Optical Measurements of Sweat for in Vivo Quantification of CFTR Function in Individual Sweat Glands

Running title: Optical sweat bioassay of CFTR function

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Highlights

Measuring CFTR function *in vivo* is challenging, and a reliable assay is needed.

An optical sweat test was developed as a linear readout of the *in vivo* CFTR function.

Sweat droplet number in CFTR-dependent sweating allowed image-analysis optimization.

Our outcome focuses on beta-adrenergic sweating, which is defective in CF.

The new outcome can be used to monitor therapy with CFTR modulators in CF patients.

Abstract

Optical measurement of CFTR-dependent sweat secretion stimulated by a beta-adrenergic cocktail (C-phase) vs. CFTR-independent sweat secretion induced by methacholine (M-phase) can discriminate cystic fibrosis (CF) patientts from controls and healthy carriers by the ratio of sweat rate in the C-phase vs. the M-phase (C/M ratio). However, image analysis is experimentally demanding and time-consuming. Here, sweat droplet number (SDN) in the C-phase, corresponding to the number of sweat-secreting glands, was a statistically significant predictor for detecting the effects of CFTR-targeted therapy. We show that in 44 non-CF subjects and 110 CF patients, SDN in the C-phase provides a linear readout of CFTR function that is more sensitive than that using the C/M ratio. In CF patients, increased SDN in the C-phase during treatment with (LUMA/IVA) was

associated with a trend toward improved lung function (FEV1). Our method is suitable for multicenter monitoring of the effects of CFTR modulators.

Keywords

Sweat glands
CFTR-targeted therapy
image analysis
cystic fibrosis

Abbreviations

Abbreviations:
CF, Cystic Fibrosis
CFTR, Cystic Fibrosis Transmembrane Conductance Regulator
SDN, sweat droplet number
C-phase, CFTR-dependent sweat secretion stimulated by a beta-adrenergic Cocktail
M-phase, CFTR-independent sweat secretion induced by Methacholine
C/M ratio, ratio of sweat rate in C-phase vs. sweat rate in M-phase
HC, healthy carriers
F, F508del mutation
M, mutations with minimal CFTR function
R, mutations with residual CFTR function
G, gating mutations
LUMA/IVA, Lumacaftor/Ivacaftor
FEV1, Forced Expiratory Volume in 1 Second

1. Introduction

Sweat Cl^- is the gold standard for the diagnosis of CF; its limitations for diagnosis and monitoring the effects of CFTR-targeted drugs include “borderline” results and intra-subject variability [1]. CFTR-dependent sweating is induced by beta-adrenergic stimulation upon increased intracellular cAMP, whereas CFTR-independent sweating is mediated by cholinergic stimulation that induces Ca^{2+}-dependent Cl^- secretion [2]. Defective CFTR affects sweat production in response to beta-adrenergic stimulation. In CF patients impaired Cl^- diffusion and NaCl reabsorption increase sweat Cl^- and Na^+ concentrations [3]. An optical ratiometric evaluation of CFTR-dependent vs. CFTR-independent sweating has been developed to support controversial diagnosis and monitoring CFTR function during CFTR-targeted treatments [4], [5], [6], [7], [8], [9]. Following implementation, we discriminated CF patients from non-CF subjects with 100% sensibility and healthy carriers (HC) from controls with 82% sensitivity and 86% specificity [4]. However, the evaluation of sweat droplets and calculation of the sweat rate based on the camera monitoring of stained sweat droplets formation are experimentally demanding, need skilled operators and dedicated software for image analysis, which are still under development. Recently, using a multilinear regression model, we showed that SDN in the C-phase is a statistically significant predictor (p-value <0.01) for the mean C/M ratio [10,11].

2. Materials and Methods

2.1. Study population

At the CF Center of Verona, we tested 44 non-CF (controls and HC) subjects and 110 CF patients following written informed consent according to the local Ethical Committee's rules (project#CFTR028). In a subgroup of 45 CF patients (F508del+/+), 17 were tested only during treatment with Lumacaftor/Ivacaftor (LUMA/IVA) (Vertex Pharmaceuticals, Boston, MA, USA) according to the clinical use (mean: 47.66 months; 25th-75th percentile: 29.10-68.85), 15 were tested without treatment, and 13 were tested with/without treatment (mean: 19.07 months; 25th-75th percentile: 7.54-30.06).

2.2. Optical ratiometric measurement of CFTR-dependent sweat

We followed a previously reported method [4] described in brief as follows: intradermal micro-injection (100 μL) of methacholine stimulated CFTR-independent sweating in the M-phase, and beta-adrenergic agonists induced CFTR-dependent sweat secretion in the C-phase following an intradermal microinjection of aminophylline and isoprenaline with atropine as a cholinergic inhibitor (Supplementary Material). Individual sweat glands were totaled by visually counting single spherical sweat droplets ("bubbles") in a water-saturated oil layer including dispersed, water-soluble blue dye particles (350 μL in a 1 cm diameter well). Single glands were mapped (ImageJ software) on pictures acquired 10 or 30 min after microinjections of methacholine or beta-adrenergic cocktail, respectively, and the sweat volume secreted per minute and the C/M ratio were calculated.

2.3. Statistical analysis

Graphpad (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. Inter-group comparisons between non-CF and CF subjects focused on differences in SDN and C/M ratios. Statistical analysis was performed using the non-parametric Mann-Whitney test applied to individual comparisons; $p<0.05$ was considered statistically significant.

3. Results

SDN was acquired by counting single sweat droplets secreted from individual glands in the C-phase. Performances of SDN and C/M ratio were compared between non-CF (n=44) and CF (n=110) subjects. The descriptive statistics are summarized in Table 1. SDN was able to discriminate between non-CF and CF subjects ($p<0.0001$) (Fig. 1a), as well as the C/M rate, ($p<0.0001$) (Fig. 1b). SDN means, but not C/M ratios, were significantly different in F508del+/+ homozygotes (1.31; 25^{th}–75^{th} percentile: 0.24–2.38; n=31) versus all the other genotypes (6.11; 25^{th}–75^{th} percentile: 2.40–9.81; n=60) (Fig. 2).

	SDN		C/M ratio	
	Non-CF (n=44)	CF (n=110)	Non-CF (n=44)	CF (n=110)
mean±sd	93.91±28.00	4.53±11.44	0.2795±0.2016	0.0090±0.0471
95% CI of mean	85.29 to 102.5	2.37 to 6.69	0.2182 to 0.3408	0.000103 to 0.01791
AUC	0.9950		0.9862	
p-value	<0.0001		<0.0001	
sensitivity%	97.27		96.36	
specificity%	100		100	

Table 1. Descriptive statistics of SDN and C/M ratio. AUC: area under the curve; CI: confidence interval.

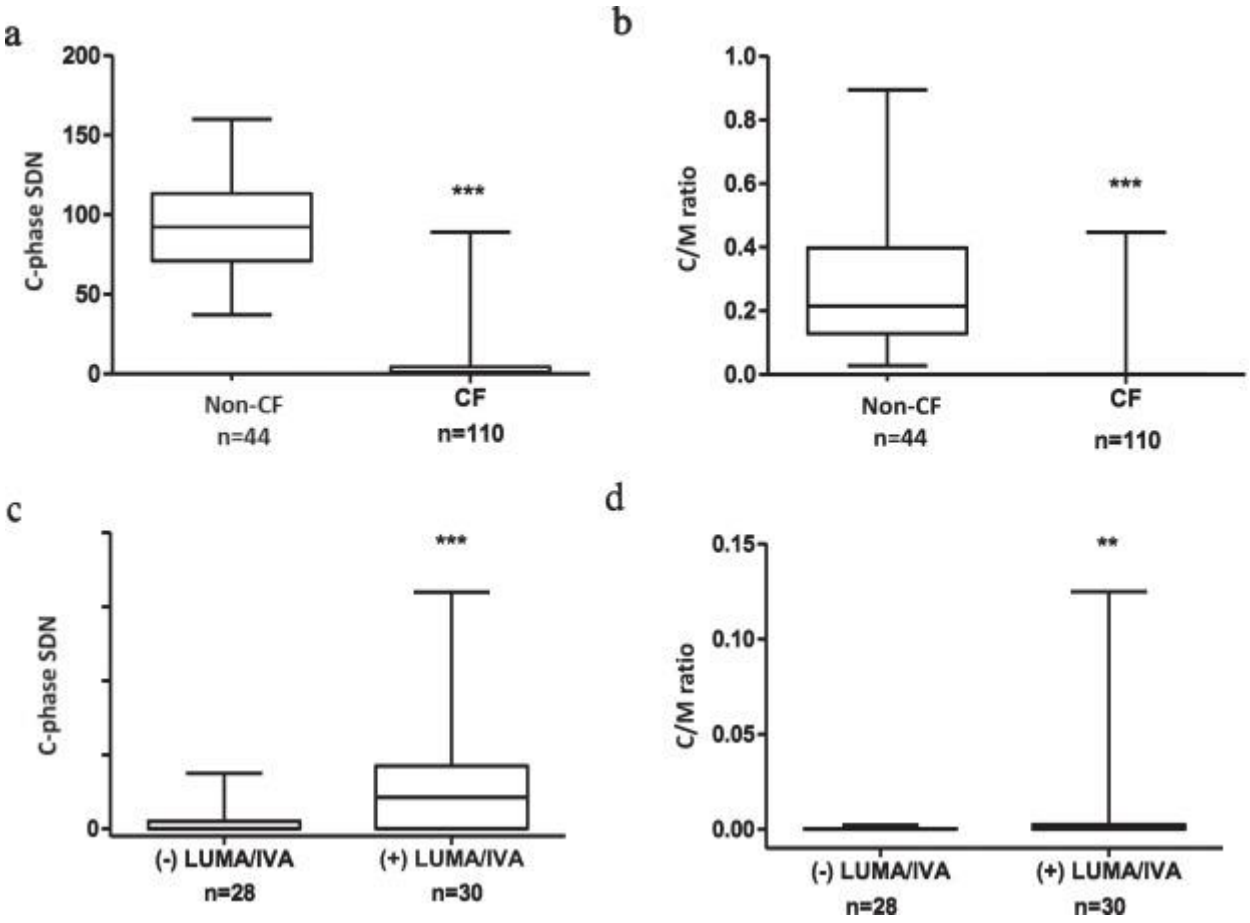

Fig. 1. **Comparison of SDN with C/M ratio.** a) Box and whisker plot (min-max) of SDN measured in the C-phase in CF patients compared to that in non-CF subjects ($p<0.0001$). b) C/M ratio in CF patients compared to that in non-CF subjects ($p<0.0001$). A comparison of SDN and C/M ratio in patients treated with LUMA/IVA. c) Changes in SDN in subjects without and with this treatment (n=28 and 30, respectively, $p=0.0004$). d) Changes in C/M ratio within the same group ($p=0.0021$). Differences were tested with the Mann-Whitney non-parametric test.

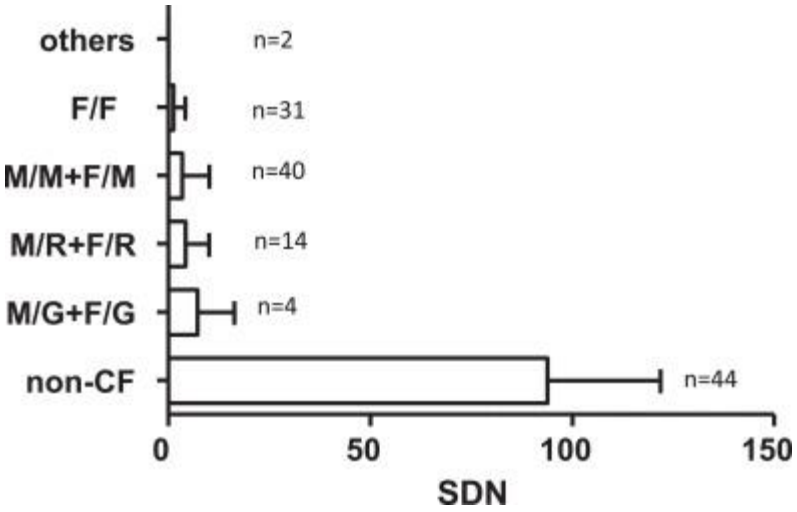

Fig. 2. **SDN in different CFTR genotypes**. SDN mean values ± SD (horizontal bars) in the C-phase of CF patients in the absence of CFTR-modulator therapy are different according to the CFTR genotypes. Abbreviations: F, F508del; M, mutations with minimal CFTR function [13]; R, mutations with residual CFTR function [14]; and G, gating mutations. SDN mean values in F/F homozygotes vs. all other CFTR genotypes are significantly different (p=0.0093), according to Mann-Whitney non-parametric test. C/M ratio mean values did not significantly differ in F/F homozygotes vs. all other genotypes (p= 0.20); data not shown.

The high sensitivity and easy detection of SDN, together with our previous identification by multilinear regression model of SDN as a good predictor of C/M ratio, suggest the use of SDN and its comparison with C/M ratio for detecting CFTR function improvement during CFTR-targeted therapy. In F508del+/+ patients tested before treatment with LUMA/IVA the mean±SD of SDN was 0.94±1.90, whereas the mean C/M ratio ranged from 0.0000 to 0.0022 (n=28). During treatment with LUMA/IVA, the mean SDN increased to 6.41±8.16 and the mean C/M ratio ranged from 0.0000 to 0.1248 (n=30). Although both analyses showed significant changes, SDN appeared to be more sensitive (n=28–30, mean diff.=5.46, 95% CI=3.15–7.77, p=0.0004; Fig. 1c) than C/M ratio (n=28–30, mean diff.=0.0057, 95% CI=-0.00251–0.013, p=0.0021; Fig. 1d). Additionally, in the subgroup of 13 CF patients with/without LUMA/IVA therapy, SDN but not C/M ratio discriminated the responsive group (p=0.0103 and 0.1993, respectively, paired test). SDN showed remarkably lower variability (mean CV% diff.=-72.8, p=0.0148) than C/M ratio.

As SDN seemed capable of identifying the response to the treatment, we sub-grouped our patients into “responders” (SDN increase >3) and “non-responders” (SDN increase ≤3) with the aim of investigating the possible association of increased SDN with improved clinical outcomes. The therapy had similar durations in responders and non-responders (mean±SD 34.6±20.58 and 35.09±25.19 months, respectively). Responders showed a trend toward improved lung function (absolute increase of % FEV1) (mean=2.01; median=1.95; n=15), which was higher than that in non-responders (mean=0.68; median=-0.74; n=13), although they had a lower decrease in sweat Cl^- than that in non-responders (17.61% vs. 20.59%, respectively; not statistically significant).

4. Conclusions

Our results demonstrate that SDN in the C-phase provides a linear readout of CFTR function, overlapping with C/M ratio, for the ability to discriminate between CF and non-CF subjects.

Of note, SDN revealed the effects of LUMA/IVA therapy on CFTR function more efficiently than the C/M ratio. The evaporimetry-based analysis of beta-adrenergic sweat did not detect significant effects of Ivacaftor in CF patients [8,12], whereas in the original optical ratiometric beta-adrenergic assay additional evaluations were required [5]. Our study proposes SDN as a rapid and sensitive outcome for detecting the response to CFTR-targeted therapies, suitable for multicenter studies. Considering the levels of lung function improvement and sweat Cl^- decrease, we expect higher changes in SDN in patients treated with more effective drugs than LUMA/IVA, such as Ivacaftor+Tezacaftor±Elexacaftor. Such changes *in vivo* could complement *in vitro* theratyping for rare genotypes that still lack approved targeted drugs. SDN represents an innovative outcome of a rapid, reproducible, robust, and relatively inexpensive optical measurement of CFTR-dependent sweating, consistent with the impact on CFTR function in different genotypes [13,14] and with clinical outcomes during CFTR-targeted treatments. It facilitates repeatability across different operators and across different sites (Supplementary Material). This might allow further improvement of this bioassay by developing a dedicated image-analysis software, by reducing time and (although minimal) invasiveness following eventual reduction of microinjections during follow-up. Normalization with the M-phase seems redundant in individual patients since we always evaluate the same glands. Cholinergic potentiation of the C-phase was reported at intermediate times, but seemed undetectable at 30 min (end of C-phase in our assay); cholinergic treatment in our assay was shorter than that in the previous study [5]. Therefore, we hypothesized that potentiation did not affect our results. Certainly, further studies are required to verify whether eventual skipping of the M-phase during follow-up might significantly affect sweat rate and/or SDN in our setting. Testing larger skin areas and both forearms every time might improve the test and compensate for the eventual lower efficiency of beta-adrenergic induction in the absence of cholinergic pretreatment.

Our study could support and facilitate the multicenter clinical application of this test by simplifying image analysis while preserving or improving test sensitivity and specificity.

Author contributions

The authors contributed as follows: Davide Treggiari: methodology, investigation, resources, and writing of the original draft; Karina Kleinfelder: methodology, investigation, and resources; Marina Bertini: investigation and validation; Gloria Tridello: formal analysis; Arianna Fedrigo: investigation and resources; Paola Minghetti: methodology, investigation, and writing; Antonella Casiraghi: methodology, investigation, and writing; Marco Cipolli: supervision and funding acquisition; Emily Pintani: data curation; Patrizia Iansa: data curation; Claudio Sorio: writing, review, and editing and supervision; Paola Melotti: conceptualization and writing, review and editing, supervision, and funding acquisition.

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Cutting Residual function of cystic fibrosis mutants predicts response to small molecule CFTR modulators

JCI Insight (2018)

Supplementary Material

Title manuscript (JCF-D-20-00460): **OPTICAL MEASUREMENTS OF SWEAT FOR IN VIVO QUANTIFICATION OF CFTR FUNCTION IN INDIVIDUAL SWEAT GLANDS**

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Evaluation of the stability of aminophylline, atropine, isoprenaline “Cocktail" for intradermal injection

This evaluation was performed in order to provide a support for the “Cocktail” preparation, useful for standardization and organization of multicenter use of the test described in this study.

The stability evaluation of aminophylline, atropine, isoprenaline “Cocktail" for intradermal injection assess chemical and physical stability of the solution, obtained by mixing the following medicinal products:

- Aminophylline 240 mg/10mL (“Tefamin” – Theophylline ethylenediamine, Recordati Spa, Italy);
- Atropine sulfate 0.5 mg/1mL (Monico Spa, Italy);
- Isoprenaline chlorhydrate 0.2 mg/1mL (Salf Spa, Italy);
- Lactated Ringer’s solution.

The final drug concentrations (280 μM atropine, 160 μM isoproterenol, and 20 mM aminophylline) were overlapping with those previously reported by Kim et al. 2016 [1].

Preparation of solution (“Cocktail”)

A single batch of 10 mL aminophylline, atropine, isoprenaline “Cocktail” were prepared by adding 3.38 mL of aminophylline, 1.6 mL of atropine sulfate and 2.0 mL of isoprenaline chlorhydrate solutions in a polypropylene centrifuge tube; then Lactated Ringer’s solution was added under vigorous shake to obtain the final concentration of 8.1 mg/mL, 0.08 mg/mL and 0.04 mg/mL, respectively. The samples split in different aliquots and stored refrigerated protected from light until analysis.

Study design

The stability was studied over a period of 48h. Samples were stored at the temperature of 2-8 °C protected from light and periodically subjected to visual inspection. Aspect and color of the solutions were noted. Samples were conditioned at room temperature by retrieving them form fridge 15 minutes before each technological and chemical measurement.

Osmolarity measurement

Measurement was performed at initial day 0. Aliquots of 0.150 mL were stored in polypropylene microcentrifuge tubes for the osmolarity measurement (K-7400S, Knauer GmbH, Germany) after approximately 24 and 48 h.

pH measurement

The remaining solution was used for pH measurement (InLab Expert Pro-ISM, Mettler Toledo Inc, Switzerland).

DLS and turbidity measurements

An additional batch of 2 mL was prepared and subjected to DLS analysis (Zetasizer Nano ZS, Malvern Panalytical Ltd, United Kingdom) to evidence the presence of possible particulate in the range from 1 to 10,000 nm. Spectrophotometric turbidity measurement was performed by acquiring a spectrum for each experimental point against lactated Ringer's solution as blank value (Lambda 25, PerkinElmer, Italy). The absorbance higher than 0.01 AU at the wavelength of 550 and 650 nm was considered as evidence of turbidity.

HPLC-UV analysis

The assay of the Cocktail components and possible degradation products was performed using a HPLC system equipped with a UV photodiode array detector (HP 1100 ChemStation system, Agilent Technologies, Italy). Chromatographic separation was achieved using a reverse-phase column (Luna® 5µm C18(2) 100 Å, 150x3.9mm, Phenomenex Srl, Italy) operating at room temperature, phosphate buffer pH 3.0/methanol (80/20, v/v) was used as mobile phase, flow rate was set at 1.0 ml/min. Injection volume was 0.020 mL. The UV-detector was set at 274 nm for the aminophylline assay (RT 7.0 min), 220 nm for the isoprenaline assay (RT 2.0 min), and 205 nm for the atropine assay (RT 13.0 min). At predetermined times, 0.100 mL samples were withdrawn from each of the three independent aliquots of 2 mL and diluted with water (1:10) just before the assay. The drug loss was estimated based on the ratio between peak areas at the specific time point and time zero. A peak at the retention time (RT) of 2.0 min (220 nm) was also observed when running the assay on Lactated Ringer's solution, consequently isoprenaline peak area was corrected by subtraction of an average value obtained from triplicate analysis of a same strength Lactated Ringer's solution diluted in water.

Moreover, Lactated Ringer's solution was also verified to be chemically stable over the same testing period.

Results and discussion

The Cocktail consisted of a clear and colorless solution and did not show any evident variation over the testing period. In the freshly prepared solutions and after 24-48 h, no particle component was found in the range 1-10,000 nm. No change in turbidity was observed over the first 24 h, while the absorbance value was found to be out of specification (> 0.01 AU) at the wavelength 550 nm after 48 h of storage. The Cocktail resulted hypotonic as osmolality was about 161 mOsm/kg; in any case, this value was stable over 48 h. The pH value of the Cocktail was ranging 8.65±0.14 over time, which is close to the reference limit value for intradermal administration. In fact, it is well-known that a pH greater than 9.0 can cause tissue necrosis, therefore generally recommended pH range for this route is 3-6 [2]. The alkaline pH can be determined by the presence of ethylenediamine, which is used for salification of theophylline, which would be otherwise poorly soluble in water; indeed, literature data suggest that theophylline precipitation can occur at pH lower than 8.0 [3]. On the other hand, isoprenaline and atropine are known to be chemically unstable at basic pH due to the increase in the oxidation [4] and hydrolysis [5], respectively. This tendency to instability of both medicinal products is also highlighted in the "Summary of Product Characteristics" in which the mixing with alkaline solutions is explicitly not recommended.

During the development of the HPLC-UV assay, linearity was demonstrated by analyzing three samples at different concentrations obtained by diluting each medicinal product: for aminophylline 1,000-500 µg/mL range was considered (R=0.998), for atropine 10-5 µg/mL (R=1.000), for isoprenaline 10-2 µg/mL (R=0.999). Repeatability was evaluated by triplicate injection on the same day (intra-day repeatability) and on two different days (inter-day repeatability) of a sample of each medicinal product diluted to a concentration close to that of the Cocktail samples. Relative standard deviations were 0.0% (intra-day) and 1.4% (inter-day) for aminophylline, 1.0% (intra-day) and 1.5% (inter-day) for atropine, 0.7% (intra-day) and 0.8% (inter-day) for isoprenaline. Preliminary, stressed samples were also prepared by diluting to about 100 µg/mL with pH 9.0 phosphate buffer and then heating at 70-80°C for 1 h to determine the retention times of the degradation products of each molecule.

The assay of the Cocktail components was preliminarily tested on a smaller aliquot of Cocktail which was diluted with water instead of lactated Ringer's solution and stored protected from light at room temperature for the first 24 h and then in refrigerated condition at 2-8 °C. After 120 h, the solution showed a significant decrease in isoprenaline content (-22.4%) as well as atropine content (-18.0%); peaks associated with the formation of degradation products (atropine: RT=1.8 and RT=16.5 min; isoprenaline: RT=1.8 and RT=3.5) were observed either at wavelength 205 and 220 nm.

Reference chromatogram at 205 nm at initial day 0 and after 48 h at 2-8 °C are reported in Figure 1.

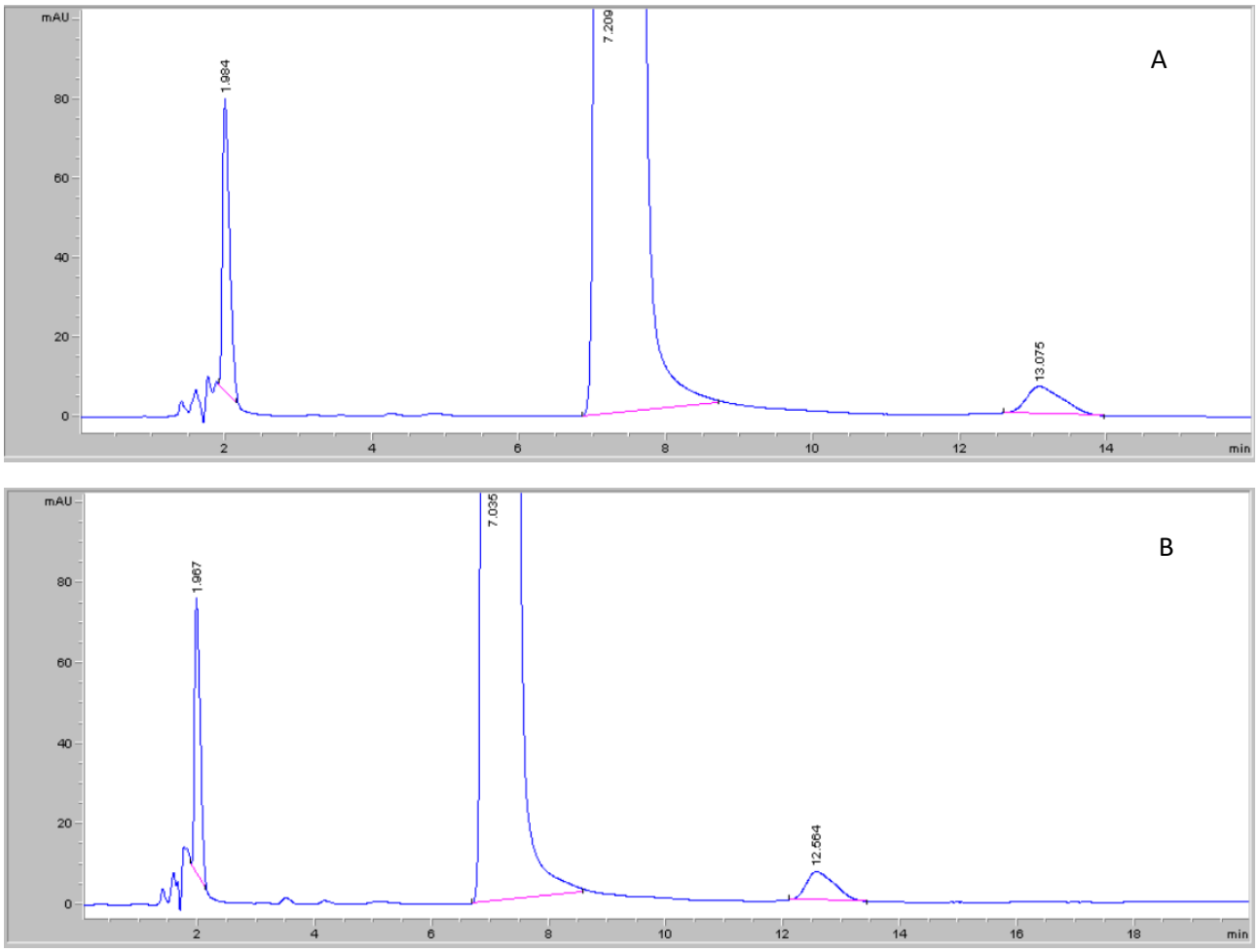

Figure 1. Reference chromatogram of the "Cocktail" at 205 nm at initial day 0 (A) and after 48h (B).

The drug loss content of each component of the Cocktail stored in refrigerated condition and protected from light are summarized in Table 1. After 7 h of storage, no signs of degradation were evident. In the case of isoprenaline, drug loss eventually became evident after 24 h, when its strength was reduced by 7.0%. This trend was confirmed at 30 h, when drug loss exceeded 10.0%. Peaks attributed to isoprenaline and/or atropine degradation products (RT=1.8 and RT=3.5) were also observed at 30-48 h.

Table 1. The drug content loss of the Cocktail stored in refrigerated condition and protected from light over time. The results are expressed as means ± SD (n = 3).

Time (h)	**% drug loss**		
	Isoprenaline	**Atropine**	**Aminophylline**
2	-0.3 ± 1.2	+0.8 ± 2.2	-0.1 ± 1.1
7	-0.8 ± 1.3	-3.3 ± 0.6	+0.2 ± 0.7
24	-7.0 ± 0.8	-3.2 ± 1.6	-2.5 ± 0.4
30	-13.9 ± 0.8	-2.5 ± 1.5	+0.2 ± 0.5
48	-17.2 ± 0.9	-2.7 ± 2.2	-0.3 ± 1.0

These results are in agreement with the results reported by Patel et al. [6], who observed a reduction by 5.9% and 49.2% after 24 h storage at room temperature of isoprenaline hydrochloride (100 mg/mL) in pH 8.0 and 9.0 buffered solutions, respectively. Moreover, this evidence emphasizes the considerable impact of solution pH on degradation rate of isoprenaline.

As far as atropine is concerned, no significant content reduction was observed over a 48 h storage period, in agreement with the work of Kedvessy et al. [7]. Indeed, an atropine sulfate solution at the concentration of 10 mg/mL presented a reduction in drug content of 4.1% and 4.7% after 72 h of storage at pH 8.0 and 9.0 buffered solutions (boric acid/sodium borate), respectively. Similarly, aminophylline was found to be stable over the same storage period.

Conclusion

The Cocktail solution consisted of a clear and colorless hypotonic solution at about pH 8.7. After preparation, it is stable stored protected from light in refrigerated condition (2-8 °C) for 24 hours. Indeed, beyond of this period of time, the isoprenaline content was reduced up to 10%, which is generally regarded as the limit for a medicinal product to be used.

All the other reagents were prepared as previously described [8]; the procedure for testing was the same as that previously implemented at our site [9] with the only update consisting of a commercially available camera holder (Kaiser Vertical Stand from Kaiser Fototechnik, Germany).

Skin damage at the site of the test and clinical signs of dehydration were absent in all the subjects.

The corresponding author is available for providing more details upon request in order to allow to set up the test in different sites.

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