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A NEW TECHNIQUE FOR SWEAT CHLORIDE DETERMINATION USING MOHR'S SILVER METHOD: DEVELOPEMENT AND ANALYTICAL VALIDATION

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ABSTRACT

This study presents a new technique for determining chloride ions in the sweat test for the diagnosis of cystic fibrosis. The technique developed is based on Mohr's silver titrimetric method. The analytical development was performed on three quality control levels (C1, C2 and C3 at 25, 45 and 65 mmol/L respectively) and validated according to the requirements of ISO15189. Repeatability was estimated by the coefficient of variation (CV) at 2.45%, 3.12% and 2.21% for C1, C2 and C3. The CV estimated the intermediate precision at 4.12%, 4.51% and 2.63% for C1, C2, C3. The bias estimated the accuracy at 0.99% for C1, 2.76% for C2 and 2.55% for C3. The assays were performed following a linear calibration curve (equation: Y=0.0001 X + (3 x 10-5) with R^2 =0.9995) between 0 and 150 mmol/L. This new technique is a simple and fast alternative using stable and less toxic reagents than the mercurimetric technique.

Keywords: cystic fibrosis; sweat test; Mohr's method; analytical validation; sweat chlorides.

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1. INTRODUCTION

Determining sweat chlorides is a medical biology test essential in diagnosing cystic fibrosis (CF) [1, 2]. It is recommended in the case of a positive neonatal screening result or a clinical signs suggestive of the disease [3,4], such as meconium ileus, fatty diarrhea or later recurrent respiratory tract infections [3,5,6]. In 2019, 7280 cases were reported in France [7] at a rate of 1/4500 newborns [8]. Cystic fibrosis is an autosomal recessive disease caused by mutations in the gene coding for the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) protein, which regulates chloride ion transport at the apical membrane of epithelial cells [9, 10]. Indeed, patients with VD have abnormally high levels of chloride in the sweat glands (exceeding 60 mmol/L) [11]. This is due to the dysfunction of the chlorine channel present in the sweat glands [10,12]. **Table 1** lists the areas of clinical interpretation.

Table 1. Clinical interpretation areas for sweat chlorides in cystic fibrosis. [4,13,14]

Chloride concentration in sweat (mmol/L)	Interpretation
<40	Negative
40 – 60	Doubtful
>60	Positive

The sweat test is a dynamic test consisting of chemical or thermal stimulation of sweating followed by chloride ion determination by different techniques [15,16], including the titrimetric method of Schales and Schales using mercury nitrate as a titrant [17]. This study presents a new titrimetric technique for determining sweat chlorides using Mohr's silver reaction. This technique uses fewer toxic reagents than Schales and Schales method.

2. MATERIEL AND METHOD

2.1. Chemical reagents

The proposed technique uses silver nitrate ($AgNO_3$, 169.87 g/mol, *Sigma Aldrich*) as titrant, potassium chromate (K_2CrO_4, 246.19 g/mol, *Prolabo*) as a color indicator, distilled water (H_2O) as dissolution solvent, and potassium chloride (KCl, 74.55 g/mol, *Panreac Quimica*) for the preparation of quality controls and the calibration of the titrant solution.

2.2. Instrumentation

The technique was optimized and validated under real conditions on patients. The sweat collection and the introduction of quality controls were done on blotting paper discs calibrated to 5 cm in diameter. The dry and impregnated blotting discs were weighed on a 10^{-5} g precision balance from *Sartorius research.* The titrating solution was calibrated with a graduated burette made of *ASPIN* class A borosilicate glass with a capacity of 10ml and a tolerance of ±0.05 mL. The titration was done with a graduated burette made of borosilicate glass ISO 385 class AS with a capacity of 1ml and a tolerance of ±0.006 mL. Experimentation, calibration and control solutions were prepared in 20 mL and 50 mL volumetric Class A vials. The control solutions were introduced with a 150 µL automatic pipette (*AHN Biotechnologie GmbH*) with a tolerance of ±0.4 µL.

2.3. Preparation of solutions

The aqueous titrant solution of $AgNO_3$ was prepared at 0.01mol/L by dissolving 0.3397 g of $AgNO_3$ in a final volume of 200 mL of distilled water. The calibration solution (S_E) is an aqueous solution of KCl at 0.01 mol/L obtained by dissolving 0.1491 g of KCl in a final volume of 200 mL. The aqueous indicator solution was prepared at 1% w/v by dissolving 0.2g of $K_2Cr_2O_4$ in a final volume of 20 mL. Two concentrated calibration solutions S_{CE} and control solutions, S_{CC}, of 0.1 mol/L KCl, were prepared independently by dissolving 0.3727 g in a final volume of 50 mL. The aqueous solutions of the calibration range (05, 10, 15, 25, 45, 65, 75) mmol/L were prepared by diluting the concentrated solution (S_{CE}) of KCl to 0.1 mol/L, summarized in **Table 3.**

Table 3. Preparation of the calibration solutions.

Concentration levels (mmol/L)	**05**	**10**	**15**	**25**	**45**	**65**	**75**
Volume from S_{CE} (mL)	1	2	3	5	9	13	15
Final volume	20 mL						

A concentrated KCl (level E) solution for the linearity limit study was prepared at 150 mmol/L by dissolving 5.592 g of KCl in a final volume of 500 mL distilled water. The linearity limit of the method was investigated over a measurement range of 0 to 150 mmol/L. Dilutions from 2

stock solutions prepared the concentration levels:

- A solution containing the analyte to be determined at a concentration more significant than that expected for the upper limit (high-level E at 150 mmol/L KCl);
- A solution containing no analyte of interest or a concentration below the expected low limit (low-level B represented by distilled water).

Different dilutions (0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150 mmol/L) were performed in 20 mL volumetric flasks and are listed in **Table 4.** Preparation of the solutions for studying the linearity limit by diluting solution E (KCl 150 mmol/L) in solution B (distilled water) for a final volume of 20 mL.

Table 4. Preparation of the solutions for studying the linearity limit by diluting solution E (KCl 150 mmol/L) in solution B (distilled water) for a final volume of 20 mL.

Dilution (%)	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
Volume B (mL)	20	18	16	14	12	10	8	6	4	2	0
Volume E (mL)	0	2	4	6	8	10	12	14	16	18	20
Concentration (mmol/L)	0	15	30	45	60	75	90	105	120	135	150

2.4. Preparation of quality controls

Quality control solutions were prepared at 25 mmol/L for C_1, 45 mmol/L for C_2 and 65 mmol/L for C_3 from the concentrated solution (S_{CC}) of 0.1mol/L KCl summarized in **Table 5**. The measurement of the titrant volume is done with a graduated burette of (1±0.006) mL. Sweat is collected on blotting paper discs calibrated to 5 cm in diameter and placed in Petri dishes. The collection device is weighed before and after sweat collection or QC deposit on a precision balance (10^{-5} g).

Table 5. Preparation of the 3 control levels.

Control levels	**C1**	**C2**	**C3**
Concentration (mmol/L)	25	45	65
Volume withdrew from S_{CC}	5 mL	9 mL	13 mL

Final volume	20 mL

2.5.Method

Mohr's method is a method for the determination of halides, including chlorides, based on their precipitation as silver chloride (AgCl) by the addition of silver nitrate ($AgNO_3$) according to the chemical reaction (a):

$$Ag^+ + Cl\text{-} \longrightarrow AgCl\downarrow \text{ (a)}$$

The end of the reaction is indicated by a color change of the reaction medium from yellow to brown as a result of the formation of the silver chromate precipitate ($Ag_2\ CrO_4\downarrow$) according to the chemical reaction (b):

$$2Ag^+ + CrO_4{}^{2}\text{-} \longrightarrow Ag_2CrO_4\downarrow \text{ (b)}$$

The developed, optimized and validated technique was carried out in 4 steps.

2.5.1. Calibration step of the $AgNO_3$ titrating solution at 0.01 mol/L : A quantity of 10 ml of 0.01 M KCl solution was placed in an Erlenmeyer flask, and 200 µL of the colored indicator was added. The titration was performed with the $AgNO_3$ solution brought by a graduated burette of (10 ± 0.05) mL until the color turned from yellow to brown. Note the titrant volume ($V_{titrant}$). The concentration of the $AgNO_3$ solution, expressed in mol/L, was calculated according to Eq. (1).

$$[AgNO3] = {}^{0.1}/_{Vtitrant} \quad (1)$$

2.5.2. Reagent blank step : A quantity of 10 ml of distilled water was added with 200 µL of the colored indicator with the titrating solution of $AgNO_3$ 0.01 mole/L, previously calibrated until the color changed from yellow to brown. Note the blank volum (V_{Blank}).

2.5.3. Determination step : A minimum quantity of 150 mg of sweat collected by thermal stimulation of sweating was collected by soaking blotting paper of 5 cm diameter, weighed before (P_1) and after (P_2) the collection. The chlorides in the sweat were dissolved in 10 mL of distilled water, and 200 µL of the colored indicator was added. The medium was titrated with the calibrated solution of $AgNO_3$ 0.01 M previously calibrated until the color turned from yellow to brown. Note the titrant volume (V_{test}) The chloride concentration, expressed in mmol/L, was calculated according to Eq. (2).

$$[chlorure] = \frac{[AgNO3].(Vtest - Vblank)}{(P2 - P1)x\ 10-3} \quad (2)$$

2.5.4. Quality control step : Three levels of quality control (C_1 at 25 mM, C_2 at 45 mM and C_3 at 65 mM) are introduced at a minimal volume of 150 µL on blotting paper preceded the assay according to the same mode and under the same operating conditions. These 3 levels correspond to the 3 medical decision zones (**Table 1.**).

The optimized technique was validated according to strategy #6 (scope B) outlined by ISO15189:2012 [18] relating to medical biology laboratories. The validation using the three levels of control focused on the parameters of repeatability, intermediate precision, accuracy, and limit of linearity [19]. The calibration curve and stability of the reagent solutions were validated according to the harmonized guideline ICHM10: 2019 [20] concerning the validation of bioanalytical tests.

3. RESULTS AND DISCUSSION

The determination of sweat chlorides by Mohr's method is a simple, fast and reliable technique according to ISO and ICH requirements and recommendations. The reactive blank step systematically eliminates the possible interference of halides, other than chlorides, that the solution water and any other material may bring in.

3.1. Repeatability was evaluated on 20 replicates of each control level under the same operating conditions. The coefficients of variation are 2.45% for C1, 3.12% for C2 and 2.21% for C3.

3.2. Intermediate precision was evaluated on 15 independent series of each control level and under different operating conditions (15 days, 15 reagent preparations, 5 different operators). The coefficients of variation are 4.12% for C1, 4.51% for C2 and 2.63% for C3.

3.3. Accuracy was evaluated on 25 replicates of each control level. The bias was estimated at 0.99% for C1, 2.76% for C2 and 2.55% for C3.

3.4. The linearity limit was studied up to 150 mmol/L with 3 trials per concentration level. The graphical representation of the determined and calculated concentrations (Y) as a function of the theoretical concentrations (X) is a straight line of equation ($Y=1.0141x + 0.2237$ with $R^2 =0.9992$) as shown in **Fig 1**.

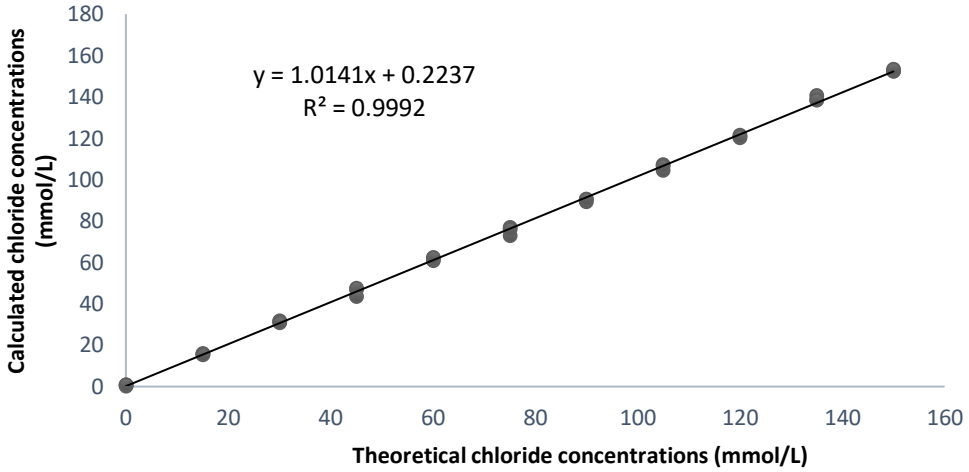

Fig 1. Linear regression curve showing the calculated concentrations as a function of the theoretical chloride concentrations (0 and 150 mmol/L)

3.5. The calibration curve was established as the average of 6 calibration curves at 7 concentrations, performed over 6 days and prepared according to Table 1. The curve is a straight line of equation ($Y=0.0001\ X + (3*10^{-5})$ with $R^2 =0.9995$) as shown in **Fig 2**.

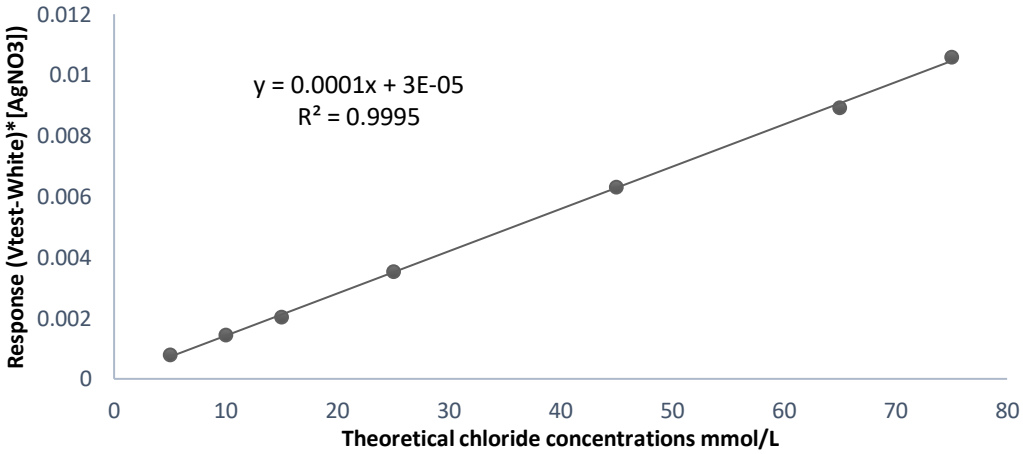

Fig 2. Calibration curve of the signal intensity as a function of the chloride concentrations introduced (5 and 75 mmol/L)

3.6. The quality control results, listed in **Table 4.**, were interpreted against a range of [mean ± 2 x standard deviation] based on the average of 30 determinations over 30 days (**Table 2.**). This time frame represents ordinary events that may affect the analytical technique.

Table 2. Concentration range of quality control solutions C1, C2 and C3.

	Theoretical value (mmol/L)	**Average value** (mmol/L)	**Standard deviation** S (mmol/L)	**Compliance interval** [C ± 2S] (mmol/L)
C1	25	25.27	0.80	23.66 - 26.88
C2	45	45.22	0.85	43.52 - 46.92
C3	65	67.5	1.36	62.93 - 68.37

3.7. The developed technique was compared with the mercurimetric technique on quality controls at the 3 concentration levels (21 at C1, 20 at C2 and 15 at C3). **Fig 3.** shows the correlation between the two quantitative variables tested. The curve is a regression line of equation (Y=1.007 X - 1.134). The results obtained by the two techniques were correlated at R^2 =0.9979.

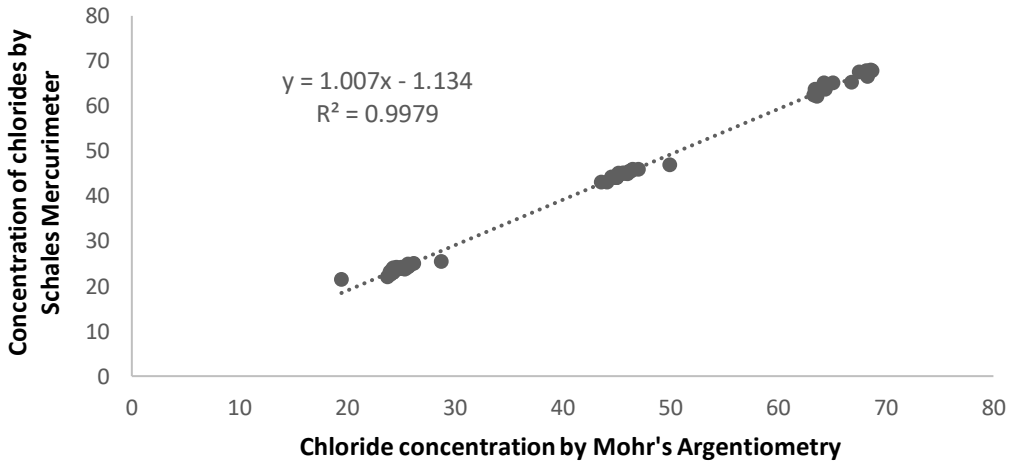

Fig 3. Correlation between quality control results measured by the silver and mercurimetric method

Four successive steps structure the proposed technique. The calibration of the titrating solution allows us to determine the reference concentration in the case where the determinations are spaced in time (exceeding the stability time of 1 month). The realization of a reactive blank allows us to subtract the interference of chlorides and other halides brought by the dissolution

solvent. Quality control at the 3 clinical decision levels guarantees the method's reliability. The actual determination can be done following the different modes of sweat stimulation (thermal or pilocarpine).

The manual titrimetric assay, from the receipt of the sweat sample to the delivery of the result, takes about 3 min per sample, making this alternative an interesting method that can be integrated into a general serial analysis organization. The total analysis time depends on the method of sweat stimulation (thermal or pilocarpine).

The reaction's endpoint (from yellow to brown) is easy to detect with the naked eye, and the technique could be adapted to automation with spectrophotometric detection in the visible. The small volumes handled require precision glassware, particularly a graduated burette of small capacity and high precision. The technique developed meets the recommendations of the analytical stage set out by various learned societies of cystic fibrosis, particularly those relating to working concentrations [14,21,22].

4. CONCLUSION

Mohr's silver method adapted to the determination of chlorides in sweat is a simple, fast, accurate and linear technique up to 150 mmol/L. The reagents used ($AgNO_3$, K_2CrO_4, KCl) and prepared in an aqueous solution in this work are available and less toxic compared to the mercurimetric method of Schales and Schales. Therefore, it is an interesting alternative for medical laboratories wishing to perform the assay and participate reliably in cystic fibrosis patients' diagnosis and follow-up strategy.

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