb, and Ti. In fact, in all determinations we analyzed simultaneously for 19 elements but never detected Cr, Co, As, Se, I, Ce, W, or Au. V, Y, and Br were also fitted, Br showing interferences with Se and Rb. We excluded results that differed from the mean for the other samples by more than 10 SD; e.g., one sample showed 3.5 ng of Pb per 1000 cells.

Influence of Blood

The broncho-alveolar washing per se always leads to a little alteration in the bronchial walls, reflected by the presence of various amounts of blood in the lavage fluid. To estimate the influence of this blood on our results, we prepared a series of blood dilutions. We chose blood with a mean value of 30.5 pg of hemoglobin per erythrocyte. The number of erythrocytes in the lavages we analyzed varied from 30 to 18300 per mm³, but mostly did not exceed 300 per mm³. Accordingly, we prepared a calibration curve for iron equivalent to that in as many as 25 050 erythrocytes. Zinc and copper were below the detection limits. Table 3 shows the results for iron, Figure 4 shows that the presence of blood did not seriously affect the analysis or the medical conclusions drawn from the results.

The major advantages of the present technique in determination of trace elements in broncho-alveolar lavage fluids are the low detection limits, the small sample requirement, the simplicity of sample preparation, and the ability to perform rapid (10 min) multi-element analyses without destroying the prepared sample. We could establish the normal inorganic composition of the lavage fluids from a wide population, information hitherto not available (7, 8). This procedure can be used to establish or confirm a diagnosis of pulmonary disease, and in wider surveys of occupational exposure of workers.

We are grateful to Messrs. Jecht and Oresans (Siemens A.G., Karlsruhe) and to Prof. Roegel (Service de Pneumologie, Centre Hospitalier Universitaire, Université Louis Pasteur, Strasbourg) for their support of our collaboration. We thank the Laboratoire d’Hydrologie of the Faculté de Pharmacie, Strasbourg, for financial support. This work was supported by grants from the Fond Spécial des Comités Départementaux contre les Maladies Respiratoires et la Tuberculose (contract no. 84 MR 10).

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