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Determination of Basic and Quarternary Ammonium Preservatives in Cosmetics with HPTLC in Combination with HPLC and Colorimetry

Key words: Cosmetics, HPTLC, HPLC, Colorimetry, Basic preservatives, Quarternary ammonium preservatives

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Introduction

Quarternary ammonium (quats) and basic compounds, such as guanidines, benzamidines and N-acetals are added to cosmetic products in order to prevent or retard microbial growth during storage and use. Legal restrictions concerning substances (positive list) and their maximum concentrations exist (1) and are the reason for our market survey. Analytical methods described for cosmetics or pharmaceuticals normally use HPLC, HPTLC or colorimetry. But quats can also be characterized and identified by their decomposition products by means of pyrolysis gas chromatography (2). Colorimetric determinations of quats make use of the Acid-Dye technique with bromothymol blue, where a lipophilic ionpair is formed (3). HPLC methods described are either based on separation by cation exchange (4) or on a combination of ionpair and reversed phase chromatography using acetate, heptane sulphonate or perchlorate as the counterion and C18 material as the stationary phase (5–8). Thin layer chromatography has also been performed by ion pair formation using acetate and separation on a reversed phase or normal phase system (8–10). In the case of aromatic structure elements HPLC detection is based on UV, otherwise on conductivity. In TLC methods identification and quantitation consist of a colour reaction with e.g. triiodide and densitometry at 400 nm (9) or bromophenol blue and densitometry at 620 nm (8). For HPLC or HPTLC, extractions are performed either with acidic methanol (8, 7) or with acetonitrile-water (5). In the most extensive work to this date (8) a combination of HPLC, HPTLC and MS is preceded by a clean up using both silicagel and an ion exchange resin. Our method is basically a modification of (8) with a simplified sample preparation which may include a clean up step based on molecule size

instead of polarity and an enhanced separation with HPTLC. For screening and balancing with colorimetry (3) was modified using a basic extraction in CH_2Cl_2 . Chlorhexidine was quantified with HPLC.

Method

Materials and instruments

Reagents

Methanol p.a. (Merck 106009), methanol gradient grade (sds 09337G16), n-hexane p.a. (Merck 104367), ammonium acetate p.a. (Merck 101116), bromophenol blue (LKB Bromma 1840-901), bromophenol blue sodium salt (Fluka 18040), hydrochloric acid 37% p.a. (Merck 100317), acetone p.a. (Merck 100014), acetic acid 100% p.a. (Merck 100063), barium bromide dihydrate > 98% (Fluka 11727), dichloromethane p.a. (SdS 0922A21), ethylacetate SupraSolv (Merck 110972), sodium carbonate water free p.a. (Merck 106392), acetonitrile LiChrosolv (Merck 1.00030), water LiChrosolv (Merck 1.5333.2500), sodium acetate p.a. (Merck 106268), demin. water., methanesulphonic acid (Merck 806022).

References (for structure formulas see (11))

Behentrimonium chloride 80% (Hoechst AG), steatrimonium bromide > 97% (Fluka 74765), cetrimonium bromide 98% (Fluka 52370), myrtrimonium bromide > 98% (Fluka 87210), laurtrimonium bromide > 98% (Fluka 44240), benzalkonium chloride 92–97% (Merck 821944), benzethonium chloride > 99% (Sigma B-8879), cetylpyridinium chloride 98–101% (Merck 2340), laurpyridinium chloride 95% (Merck 820546), hexetidine 94% (Aldrich 25,918-7), chlorhexidine digluconate in water 20% (Sigma C-9394), hexamidine diisethionate 99% (l'Oreal), domiphen bromide 97% (Aldrich 24,748-0).

Columns and plates

SCX 500 mg 3 ml (Varian 1210-2040), CBA 500 mg 3 mL (Isolute 520-0050-B) Sephadex LH 20 (Pharmacia Biotech 17-0090-01), RP2 F_{254S} 10 × 10 cm (Merck 1.13726), Si 60 10 × 10 cm F₂₅₄ (Merck 1.05564), HPLC column: Hypersil CPS 5 μm with precolumn (250 × 4.0 mm and 5 × 4.0 mm) (Knauer).

Instruments

TLC applicator (CAMAG Linomat IV), horizontal developing tank (CAMAG), dipping unit (Baron DC Tauchfix), TLC scanner (Zeiss KM3), UV/VIS spectrophotometer (Perkin Elmer Lambda 1), HPLC pump (Waters 600 MS), column oven (Waters Code 600), UV detector (Waters 486), Maxima chromatography software.

TLC and HPLC procedures

Solvent for RP2 plates

Disolve 3.84 g ammonium acetate in 100 ml methanol. Take 70 ml of this solution and add 30 ml of water.

Solvent for Si 60 plates

Disolve 3.84 g ammonium acetate in 100 ml methanol. Mix 9 parts with 1 part of ethylacetate (v/v).

Dipping solution

Disolve 50 mg bromophenol blue in 100 ml methanol and add 0.4 ml acetic acid. Store at 4 °C.

HPLC parameters

Isocratic eluent: 327 g acetonitrile, 166 g methanol, 370 g water, 2.9 g methanesulphonic acid, 4.1 g sodium acetate. Adjust to pH 5.0 with acetic acid. Column temperature 40 °C, flowrate 0.8 ml/min (see also (8)).

Calibration solutions

Prepare a 25 ml solution of 50 mg reference compound in methanol. Store at 4 °C. Due to their instability chlorhexidine standards must be prepared on a daily basis.

Sample preparation

Weigh a 1 g aliquot of cream or gel in a 10 ml testtube, add 2 ml methanol (for hexamidines use 0.1 M methanolic HCl) and homogenize on a vortex and, if necessary in an ultrasonic bath. Very pasty creams should be sonicated for at least 20 minutes. After centrifugation load a conditioned SCX column (condition with 1 ml water) with the supernatant. Repeat procedure with the residue and 2 ml solvent. For chlorhexidines use CBA instead of SCX. To avoid foaming, shampoos should be diluted in 4 ml of the corresponding solvent and stirred not shaken. In the case of aqueous and alcoholic samples such as mouthwashes or hair lotions, 2 ml aliquots are loaded directly on the column. Wash column with 2 ml of water, methanol, hexane and acetone. Elute analytes with 4 ml methanolic HCl 10% w/v (SCX column) or with 4 ml methanolic barium bromide 0.25% w/v (CBA column). Evaporate to dryness at 65 °C in an evaporator under N₂ and redissolve residue in 1 ml methanol. A further clean up can be helpful when, in the subsequent HPTLC analysis, matrix components interfere with analytes: Evaporate the sample solution again to a minimum volume. Fill ¾ of a Pasteur pipet with Sephadex which has been suspended in methanol for at least 2 h and elute transferred sample with methanol. In a first run fractions of 0.5 ml should be taken as an orientation. A second run is

then performed with an optimized fraction size depending on matrix components and analytes involved.

Plate conditioning

Before sample application RP 2 plates must be activated for 1 hour at 120 °C. Before elution condition for 15 minutes in the developing tank.

Chromatography

Apply 1 to 5 µl of standard and 5 µl of sample solution with the following parameters: Plate width 100, Start pos. 7, Band 4, Space 4, Sec/µl 13, Tracks 11, Start 1.2 cm. Elute over a distance of about 5 cm and check for UV activity after drying at 120 °C for at least 15 min. Derivatise for 1 sec. in the dipping solution. Dry with hot air before scanning.

Quantitation

Scanner parameters: orifice 3.5, wavelength 620 nm, monochromator -0.05, emission mode. All analytes except chlorhexidine and its corresponding salts are quantified with external standards as described above. Calibration curves are linear except for myrtrimonium salts and hexetidine, where reponse/concentration curves are best described with a cubic function. Because of matrix effects chlorhexidines must be quantified with HPLC.

Confirmation

To distinguish between compounds with similar *rf* values, a confirmational analysis on a silica gel 60 plate must be performed: Preclean plate first with methanol. Dry at 90 °C for 15 min. Then preclean with solvent. Dry again for 40 min and condition for 15 minutes before performing elution. Dry at 120 °C for 30 min and check for UV activity at 254 nm before derivatization.

Colorimetric procedures

Bromophenol blue solution 0.1%

Dissolve 0.1 g bromophenol blue sodium salt in 100 ml water.

Sample preparation

Dissolve or suspend a 1 g aliquot in 100 ml water. Dilute 10 ml of this solution to 50 ml with water and pour in a 100 ml extraction funnel. Add 1 ml bromophenol blue solution and shake well. Then add exactly 25 ml CH₂Cl₂ and 1 ml 10% Na₂CO₃ and shake vigorously for 1 min. After phase separation, discard first ml. Wash cuvet with the second ml and use the third ml for colorimetric determination.

Calibration and quantitation

Dilute 0 (blank), 50, 150, 250, 500 µl aliquots of a 10^{-3} M solution of quats to 50 ml with water. Extract mit CH_2Cl_2 as described. Measure at 605 nm against CH_2Cl_2 .

Quality control

As a quality control 2 pharmaceutical preparations (mouthwash and wound ointment) with declared quantitative compositions of cetylpyridinium chloride were analyzed. The results obtained corresponded well with the indicated concentrations. All samples analyzed during the market survey were spiked with the compound of interest and the recovery rates determined. Rates normally ranged between 87 and 120%. Relative precision normally varied between 3 and 10% depending on sample homogeneity. UV active compounds were also quantified at 270 nm and the results compared with those obtained by colour reaction. On the average a deviation of $\pm 18\%$ was found.

Results and discussion

HPTLC data for the compounds of interest are given in table 1 and 2. Also included are the following five compounds we had suspected to interfere with determination due to their functional groups but actually posed no problems: methyl anthranilate, a fragrance component, shows no colour reaction. Under UV only a diffuse spot with an undefined *rf* value is observed. Denatonium benzoate, which is used as a denaturant, has a *rf* value well above the region of interest. The antistatic agents cocamidopropyl betaine, hydroxy-propyltrimonium hydrolysed wheat protein and sodium cocoyl hydrolysed soy protein did not interfere with the colour reaction nor were they detectable under UV. Of the 13 compounds of interest, hexetidine, domiphen and cetylpyridinium on one hand, and myrtrimonium, benzethonium and benzalkonium on the other hand have the same or similar *rf* values. In the first case hexetidine does not show UV activity and gives a somewhat different colour reaction. Cetylpyridinium and domiphen can easily be distinguished on Si 60. In the second case, myrtrimonium shows no UV activity whereas benzalkonium characteristically has 2 spots in contrast to benzethonium. These two compounds also differ in their retention on Si 60.

The procedure was used for supervising existing limits in 21 cosmetic products whose components were qualitatively declared on their labels. The results are compiled in table 3. Samples containing less than 10% of the legal limit showed poor recovery rates but such low concentrations were not of interest for this study. Most samples could be analysed without problems. In the case of hexamidine a further clean up step using Sephadex efficiently eliminated interferences as is shown in figure 1. This is probably due to the capability of this resin to also separate compounds according to their molecular size. Analytical difficulties were encoun-

Table 1. HPTLC characteristics of quarternary ammonium and basic compounds on a RP 2 phase

Compound	rf value	Spot colour	UV detection 254 nm
laurtrimonium bromide	0.46	blue	not detectable
myrtrimonium bromide	0.33	blue	not detectable
cetrimonium bromide	0.21	blue	not detectable
steartrimonium bromide	0.14	blue	not detectable
laurylpyridinium chloride	0.38	blue	intense fluorescent
cetylpyridinium chloride	0.18	blue	intense fluorescent
benzethonium chloride	0.33	blue	fluorescent
benzalkonium chloride	0.35	blue	fluorescent (2 spots)
domiphen bromide	0.18	blue	fluorescent
behentrimonium chloride	0.04	blue	not detectable
hexamidine diisethionate	0.50	blue	intense fluorescent
chlorhexidine digluconate	0.25	blue	intense fluorescent
hexetidine	0.18	bluish purple	not detectable
possible interfering compound			
denatonium benzoate	0.72	blue	intense fluorescent
cocamidopropyl betaine	not detectable	not detectable	not detectable
hydroxypropyltrimonium hydrolysed wheat protein	not detectable	not detectable	not detectable
sodium cocoyl hydrolysed soy protein	not detectable	not detectable	not detectable
methyl anthranilate	not defined	not detectable	fluorescent

Table 2. HPTLC characteristics of quarternary ammonium and basic compounds on a Si 60 phase

Compound	rf value	Spot colour	UV detection 254 nm
laurtrimonium bromide	0.22	blue	not detectable
myrtrimonium bromide	0.23	blue	not detectable
cetrimonium bromide	0.23	blue	not detectable
steartrimonium bromide	0.24	blue	not detectable
laurylpyridinium chloride	0.24	blue	fluorescent
cetylpyridinium chloride	0.24	blue	fluorescent
benzethonium chloride	0.29	blue	fluorescent
benzalkonium chloride	0.40	blue	fluorescent
domiphen bromide	0.33	blue	fluorescent
behentrimonium chloride	0.24	blue	not detectable
hexamidine diisethionate	0.42	blue	fluorescent
chlorhexidine digluconate	0.44	blue	fluorescent
hexetidine	0.66	bluish purple	not detectable

Table 3. Quarternary ammonium and basic preservatives in cosmetic products (all products with qualitatively declared compositions)

Sample	Analyte	Concentration	analytical problems	Limits (1)
cleansing lotion	hexamidine	<0.01%		0.10%
night cream	chlorhexidine	0.01%		0.30%
	hexamidine	0.04%		0.10%
baby cream	cetylpyridinium	0.07%		***
hair rinse	chlorhexidine	0.03%		0.30%
	cetrimonium	—	*	0.10%
face cream	cetrimonium	0.03%		0.10%
baby cream	chlorhexidine	0.02%		0.30%
baby cream	cetylpyridinium	0.10%		***
baby cream	cetylpyridinium	0.13%		***
baby cream	cetylpyridinium	0.07%		***
make up remover	chlorhexidine	0.07%		0.30%
make up remover	chlorhexidine	0.02%		0.30%
cleansing lotion	myrtrimonium	0.40%		0.10%
	chlorhexidine	0.03%		0.30%
tooth paste	domiphen	0.10%	**	***
mouth wash	cetylpyridinium	0.03%		0.05%
mouth wash	cetylpyridinium	0.04%		0.05%
cleansing lotion	chlorhexidine	<0.01%		0.30%
hair wave set	cetrimonium	0.04%		0.10%
hair rinse	cetrimonium	0.70%		0.10%
face mask	myrtrimonium	0.09%		0.10%
hair conditioner	cetylpyridinium	1.90%		***
bubble bath	cetrimonium	—	**	0.10%

* interfering components render quantitation of cetrimonium impossible

** counterion masks analyte

*** new legal limits not yet defined

tered in 3 samples: in the case of a hair rinse, purple spots of unknown matrix components interfered with the determination of the declared cetrimonium compound (blue spot). In a toothpaste and a bubblebath, matrix components acting as counterions irreversibly masked the analytes. Attempts to analyse these samples with the alternative colorimetric procedure only proved to be successful for domiphen in toothpaste. As an unspecific method for quats, colorimetry should only be used, when one compound is involved or to balance results obtained by HPTLC. With HPTLC chlorhexidines can only be determined qualitatively using CBA cartridges. Quantitative determination with HPLC is superior and should therefore be preferred.

Of the 13 compounds analysed, 6 were actually detected in cosmetics. Chlorhexidine and cetylpyridinium were each found in 7, cetrimonium in 4, myrtrimonium and hexamidine in 2 and domiphen in 1 product.

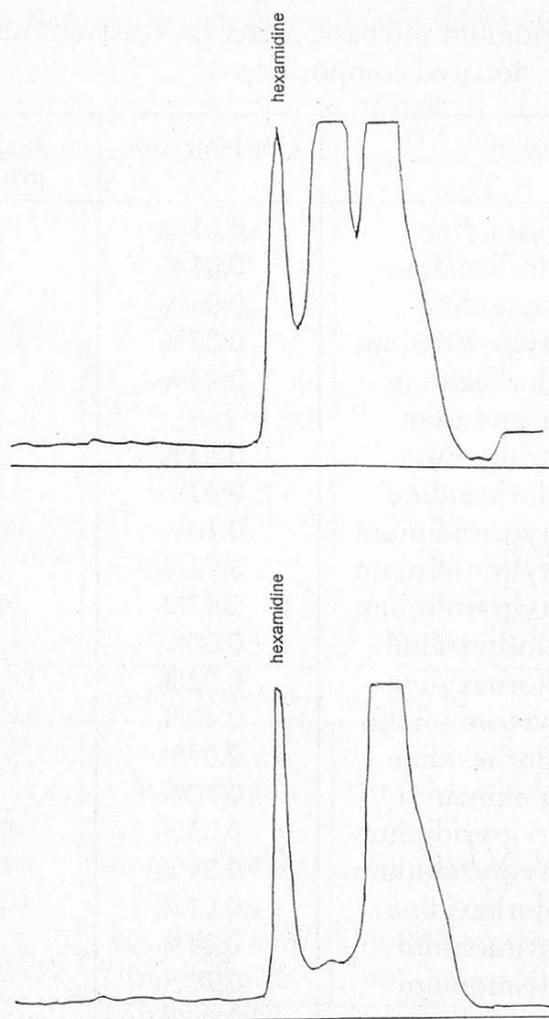


Fig. 1. HPTLC densitometry of a fatty cream containing hexamidine diisethionate with interference (top) and after clean up with Sephadex (bottom).

It was shown, that a combination of the 3 procedures HPTLC, HPLC and colorimetry can successfully be applied in most cases to supervise legal limits. Interpretation of the data regarding legal restrictions, however, is not always easy, because limits are only applicable when the compounds of interest are used as preservatives.

Many quats, however, are multifunctional and can also be used e. g. as an antistatic or deodorant agent or as a surfactant. Two samples contained quats in concentrations which surpassed the legal limit by a factor of 4 and 7. Yet for the cleansing lotion and hair rinse in question, myrtrimonium respectively cetrymonium salts were applied as a cosmetic biocide respectively as an antistatic agent. In the case of a hair conditioner cetylpyridinium was applied at concentrations exceeding the limit by over a factor of 6. Eventhough the compound was used as an antistatic agent and not as a preservative, comprehensive toxicological data prohibit its use in higher concentrations.

Summary

A method is described which allows the determination of 3 basic and 10 quarternary ammonium preservatives in cosmetics such as creams, lotions, toothpastes and mouthwashes. The method is mainly based on a HPTLC procedure, which is supplemented by HPLC and colorimetry. After extraction with methanol, a clean up step was performed using a SCX or CBA cartridge followed, if necessary, by elution on a Sephadex column. For separation activated RP2 HPTLC plates with methanolic ammonium acetate as a mobile phase were used. Detection and densitometric quantitation consisted of a colour reaction with bromophenol blue and scanning at 620 nm. Confirmation was done on Si 60 plates. For chlorhexidines reliable detection and quantitation could only be performed with HPLC. Interfering matrix components could easily be distinguished from the analytes either by their differing colour reaction or retention time. Colorimetry was applied for screening and sometimes for balancing. A market survey including 21 products showed that the compounds most often used are cetylpyridinium chloride in mouthwashes and baby creams, cetrimonium salts in shampoos, bubblebaths and face creams and chlorhexidine salts in fatty creams and alcoholic cleansing products.

Zusammenfassung

Es wird eine Methode beschrieben, mit welcher 3 basische Konservierungsstoffe und 10 quarternäre Ammoniumsalze in Kosmetika wie Cremes, Lotions, Zahnpasten und Mundwässer bestimmt werden können. Nach einer Extraktion mit Methanol wurde ein Reinigungsschritt mit einer SCX- oder CBA-Säule und anschließend, falls nötig, mit einer Sephadexsäule durchgeführt. Zur Auftrennung der Analyten wurden aktivierte RP2 HPTLC-Platten mit methanolischem Ammoniumacetat als mobile Phase verwendet. Die Detektion und die densitometrische Quantifizierung bestanden aus einer Farbreaktion mit Bromphenolblau und Scannen bei 620 nm. Die Identitäten wurden auf Si-60-Platten bestätigt. Chlorhexidine konnten nur mit HPLC zuverlässig nachgewiesen und quantifiziert werden. Begleitstoffe der Matrices konnten aufgrund ihrer unterschiedlichen Färbung und Retentionszeiten leicht von Analyten unterschieden werden. Colorimetrie wurde als Screening und fallweise auch zur Bilanzierung eingesetzt. Eine Marktüberwachung an 21 Produkten zeigte, dass Cetylpyridiniumchlorid in Mundwässern und Babycremes, Cetrimoniumsalze in Shampoos, Schaumbädern und Gesichtscremes und Chlorhexidine in Fettcremes und alkoholischen Reinigungsprodukten die am häufigsten verwendeten Substanzen sind.

Résumé

La présente méthode permet de déterminer 3 agents de conservation basiques et 10 agents de conservation d'ammonium quaternaire dans les cosmétiques, par exemple dans les crèmes, lotions, dentifrices ou dans les solutions bucco-dentaires. Après extraction au méthanol, une purification est exécutée au moyen d'une colonne de SCX ou CBA suivie, si nécessaire, d'une élution sur une colonne Sephadex. Pour la séparation par HPTLC, des plaques RP2 activées sont utilisées avec une solution au méthanol d'acétate d'ammonium comme phase mobile. La détection et la quantification densitométrique se font à l'aide d'une réaction colorée au bromo-phénol bleu et d'une mesure à 620 nm. Une confirmation été exécutée avec des plaques

Si 60. Pour les chlorhexidines, une bonne détermination n'est possible que par HPLC. Les composants matriciels interférant peuvent facilement être différenciés des analytes soit par leur réaction colorée distincte, soit par leur temps de rétention. La colorimétrie a été appliquée pour établir un bilan quantitatif et comme screening.

Une surveillance du marché portant sur 21 produits, a montré que les agents de conservation le plus souvent utilisés sont le chlorure de cétalpyridinium dans les solutions bucco-dentaires et les crèmes pour bébés, des sels de cétrimonium dans les shampoings, bains-mousse et crèmes faciales et les sels de chlorhexidine dans les crèmes grasses et les lotions pour le visage à base alcoolique.

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