Development of a Screening System for Cystic Fibrosis

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We have developed a simple method for detecting high concentrations of chloride in sweat from ambulatory subjects, a measurement useful in the detection of cystic fibrosis. The method is based on the standard approach of stimulating sweat generation through iontophoresis of pilocarpine nitrate into the skin, followed by collection and analysis of the sweat for chloride concentration. The sweat-stimulating reagents are contained in polymeric gel pads, which are used in conjunction with a small battery-powered stimulator. The chloride analysis is subsequently done on the stimulated site by use of a thin test patch that picks up a fixed amount of sweat and changes color if the chloride concentration is higher than a predetermined value. The successful completion of a test is indicated by a fill tab, which changes color when the appropriate amount of sweat has been picked up by the chloride test patch.

Additional Keyphrases: sweat chloride - methods for outpatient testing - "kit" methods - pediatric chemistry - iontophoresis

Cystic fibrosis (CF) is a recessive trait that is carried by about one in 20 Caucasians. Screening for CF is not routine, despite its being the most common genetically inherited disorder in the United States, far more prevalent than phenylketonuria or other well-known genetic diseases. If totally untreated, afflicted children usually have a life expectancy not more than into the preteen years. With early diagnosis and treatment, on the other hand, their quality of life can be remarkably improved and longevity can be increased significantly.

Complete diagnosis for CF involves several tests. However, the primary test is analysis of the sweat chloride concentration (1). Normal individuals have sweat chloride concentrations below 30 mmol/L (96% below 30 mmol/L, the remainder below 60 mmol/L) with a mean value between 20 and 30 mmol/L (2, 3). However, individuals with CF have sweat chloride concentrations between 60 and 160 mmol/L with many exceeding 100 mmol/L (2, 3).

Most of the diagnostic techniques in use today involve the generation of sweat by iontophoresis with pilocarpine nitrate. The drug, at a concentration of about 0.2 mg/L, and an inert electrolyte solution are usually contained in gauze pads taped to the patient’s arm and connected to an electrical stimulator by wires. The stimulator requires current from a standard electrical outlet. The current and time typically necessary to drive an adequate amount of the pilocarpine nitrate into the skin are 1.5 mA for 5 min. Once the iontophoresis of the pilocarpine nitrate is complete, the wires and gauze pads are removed, the stimulated area is washed with distilled water, and a clean gauze pad is placed under a plastic cover and taped over the stimulated area of skin. Up to 100 mg of sweat is generally collected for analysis over a 30-min period (3). The sample can be analyzed for either sodium or chloride content; however, chloride is most often measured. Several techniques are available for chloride analysis, including titration (3, 4), coulometry (3), potentiometry (5, 6), and osmometry (7).

The accuracy of each of the above methods is adequate for the needs of the test. However, the instrumentation, while not complicated by today’s standards, is somewhat expensive and requires specially trained personnel. Thus, the standard tests must be performed at special locations (CF centers, hospitals, etc.) where the instrumentation and personnel are available. Several CF screening tests have been developed and reported earlier in the literature (8–14). However, according to Gregg and Boucher, none of these has been widely accepted (15). Gregg and Boucher also reported a new test method based on a commercially available chloride test strip (15), but in 18 years since their publication this method also has not found wide acceptance. Therefore, we felt it useful to develop a screening technique that might better meet the needs of clinicians. The availability of such a test method would not only make testing procedures simpler and faster, but also, by allowing a subject to be ambulatory, would make available a method more suitable for general screening of young children.

In the method we report here two components are used. The first is a battery-powered device that contains two adhesive gel pads impregnated with the chemicals necessary to stimulate sweat formation. (This device was described in detail by W. Warwick et al. at the Sept. 1981 meeting of the International Cystic Fibrosis Association in Bern, Switzerland.) The second is an absorbent patch that responds with a color change to chloride concentrations above a threshold value. The patch is applied directly over the area of skin stimulated for sweat formation and absorbs sweat directly, thereby eliminating the steps of collecting and transferring the sample.

Materials and Methods

One of the difficulties in designing a suitable test method is the small volume of sweat generated by any of the stimulating methods. A sample size of 100 mg is obtained in the best cases, but 50 mg or less is obtained from some individuals. We chose a standard test size of 50 mg for the initial patch design. Later, we also developed a smaller patch designed to hold 30 mg. Both test patches are similar except for their size. In this paper, we refer to the larger (50-mg) patch except where noted.

The test patch is shown in top and cut-away views in Figure 1. The sweat enters the test patch through a small hole in its center and diffuses radially through two circular regions. The inner circle is a blanking region; the region surrounding it is the indicator ring. The two regions are designed to absorb a total of 50 mg of sweat, of which 37 mg is finally present in the outer ring and 13 mg in the inner circle.

The blanking region holds reagents that complex an amount of chloride equivalent to the threshold concentration of chloride in the first 37 mg of sweat entering the patch. Thus, as the sweat enters the test patch from the center of the blanking circle, chloride is complexed by these

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reagents and effectively removed from solution. If the concentration of chloride is smaller than or equal to the threshold concentration, all of the chloride present is complexed. If, on the other hand, the chloride concentration is greater than this threshold value, the excess chloride is carried radially to the indicating ring of the patch. When the solution migrating into this region contains chloride, a color change occurs, indicating a positive test result. Migration of the solution into this area continues until the entire circular area of the patch is filled by sweat. Note that chloride present in the last 13 mg of sweat entering the patch is not complexed nor used as part of the test. The part of the sweat sample that eventually enters the indicating ring is the "active sample," and the part that remains in the blanking circle is the "inactive sample."

We found it necessary to know when the test patch had collected the proper amount of sweat and could be read. However, simply watching to determine when the patch was full was ambiguous. Thus we added a third region, a small tab at the perimeter of the indicating ring. When the third region fills with sweat, it changes color regardless of whether there is chloride in the sample.

Description of the Test Patch

The blanking circle is impregnated with silver phosphate. As the sweat enters this circle, the chloride is removed by the following reaction:

$$3\text{Cl}^- + \text{Ag}_3\text{PO}_4 + \text{H}_2\text{O} \rightarrow 3\text{AgCl} + \text{HPO}_4^{2-} + \text{OH}^- \quad (1)$$

The amount of silver phosphate is sufficient to react with the threshold concentration of chloride in the active portion of the sample.

Chloride in excess of the threshold amount is free to be carried to the indicating ring as more sweat enters the patch. Silver chromate in the ring reacts with this chloride to give a brown to white color change.

$$2\text{Cl}^- + \text{Ag}_2\text{CrO}_4 \rightarrow 2\text{AgCl} + \text{CrO}_4^{2-} \quad (2)$$

brown → white

The indicating ring also contains some soluble chromate. As the sweat fills the test patch, this excess chromate is carried into the fill indicator tab, which contains silver nitrate. The color change indicating test completion is a result of reaction 3.

$$\text{CrO}_4^{2-} + 2\text{AgNO}_3 \rightarrow \text{Ag}_2\text{CrO}_4 + 2\text{NO}_3^- \quad (3)$$

white → brown

A critical step in the fabrication of a patch is controlling the amount of silver phosphate in the center blanking circle. Dry, preweighed sheets of Whatman No. 20 Chrom paper (Whatman, Inc., Clifton, NJ 07014) are soaked in a silver nitrate solution and reweighed. The sheet is then soaked in a trough of sodium phosphate and allowed to dry at room temperature. Center blanking circles containing silver phosphate are punched out just before assembly.

A similar procedure is used to prepare the brown (silver chromate) paper used for the indicating ring. The amount of reagents impregnated into the paper in this area is not critical. After being impregnated with silver nitrate, the sheets of No. 20 Chrom paper are soaked in a potassium chromate solution. Excess soluble chromate is included for reaction with the fill tab indicator. Indicating rings of the desired dimensions are cut from the silver chrome sheets prior to assembly. The fill indicator tabs are cut from a sheet of No. 20 Chrom paper impregnated with silver nitrate.

The patches are assembled as shown in Figure 2. The blanking circle (D) is placed in the center of the indicating ring (C). A piece of polyester tape (A) is placed over the assembly and a fill tab (B) is laid in place. A second piece of polyester tape (E) with a 1-mm-diameter hole in the center is placed over the back of the patch, aligning the hole in the tape with the center of the patch. This is followed by a piece of double-stick tape (F) with a 1-mm hole in its center and a piece of spunbonded polyethylene material (G) with a series of 16 grooves radiating from its center hole. The 1-mm hole through the back pieces (E–G) is filled with a plug of filter paper (H). Patches are stored in polyethylene-lined foil pouches to protect them from light.

All solutions used in fabricating the patches and in performance-evaluation studies were prepared from reagent-grade chemicals and distilled water. The chloride solutions used in testing the patches (30, 40, 45, 50, 55, 60, 70, and 80 mmol of NaCl per liter) were standardized for chloride against a mercuric nitrate solution, with diphenyl carbazone as the indicator.
Results

To determine the performance characteristics of the test patches, we first simulated sweating with a series of solutions of known chloride concentrations. Preweighed patches were randomly assigned to eight different chloride concentrations: 30, 40, 45, 50, 55, 60, 70, and 80 mmol/L (n = 35 each). To simulate sweating skin, we used chloride-free sponges (Wellco TC-205; Wellco, Inc., Apex, NC 27502) saturated with the various test solutions. The assigned preweighed patches were randomly placed onto the appropriate sponges. The chloride solution wicked into the patch in about 20 min. When the fill-indicator tab turned brown, the fill time was recorded, the patch was removed from the sponge, the excess solution was wiped off, and the patch was reweighed.

Five observers read the results of the test as positive or negative by each of three methods: Method A: any break (i.e., color change) of the inner perimeter of the indicating ring is positive; Method B: 50% or more break of the inner perimeter of the indicating ring is positive; Method C: complete break of the inner perimeter of the indicating ring is positive. Any test not read as positive was said to be negative. There were no "no tests." Table 1 gives the results for this study for the three methods of reading the 280 patches.

Before proceeding to an extensive clinical study, a small trial was conducted at the University of Minnesota CF Center (16). The 50-mg size patch was tested, according to the procedure discussed above, on 20 patients (ages 11 months to 30 years), 10 of whom were known to be normal and 10 diagnosed as having CF. The patches collected an average (±SD) of 49.5 ± 3.3 mg of sweat in an average fill time of 35 min (range 22–67 min). All 10 normal subjects gave a negative test by Method A (i.e., no portion of the brown indicating ring had changed color). Nine of the diagnosed CF patients gave a positive reading by Method A. One of the known CF patients gave insufficient sweat for a test.

Several CF centers in the U.S. are now participating in the evaluation of the stimulating device and patches in a clinical environment. They are correlating the results of these tests with their normal procedures. The results obtained so far compare favorably with accepted procedures. A detailed analysis of these data will be published by the clinical investigators at the conclusion of their study.

Discussion

The patches were designed with a 45 mmol/L threshold value; i.e., a patch would give a negative reading for chloride concentrations <45 mmol/L and a positive reading for chloride concentrations >45 mmol/L. This is approximately the midpoint in the bimodal distribution of sweat chloride concentrations found in normal and CF individuals (17), and was chosen in an attempt to identify all CF individuals and minimize the number of normal individuals who should undergo further evaluation as a result of a positive test result.

For the patch to show a color change with chloride concentrations greater than the threshold value, we had to design it to complex all the chloride in the sample up to this value. The amount of blanking reagent that should be incorporated in the inner circle might, at first, seem to be the amount that would react with the chloride in 50 mg of sweat. However, the amount actually required is the amount that will react with the chloride in the first 37 mg, or the active portion of the sweat sample. This is adequate because the last portion (approximately 13 mg) of the sweat to enter the test patch migrates as far as the periphery of the blanking circle but does not enter the indicating region of the patch; thus, its chloride concentration is irrelevant to the test.

The higher the concentration of chloride in the sweat, the smaller the volume of sweat required to react with all the silver phosphate in the blanking region. Therefore, excess chloride will start migrating into the indicating ring of the patch earlier in the test, and progressively higher concentrations of chloride will produce proportionately larger areas of color change (Figure 3).

The rate of migration of the sweat through the patch seems to be reasonably constant. However, because it was difficult to determine exactly when the patch was completely filled, we added the fill tab at the edge of the indicating ring. Some of the soluble chromate in the indicating ring is carried with the sweat solution, regardless of the chloride concentration, to the fill tab. Knowing when the patch has

Table 1. Results of Laboratory Evaluation of the Test Patch for CF

<table>
<thead>
<tr>
<th>CI, mmol/L</th>
<th>Positive readings, %</th>
<th>Observers agreement, %</th>
<th>Positive readings, %</th>
<th>Observer agreement, %</th>
<th>Positive readings, %</th>
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<td>82.9</td>
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<tr>
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</table>

*See text for definition of test reading methods.

aPercent of all observers in agreement.
been filled to the proper level is important because the patch should be read before any significant amount of the unblanked chloride from the inner circle can diffuse outward and give a false-positive result.

Most of the difficulties in designing the patch were incurred because the patch was required to respond to concentration, which meant it had to pick up a constant amount of sweat. Furthermore, the amount of sweat available was relatively small. Therefore, the paper used in the patch had to meet three requirements. First, the diffusion rate had to be slow enough to permit time for the chloride-complexing reactions to take place. If the paper filled too rapidly, chloride that should have been "blanked" in the center circle would not have time to react with the silver phosphate and would reach the indicating ring, giving a false-positive result. The paper also had to have uniform flow characteristics so the sample would diffuse simultaneously and uniformly in all directions as it filled the patch. This characteristic was also necessary to allow for uniform impregnation of reagents during the fabrication steps. Finally, we needed a paper that would not absorb large amounts of water per unit volume. The size of the test patch should be 5 or 6 cm² at most and should hold no more than 50 mg of sample. Using these guidelines, we concluded that Whatman No. 20 Chrom paper performed satisfactorily. This paper also has sufficient strength to permit the gentle handling necessary during the impregnation process. This paper held 9.9 (SD 0.5) µL of liquid per square centimeter and therefore a circle 2.45-cm in diameter (5.1 cm²) would absorb 50.3 µL. The 1.27-cm diameter of the inner blanking circle was chosen on the basis of convenience and appearance.

The average fill-time (±SD) obtained from the laboratory studies (19.6 ± 5.4 min) should be considered the fastest realizable fill-time because of the instantaneous supply of sample. In actual use, the patch is applied to dry skin after stimulation, and the filling rate is dictated by the rate of sweating and transfer of the sweat to the center opening of the patch. The material for the back of the patch, a spunbonded hydrophobic polyethylene sheet, and the series of lines radiating from the center hole, were chosen to facilitate the collection of sweat from the stimulated skin. Even so, not all the sweat produced in a stimulated area is transferred into the patch.

Early indications from the preclinical and clinical studies were a review of the literature suggest that approximately 10% of the children tested will produce insufficient sweat to fill a 50-mg patch. Therefore, we also designed a miniaturized patch to hold 30 mg of sweat. Neither the chemistry nor the principles were changed, and the results of another laboratory study on 280 of these patches were similar to that in Table 1. This patch has not been evaluated as extensively as the larger one; however, we believe it will perform very well, with an accuracy equivalent to the larger patch.

We were also concerned about the uniformity of impregnation of the silver phosphate in the blanking region. We chose silver phosphate because it is relatively insoluble in water and would not migrate out of the blanking region with the solvent front. Electron microscopy of the silver phosphate-impregnated paper showed uniform crystals of 0.25 to 0.50 µm size. Energy dispersive spectroscopy confirmed that the distribution of silver throughout the paper was uniform.

The laboratory simulations of sweat generation were the easiest way to characterize patch performance. Because there was complete control over the concentration of chloride in the samples, we could evaluate performance at relatively close intervals of chloride concentration. As the

![Graph](image)

**Fig. 4.** Plot of percent of positive readings vs chloride concentration for Methods A (O ---- O), B (Δ - - Δ), and C (□ - - □)

See text for discussion of the methods.

data in Table 1 show, recognition of a positive test is a function of both the reading method and the chloride concentration. For the very low concentration of chloride none of the patches would be expected to be positive and, indeed, virtually all of the observers agreed with each other. Similarly, for the highest concentration of chloride, all the patches would be expected to be positive, and the observers again agreed. In the middle, where the concentrations of chloride were closer to the threshold value, observers disagreed most often. Moreover, the concentration of chloride at which there was most disagreement among observers moved from lower to higher values for reading Methods A through C, respectively. Thus, each reading method was best for certain chloride concentrations. When performance was analyzed by plotting the percent of positive tests vs the concentration of chloride, three sigmoidal curves were obtained (Figure 4). Ideally, these curves should be congruent and should approximate a step function with its transition occurring at 45 mmol/L. However, variations in the patches produce sigmoidal plots distributed along the concentration axis as in Figure 4. The inflection points for Methods A and B were 41 (SD 5) and 52 (SD 5) mmol/L, respectively; the curve for Method C was difficult to characterize, but the inflection point appears to be near 65 mmol/L.

The differences among the three curves are an indication of the following variations and limitations of the patches: amount of silver phosphate impregnated in the blanking circle; uniformity of fabrication; interferences with radial flow; variations in fill weight; and diffusion of free chloride from the inactive portion of the sample into the indicating ring. However, as the data show, the patches performed very adequately for the test needs. Only the region between where Method A is positive and Method C is negative leaves doubt and might require retesting.

We initiated this work because we think there is a need for a simple method of testing for cystic fibrosis in ambulatory subjects. The test developed appears feasible for testing and even routine screening of young children, as confirmed by the results presented here. This accurate and simple test patch for evaluating the chloride concentration in sweat after iontophoretic stimulation by pilocarpine nitrate should become a very useful diagnostic tool.

The development of the screening system was the joint effort of the following people, all at Medtronic: Terri Buyck, Pat Cahalan, Al Jevne, Rita Hirsch, Gary Lattin, Paul Sorensen, Richard Spevak, and the authors.
References