

Colorimetric Method for Quantifying Human *Stratum Corneum* Removed by Adhesive-Tape-Stripping

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Tape-stripping of the skin is a useful method for removing the *stratum corneum* and obtaining more information about the function of this skin layer as the main barrier for skin penetration. The amount of *stratum corneum* removed is of relevance in establishing the concentration profile of chemicals within the *stratum corneum* after topical application. Weighing is the preferred method for measuring the amount stripped, but because it is often subject to artifacts, alternative methods are sought. We present a simple, colorimetric method for determining the amount *stratum corneum* removed by sequential adhesive-tape-stripping of human skin *in vivo*. The method is based on quantification of the sodium hydroxide soluble protein fraction using a commercially available protein assay similar to the Lowry assay. The method is shown to be an accurate and reproducible alternative to weighing, also demonstrating uniform removal of *stratum corneum* layers following the very initial strips. Key words: colorimetric protein assay; quantitative methods; D-Squame[®] disk.

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Stratum corneum (SC), the outermost skin layer, can be removed sequentially by repeated application of appropriate adhesive tape; this procedure is commonly known as SC tape stripping (1, 2). Layers of corneocytes can be removed, and the characteristics of the revealed part of the SC can then be investigated (3, 4).

Since transport across the SC is believed to be a rate-determining step in penetrating the skin, SC tape-stripping allows investigation of the barrier function of this skin layer. Hence, after applying substances topically, SC tape-stripping is a useful procedure for determining the concentration profiles of the substances within the SC (5). In order to obtain concentration profiles, the amount of substance measured should be normalized with respect to the amount of SC tissue removed in each individual tape strip. It is not necessarily adequate, however, to relate the amount of drug to the tape strip number or to pooled tape strips, since the amount of SC tissue removed by tape-stripping might not be constant for each individual strip (6). Additionally, marked differences in the adhesive properties of tapes commonly used for stripping (7) result in different amounts of SC tissue removed per surface unit. Consequently, it is necessary to measure the amount of SC removed with each strip. Weighing is the preferred method (8), but it is time-consuming and often subject to artifacts due to absorption

and desorption of moisture during weighing of the tape before and after stripping (6, 9). To circumvent this problem, alternative methods for determining the removed amount of SC tissue are being researched (9–13).

An aim was to establish a colorimetric method for determining the amount of SC removed by tape-stripping based on the quantification of the sodium hydroxide soluble SC protein fraction, using a commercially available protein assay similar to the Lowry assay (14, 15). The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent and spectrophotometrical measurement of the reaction products with maximum absorbance at 750 nm.

MATERIALS AND METHODS

Study population

Five healthy Caucasian volunteers (designated A, B, C, D and E; four males and one female; 35 ± 15 years, ranging from 26 to 62 years old) participated after giving their informed consent. They were of Fitzpatrick skin type II or III and free of active skin diseases in the test area. The study was approved by the University of California Committee on Human Research.

Chemicals

The following solutions and reagents were utilized: NaOH, HCl, hexane (highest purity available, purchased from Fisher Scientific[®], U.S.A.), bovine pancreas trypsin type III and PBS purchased from Sigma[®], U.S.A., and deionized water prepared by using Milli Q (Millipore[®], U.S.A.). The Bio-Rad DC protein assay kit includes reagent A (an alkaline copper tartrate solution), reagent B (dilute Folin reagent) and reagent S (sodium dodecyl sulfate solution). Bovine serum albumin (BSA) standard and bovine gamma globulin standard were also purchased from Bio-Rad.

Adhesive tape

As adhesive tape, D-Squame[®] disks (CuDerm[®] Corporation, Dallas, TX) with a diameter of 2.2 cm (area 3.80 cm²) were used. As a synonym for the D-Squame[®] disk, the word “disk” is used here.

SC donors

Human skin for preparation of SC was taken from 3 male cadavers (46 ± 12 years, ranging from 33 to 56 years) no later than 24 h after death and stored for a maximum of 1 month at -20°C before use. The skin samples were from leg and buttocks.

SC standard dispersion

Human SC sheets were prepared as follows (16). The epidermis was heat-separated from pieces of human skin for 30 sec in a 60°C water bath. Then the epidermis was treated in 0.0001% (w/v) bovine pancreas trypsin type III in PBS overnight at 37°C . After washing the SC sheets several times in distilled water, they were air-dried and stored under vacuum at room temperature. Before use, the SC was rinsed twice in ice-cold hexane to remove skin contaminating lipids.

In order to prepare a human SC standard dispersion, dry SC was dissolved in 1 M NaOH under stirring for 24 h at room temperature,

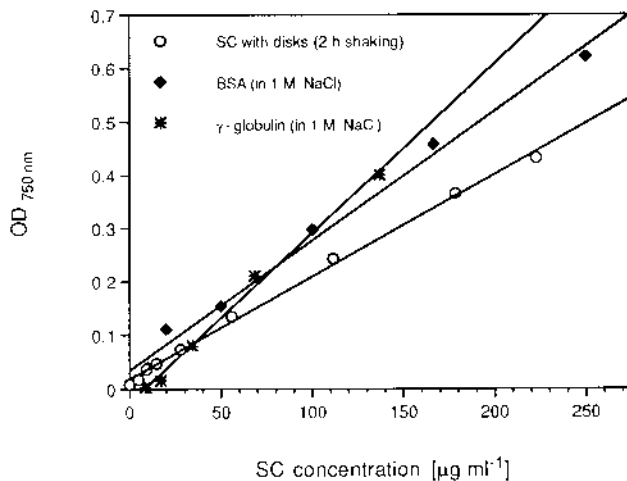


Fig. 1. Human SC calibration curve showing linearity between the SC concentration in 1 M NaCl and optical density measured at 750 nm. The protein standard curves BSA and bovine gamma globulin served as comparison.

sonicated for 1 to 2 h and finally stirred for another 24 h at room temperature. The resulting SC dispersion contained up to $500 \mu\text{g SC ml}^{-1}$ 1 M NaOH and was slightly turbid. A series of standard dilutions with 1 M NaOH, ranging from 0 to $500 \mu\text{g SC ml}^{-1}$ 1 M NaOH, was prepared for obtaining the SC calibration curve.

SC calibration curve

Freshly prepared SC standard dilutions were used for establishing the human SC calibration curve. Unfiltered and filtered (through a $0.8 \mu\text{m}$ disposable sterile syringe cellulose acetate membrane filter; Corning, U.S.A.) SC standard dilutions were neutralized with aliquot volumes of 1 M HCl, vortexed for 5–10 sec and then analyzed according to the Bio-Rad DC protein microassay protocol (15). Spectrophotometrical measurement was performed at 750 nm on a Hitachi U-2001 UV/Vis Spectrophotometer.

To investigate the influence of the disks on the protein assay, they were placed in 20 ml glass vials (Fisherbrand® scintillation vials; Fisher Scientific®, U.S.A.) with the adhesive side facing the screw caps. They were then covered with 1 ml of the unfiltered standard dilution series and shaken for different time intervals on a Gyrotory® G76 (New Brunswick Scientific Co., U.S.A.) shaker. After neutralization with equal amounts of 1 M HCl and vortexing for 5–10 sec, the Bio-Rad DC protein microassay was performed (15).

Validation of the colorimetric assay using disks

For validating of the colorimetric assay, tape-stripping was performed on the volar forearm in the center between the cubital fossa and wrist of three volunteers (C, D, and E). A marked area was stripped sequentially seven times. By applying different pressure on each disk, different amounts of SC were removed. The strips were weighed using a Mettler AT 20 balance (precision: $\pm 2 \text{ mg}$) and placed in glass vials as described above; 1 ml of 1 M NaOH was added, the vials were sealed, and shaken over different time intervals. After neutralization with 1 M HCl and vortexing for 5–10 sec, the protein assay was performed as described previously.

To check whether all sodium hydroxide soluble SC protein fraction was extracted after one extraction in 1 M NaOH, the disks were removed after neutralization and placed in a new glass vial; 1 ml 1 M NaOH was added and shaken for 1 h. After neutralization with 1 ml 1 M HCl the protein assay was repeated.

Sequential tape-stripping using disks

Tape-stripping was performed on the volar forearm in the center between the cubital fossa and wrist of three volunteers (A, B, and C).

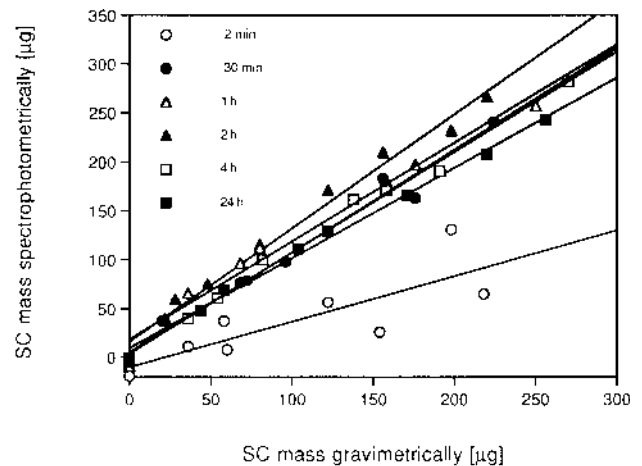


Fig. 2. Validation of the colorimetric assay using disks: the amounts of SC removed as determined gravimetrically versus the amounts of SC determined spectrophotometrically for different shaking times are shown (data for volunteer C). The proportionality between these different quantitative methods indicates the relevance of the presented colorimetric assay.

The marked area on the skin of the volunteers was sequentially stripped 20 times after applying constant and uniform pressure for 5 sec. After gentle removal from the skin, the disks were placed in glass vials as described above and immersed in 1 ml 1 M NaOH. Neutralization and protein assay were performed after shaking the vials for 2 h. No weighing was done.

RESULTS

SC calibration curve

Fig. 1 shows a human SC calibration curve. Table I summarizes the slopes and intercepts of the linear best-fits for the calibration curves; averaged values for the different human SC samples are given. Linearity between the SC concentration in 1 M NaCl and the optical density measured at 750 nm ($\text{OD}_{750 \text{ nm}}$; corresponding to the absorption maximum of the reaction product from protein in alkaline tartrate solution and Folin reagent) was demonstrated. Generally, for the SC calibration curves using different human SC sources, regression coefficients (r^2) higher than 0.99 were obtained for the

Table I. Slope and intercept of the linear best-fit for different protein calibration curves (n =number of different SC donors or samples, respectively)

	Slope (mean $\pm$ SD)	Intercept (mean $\pm$ SD)
SC without disks ($n=2$)	0.0019 ± 0.0002	$+0.0183 \pm 0.0101$
SC with disks after 2 h shaking ($n=3$)	0.0019 ± 0.0002	$+0.0212 \pm 0.0068$
BSA in 1 M NaCl ($n=1$)	0.0024	$+0.0337$
Bovine gamma globulin in 1 M NaCl ($n=1$)	0.0032	-0.0266

linear best-fits in the SC concentration range from 0 to 250 µg human SC ml⁻¹. Neither filtering the SC standard dilutions nor immersing the disks for different times up to 24 h in the dilutions resulted in any significant change in the calibration curves (data not shown). For comparison, two different protein standards, BSA and bovine gamma globulin, were prepared and measured under the same conditions as the human SC (see Fig. 1 and Table I). Calibration curves for BSA and gamma globulin showed different slopes, however, compared to the human SC calibration curve.

Only minor interindividual differences seem to exist for establishing SC standard curves with different SC sources under the conditions investigated, as can be concluded from the good reproducibility. Therefore, calculations of the amount of SC removed by disk tape-stripping were based on using the calibration curve (1) obtained after shaking immersed disks in 1 M NaOH for 2 h:

$$\mu\text{g SC ml}^{-1} = (\text{OD}_{750 \text{ nm}} - 0.0212) (0.0019)^{-1} \quad (1)$$

Validation of the colorimetric assay using disks

Validation of the assay was performed in order to investigate whether the amount of SC removed by disk tape-stripping,

and determined by the spectrophotometric assay described, corresponds to the amount measured gravimetrically for each tape strip. In particular, the influence of shaking time on the extraction efficiency was investigated. Skin of three human volunteers was stripped with the disk, different pressures being applied in order to remove different amounts of SC tissue. After weighing, the spectrophotometric assay was carried out. A well-defined linear relation was found between the mass of SC removed after disk tape-stripping and the amount of SC determined spectrophotometrically (Fig. 2, Table II). Correlation coefficients (r^2) higher than 0.95 were obtained for the linear best-fits after shaking times of 30 min and longer. Taken from the slopes of the best-fit curves, after 2 h shaking, an extraction recovery of $96\% \pm 18\%$ was obtained, indicating that most SC protein was removed from the tape. Further extractions with fresh 1 M NaOH following the first immersion of the disks for 2 h or longer showed that only negligible ($<5 \mu\text{g}$) amounts of SC protein could be extracted. Consequently, a shaking time of 2 h seems optimal for removing most SC protein from the tape. Shorter immersion times might result in SC protein left on the tape, whereas longer times do not significantly change the extraction efficacy. Note, however, that even after immersion times of up to 24 h, the typical imprint of the stripped SC pattern can still be observed on the disks.

Sequential tape-stripping using disks

As an example of a possible application of the presented method, a repeated tape-stripping study was performed. Three male Caucasian volunteers (A, B, and C) were sequentially tape-stripped with the disk 20 times on each site. The amount of SC removed was determined spectrophotometrically. Fig. 3 shows the amount of SC determined for each single strip for the three volunteers, as well as the cumulative amount of SC stripped. With the exception of the first few strips, particularly the first, which removed higher amounts of loose, superficial SC, the disks removed a constant amount of SC up to strip

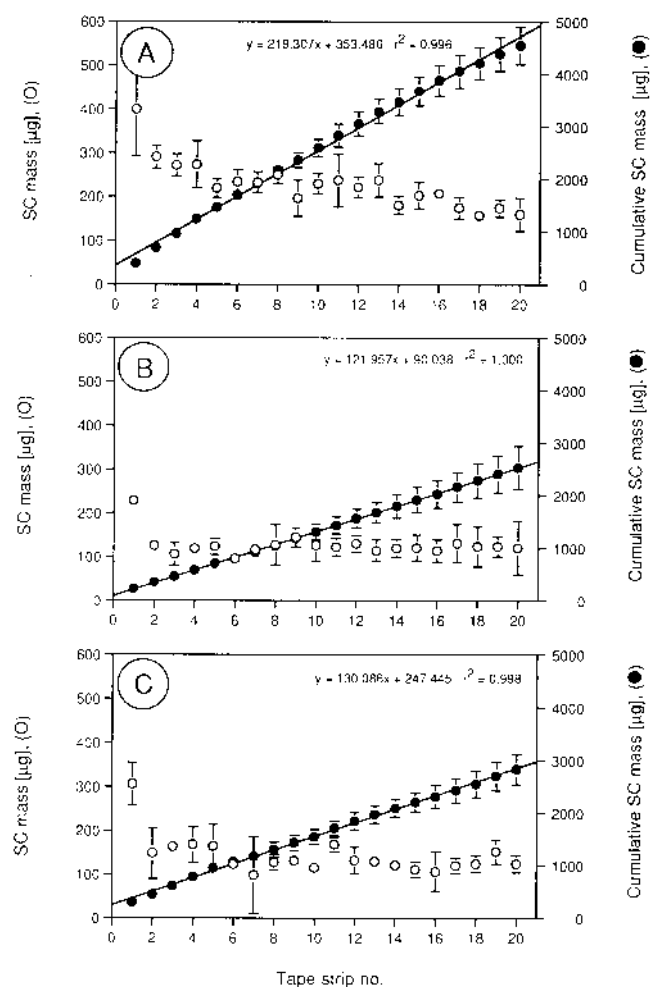


Fig. 3. SC removed by sequential tape-stripping for three human volunteers (A: 62 years old, B: 27 years old, and C: 31 years old) as assessed by spectrophotometric assay (mean $\pm$ SD are shown; 3 measurements).

Table II. Validation of the colorimetric assay using disks; the extraction recovery and the correlation coefficient (r^2) of the linear best-fit of the plot SC amount determined gravimetrically versus SC amount determined spectrophotometrically in function of shaking time are shown (n=number of volunteers tested)

Shaking time	Extraction recovery (%) (mean $\pm$ SD)	Correlation coefficient r^2 (mean $\pm$ SD)
2 min (n=1)	47	0.66
30 min (n=3)	86 ± 16	0.97 ± 0.01
1 h (n=3)	89 ± 10	0.96 ± 0.03
2 h (n=3)	96 ± 18	0.98 ± 0.00
4 h (n=3)	93 ± 10	0.99 ± 0.01
24 h (n=1)	92	0.99

number 20, as assessed spectrophotometrically. For volunteer A (age 62 years, dark sun-tanned skin) up to 200 μg SC per strip (corresponding to about 50 $\mu\text{g cm}^{-2}$) was removed, whereas for volunteers B and C (ages 26 and 31 years, medium sun-tanned) only about 120 μg SC was removed per strip (corresponding to about 30 $\mu\text{g cm}^{-2}$).

DISCUSSION

This work describes a new and simple method for quantifying human SC removed by adhesive tape-stripping. As an alternative to weighing, a colorimetric method based on a commercially available protein assay similar to the Lowry assay was applied.

We used preformed disks of uniform size as a model for adhesive tape. The SC protein assay was performed after the SC stripped disks were immersed in a sodium hydroxide solution, and neutralization with hydrochloric acid. Immersion in the sodium hydroxide solution resulted in complete removal of the SC protein fraction from the tape. However, most of the more hydrophobic lipids appeared still to adhere to the adhesive, resulting in the typical imprint of the SC pattern, recognizable after immersion of the disk. The assay uses human SC as standard; the protein standards like BSA or bovine gamma globulin do not give accurate results under the present conditions.

Compared to weighing, the colorimetric assay as described has some advantages. The weighing of tape strips, which is time-consuming and laborious because the strips have to be weighed before and after stripping under constant hydration conditions, can be omitted. Additionally, when topically applied substances have penetrated the skin, the weighing of SC may become unreliable, making it difficult to obtain accurate concentration profiles for penetrating substances (6).

The study of repetitive tape-stripping showed that the colorimetric assay is a useful method for determining the amount of SC removed by each strip. The results from earlier studies using the gravimetric method could be confirmed, which also demonstrated a constant amount of SC removed with each tape strip (8, 10). Only the initial (2 to 3) tape strips, which remove larger amounts of superficial, loosened SC, depart from this norm. Normalization of penetration gradients of xenobiotics absorbed in the skin thereby becomes straightforward.

This method could also be used to investigate differences in the SC composition between ethnic, age, and gender groups. Furthermore, the method might be of interest in investigating skin disorders such as keratoses and possibly help in the ongoing discussion towards establishing an appropriate method for evaluating the bioequivalence of topical dermatological dosage forms (17).

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REFERENCES

1. Pinkus H. Examination of the epidermis by the strip method of removing horny layers. *J Invest Dermatol* 1951; 16: 383–386.
2. Blank IH. Further observations on factors which influence the water content of the stratum corneum. *J Invest Dermatol* 1953; 21: 259–271.
3. Van der Valk PGM, Maibach HI. A functional study of the skin barrier to evaporative water loss by means of repeated cellophane-tape stripping. *Clin Exp Dermatol* 1990; 15: 180–182.
4. Kalia YN, Pirot F, Guy RH. Homogeneous transport in a heterogeneous membrane: water diffusion across human stratum corneum *in vivo*. *Biophys J* 1996; 71: 2692–2700.
5. Schaefer H, Stüttgen G, Zesch A, Schalla W, Gazith J. Quantitative determination of percutaneous absorption of radiolabeled drugs *in vitro* and *in vivo* by human skin. *Curr Probl Dermatol* 1978; 7: 80–94.
6. Tsai JC, Cappel MJ, Weiner ND, Flynn GL, Ferry J. Solvent effects on the harvesting of stratum corneum from hairless mouse skin through adhesive tape stripping *in vitro*. *Int J Pharm* 1991; 68: 127–133.
7. Tsai JC, Weiner ND, Flynn GL, Ferry J. Properties of adhesive tapes used for stratum corneum stripping. *Int J Pharm* 1991; 72: 227–231.
8. Bommannan D, Potts RO, Guy RH. Examination of stratum corneum barrier function *in vivo* by infrared spectroscopy. *J Invest Dermatol* 1990; 95: 403–408.
9. Martin E, Neelissen-Subnel MTA, De Haan FHN, Boddé HE. A critical comparison of methods to quantify stratum corneum removed by tape stripping. *Skin Pharmacol* 1996; 9: 69–77.
10. Henn U, Surber C, Schweitzer A, Bieli E. D-Squame[®] adhesive tapes for standardized stratum corneum stripping. In: Brain KR, James VJ, Walters KA, eds. *Prediction of percutaneous penetration*, vol. 3b. Cardiff: STS Publishing, 1993: 477–481.
11. Flamand N, Justine P, Bernaud F, Rougier A, Gaetani Q. *In vivo* distribution of free long-chain sphingoid bases in the human stratum corneum by high-performance liquid chromatographic analysis of strippings. *J Chromatogr B* 1994; 656: 65–71.
12. Lodén M, Boström P, Knećzke M. Distribution and keratolytic effect of salicylic acid and urea in human skin. *Skin Pharmacol* 1995; 8: 173–178.
13. Rogers J, Harding C, Mayo A, Banks J, Rawlings A. Stratum corneum lipids: the effect of ageing and the seasons. *Arch Dermatol Res* 1996; 288: 765–770.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265–275.
15. Bio-Rad Laboratories. DC Protein Assay: Microassay Procedure.
16. Kligman AM, Christophers E. Preparation of isolated sheets of human stratum corneum. *Arch Dermatol* 1963; 88: 702–705.
17. Shah VP, Flynn GL, Yacobi A, Maibach HI, Bon C, Fleischer NM, et al. Bioequivalence of topical dermatological dosage forms – Methods of evaluation of bioequivalence. *Pharm Res* (in press).