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Journal of Chromatography A, 677 (1994) 377–384

JOURNAL OF CHROMATOGRAPHY A

Quantitative analysis of quaternary ammonium antiseptics using thin-layer densitometry

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First received 14 March 1994; revised manuscript received 5 May 1994

Abstract

A thin-layer chromatography method for quantitative analysis of quaternary ammonium antiseptics is described. Silanized silica gel was used as the stationary phase. The mobile phase consisted of methanol–25% (m/v) sodium acetate solution–acetone (65:35:20). The method is able to separate the chain homologues of benzalkonium chloride, cetylpyridinium chloride and cetrimide. Detection was performed using a colour reaction with potassium triiodide solution. The different homologues were quantified using UV densitometry at 400 nm. A number of commercial samples was analysed using this method. From the results it appears that it is worthwhile to have a limit test for the composition of quaternary ammonium antiseptics in pharmacopoeial monographs, the more so as the antibacterial activity depends on it.

1. Introduction

Quaternary ammonium antiseptics such as benzalkonium chloride (BEC), cetrimide (CTB), cetyltrimethylammonium bromide (C_{16}-CTB) and cetylpyridinium chloride (CPC) are cationic surfactants with bactericidal activity which are frequently used in pharmaceutical preparations. The structures of BEC, CTB, C_{16}-CTB and CPC are shown in Fig. 1. Commercial samples of BEC and CTB are mixtures of homologues with different chain lengths. The antibacterial activity of these homologues is different [1–3]. Homologues of BEC, CTB and CPC have been separated qualitatively using paper chromatography [4]. Quantitative analysis of the chain homologues of BEC has been carried out by gas chromatography (GC), following a modified Hofmann degradation (see [5–7]), or directly [8,9], by GC–mass spectrometry [9], by mass spectrometry [10,11] and by liquid chromatography (LC) [12–19]. Quantitative analysis of CPC by GC has also been reported [20], as well as by LC [21]. The separation of CTB homologues by GC has been described [22], but LC methods were not described, probably due to the lack of a chromophore. Recently, capillary electrophoresis (CE) was described for the separation of homologues of BEC, using direct UV detection, and of homologues of CTB, using indirect UV detection [23]. Quantitative densitometric thin-layer chromatography (TLC) methods have been reported for the determination of cetalkonium chloride, which corresponds to BEC

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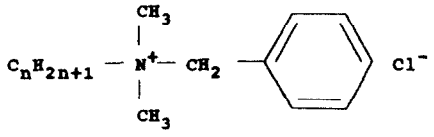
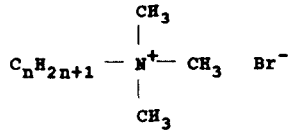
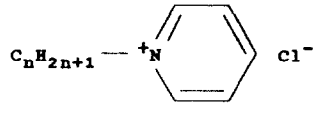
0021-9673/94/$07.00
SSDI 0021-9673(94)00448-I



Benzalkonium chloride (BEC) n = 8 to 18



Cetrimide (CTB) n = 12 to 16
Cetyltrimethylammonium bromide (C_{16}-CTB) n = 16
Cetrimonium bromide (C_{16}-CTB) n = 16



Cetylpyridinium chloride (CPC) n = 16

Fig. 1. Structures for quaternary ammonium antiseptics.

with a C_{16} chain [24] and of BEC [25]. However, no separation of homologues was obtained.

Here a TLC method is described which enables the separation of the chain homologues of BEC, CPC and CTB. Detection is performed using a colour reaction with KI_3. Densitometry (UV 400 nm) is used to quantify the different homologues. The composition of different commercial samples is reported.

2. Experimental

2.1. Chemicals

Methanol and acetone were from Rathburn (Walkerburn, UK). Dichloromethane was from Janssen Chimica (Beerse, Belgium). Water was distilled twice from glass apparatus. Sodium acetate trihydrate and tetrabutylammonium hydrogen sulphate (TBA) of reagent grade were from Janssen Chimica. Potassium iodide and iodine of reagent grade were from Merck (Darmstadt, Germany).

Precoated silanized silica gel layers on glass (20 × 10 cm) (DC Fertigplatten Kieselgel 60 silanisiert) were obtained from Merck.

2.2. Standards and samples

House standards of the C_{12}, C_{14}, C_{16} and C_{18} homologues of BEC, CTB and CPC were prepared in the laboratory. The substances were synthesized by reacting the appropriate alkylbromide with triethylamine, benzyldimethylamine or pyridine [1]. When necessary, the bromides were transformed into the corresponding chlorides. Therefore the tetraphenylborate salts were precipitated [26]. These derivatives were treated with excess potassium chloride. The resulting quaternary chlorides were recovered from the filtrate by extraction with chloroform [27]. The house standards were analysed by Karl Fischer titration of water and by non-aqueous titration of halogenides with perchloric acid, in acetic acid containing mercuric acetate as the solvent.

Commercial samples of BEC, CTB, C_{16}-CTB and CPC were obtained from Aldrich (Milwaukee, MI, USA), BDH (Poole, UK), Federa (Brussels, Belgium), Flandria (Ghent, Belgium), Janssen Chimica, Merck, Pharmachemic (Antwerpen, Belgium) and Schuchardt (Hohenbrunn, Germany).

2.3. Antibacterial activity

The antibacterial activity of the house standards was determined by a quantitative suspension test [28] towards *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442 and *Escherichia coli* ATCC 11229. At time zero 0.1 ml of bacterial suspension was added to 10 ml of disinfectant solution or of water (control). After a medication time of 20 min (for *S. aureus*) or 60 min (for *P. aeruginosa* and *E. coli*), 1 ml was transferred into 9 ml of neutralizing solution containing lecithine (2%), polysor-

bate 80 (2%) and sodium thiosulphate (0.5%) [29]. Aliquots (1 ml) of this mixture and of its tenfold dilutions were brought in subculture and the colony-forming units (cfu) were counted after incubation at 37°C. The germicidal effect was expressed as the logarithm of the ratio of the number of cfu per ml in the medication mixture without disinfectant to the number of cfu per ml after medication with the disinfectant. The concentration of the disinfectant solutions examined was 10^{-4}% (m/v) in water for *S. aureus* and $5 \cdot 10^{-4}$% (m/v) for *P. aeruginosa* and *E. coli*.

2.4. TLC method

The TLC plates were activated by heating at 110°C for 1 h. A narrow band of the layer was removed from both sides of the plate supposed to be in vertical position during development. A dipping solution was prepared by mixing 1 volume of an aqueous solution containing 4% (m/v) potassium iodide and 2% (m/v) iodine (KI_3 solution) and 19 volumes of a mixture methanol–water (1:1). A solution of TBA (1.00 g/l) in methanol–dichloromethane (1:1) was used as an internal standard (I.S.). Commercial samples were dissolved in I.S., in a concentration of 10.0 mg/ml. House standards of the chain homologues were dissolved in I.S. in concentrations chosen to correspond to the amounts present in the commercial samples. Aliquots of 2.0 μl were applied to the TLC plate with a microsyringe (Hamilton, Bonaduz, Switzerland) starting at 20 mm from the edge and at 20 mm from the bottom of the plate. The distance between the lanes was 10 mm, so that 17 spots could be applied per plate. The chromatographic chamber was lined with paper and equilibrated with the mobile phase methanol–25% (m/v) aqueous sodium acetate trihydrate solution–acetone (65:35:20) for at least 1 h prior to use. The plate was developed at room temperature over a distance of 10 cm. After development the plate was dried in the air (5 min) and in an oven at 105–110°C (5 min). Detection of the spots was performed by dipping the plate for 10 s horizontally in a laboratory-made container, containing 200 ml of dipping solution. The TLC plate was allowed to dry on the bench for 15 min and was heated in an oven at 105–110°C for 15 min. Yellowish-brown spots on a colourless background were obtained. The plate was stored at room temperature for 1 h in order to let the colour stabilize. Then the chromatograms were analysed with a CS-990 TLC scanner (Shimadzu, Kyoto, Japan) using the following parameters: zigzag swing width: 10 mm; scan step in the *Y*-direction: 0.1 mm; beam size: 1.2 mm × 1.2 mm; absorption, reflection mode with $\lambda = 400$ nm; linearizer SSX: 3; background correction: off; drift-line integration: 0.3. The ratios of the peak area of each homologue in the commercial samples and the peak area of the I.S. were compared to the ratios obtained for the corresponding spots of the house standards.

3. Results and discussion

For quantitative work, chromatographic methods need reference substances. Laboratory standards for the C_{12}–C_{18} homologues were prepared and analysed. The results for titration and for antibacterial activity of the laboratory standards are reported in Table 1. The result of the titration of the halogenides is expressed as the percentage of the corresponding homologue. For all the samples the total mass explained by this titration and the water content is between 97 and 101% except for C_{12}-CTB where the high titration value is probably due to the presence of some inorganic halogenide. The chromatographic purity of the homologues was checked by the TLC method described in this paper; no impurities were observed. In all further experiments using solutions of the laboratory standards the titration value was taken into account to calculate the concentration of those standards for which the titration value was below 100. The results in Table 1 show that the longer-chain homologues show a stronger germicidal activity. This confirms earlier observations [1,2]. It means that the germicidal activity of a quaternary ammonium compound depends upon the composition of the sample and therefore this composition should be limited by official compendia. The

Table 1
Chemical analysis and antibacterial activity of the laboratory standards

Laboratory standard	Content (non-aqueous titration)[a]	Water (Karl Fischer titration)	Germicidal effect		
			S. aureus	*P. aeruginosa*	*E. coli*
C_{12}-BEC	88.3 (0.3)	9.3	0.12	0.13	0.41
C_{14}-BEC	89.4 (0.3)	8.6	0.80	1.93	2.28
C_{16}-BEC	90.3 (0.1)	8.0	1.73	>4	>4
C_{18}-BEC	93.8 (0.3)	6.5	1.98	>4	>4
C_{12}-CTB	104.0 (0.1)	0.4	0.07	0	0.08
C_{14}-CTB	100.0 (0.1)	0.0	0.14	0.11	0.69
C_{16}-CTB	99.8 (0.3)	0.1	1.99	2.72	1.97
C_{18}-CTB	99.4 (0.2)	0.5	2.27	2.03	4.00
C_{12}-CPC	94.0 (0.3)	6.0	0.15	0	0.11
C_{14}-CPC	92.8 (0.4)	5.4	0.23	1.18	1.54
C_{16}-CPC	92.0 (0.4)	4.9	2.12	3.33	3.42
C_{18}-CPC	96.4[b]	4.7	2.14	4.22	>4

[a] Relative standard deviations (%) in parentheses.
[b] This sample was titrated only once.

European Pharmacopoeia (Ph. Eur.) does not prescribe such limits for the composition of BEC, CTB or CPC [30]. The United States Pharmacopoeia (USP) prescribes a limit test for the composition of BEC, using LC [31].

This paper describes an easy to perform TLC test which does not need a chromophore for detection. Originally the TLC method was developed in this laboratory for identification of CTB and the method was adopted by the Ph. Eur. for this purpose [30]. The composition of the mobile phase described here is slightly adapted by increasing the amount of methanol so that the method is able to separate the homologues of BEC, CTB and CPC. R_F values are reported in Table 2. TBA, used as I.S., is well separated ($R_F = 0.65$). Much effort was put in the development of a detection method suitable for quantitative analysis.

Table 2
R_F values for the different homologues

	C_{12}	C_{14}	C_{16}	C_{18}
BEC	0.40	0.31	0.22	0.15
CTB	0.45	0.36	0.26	0.12
CPC	0.43	0.33	0.24	0.17

R_F TBA = 0.65

3.1. Detection method

First, the plates were sprayed with a solution consisting of a methanol–KI_3 (1:1) solution. The major drawback of spraying was the background colour not being uniform. Unstable baselines were obtained and integration was difficult to standardize. Another disadvantage of the spraying method was the long time needed for stabilization of the colour before a plate could be scanned. A dipping method was then developed using a solution consisting of 1 volume of KI_3 solution and 19 volumes of methanol–water (1:1). The TLC plate was dipped horizontally in the solution for 10 s, which was determined to be the optimal time. When after dipping the plate was allowed to dry on the bench for 15 min and was heated in an oven at 105–110°C for 15 min, the background colour had disappeared completely. The time required for stabilization of the colour of the spots was checked by scanning a plate with 17 chromatograms of the same sample after 0.5, 1, 2 and 4 h. The ratio between the

Table 3
R.S.D.s on the area of BEC homologues in a commercial sample using two detection techniques

Homologue	R.S.D. (%)	
	Spraying	Dipping
C_{12}	7.0	4.3
C_{14}	9.5	5.8
C_{16}	10.0	9.0
C_{18}	31.0	9.3

The sample contains 74% (m/m) C_{12}-BEC, 19% C_{14}-BEC, 4% C_{16}-BEC and 3% C_{18}-BEC. The plate was scanned 3 h after spraying or dipping. Number of analyses: 17.

area of the homologues and the area of the I.S. was constant after 1 h of stabilization. The results obtained by the dipping method were compared to those obtained with the spraying method. The relative standard deviations (R.S.D.s) on the ratio area homologue/area I.S. for the different homologues of a commercial BEC sample using both detection methods are compared in Table 3. The R.S.D. for the dipping method was much lower than that of the spraying method.

Cetylpyridinium chloride is the only compound absorbing sufficiently in UV at 254 nm, so that the spots can be detected without previous dipping of the plate. Two commercial CPC samples containing only C_{16}-CPC were assayed using scanning before ($\lambda = 254$ nm) and after ($\lambda = 400$ nm) dipping. Here a solution of C_{12}-CPC laboratory standard (5.0 mg/ml) in methanol–dichloromethane (1:1) was used as internal standard solution, since TBA is not absorbing at 254 nm. The content (in %, m/m) obtained by scanning at 254 nm was 90.8% for both samples, showing a R.S.D. of 1.6% (sample 1) and 1.4% (sample 2) for a number of 6 analyses. After dipping and scanning at 400 nm, the content was 91.1% for sample 1 (R.S.D. = 3.3%) and 90.5% for sample 2 (R.S.D. = 2.1%). These results were similar, but the variation was higher after dipping, which is normal since an additional colour reaction had to be performed. Scanning of BEC in UV at 254 nm was found to be unsuitable, due to lack of sensitivity. Scanning of BEC or CTB at lower wavelengths (205–210 nm) was possible but the results were not as good as with the dipping method, due to the higher noise and baseline drift.

3.2. Linearity and detection limits

The linearity of the method was examined for the homologues most frequently present in commercial samples of BEC, CTB and CPC. Results are shown in Table 4, where y = area homologue/area I.S.; x = amount (μg) of sample applied to the plate; r = coefficient of correlation; $S_{y,x}$ = standard error of estimate; MR = range of mass (μg) examined; number of analyses = 18; number of concentrations = 6.

Table 4
Regression lines

Compound	Slope	Intercept	r	$S_{y,x}$	MR (μg)
C_{12}-BEC	0.89	−1.24	0.996	0.060	4–20
C_{14}-BEC	0.60	−0.40	0.960	0.020	4–16
C_{16}-BEC	0.44	−0.04	0.985	0.003	1–3
C_{18}-BEC	0.43	−0.08	0.937	0.001	1–3
C_{12}-CTB	0.60	−0.21	0.980	0.005	1–4
C_{14}-CTB	0.61	0.01	0.994	0.030	8–20
C_{16}-CTB	0.45	1.02	0.935	0.161	12–24
C_{16}-CPC	0.56	0.95	0.954	0.189	12–24

See text.

The mass ranges were chosen with respect to the amounts usually present in commercial samples. In the assay of commercial samples the calibration curves are not used, but standard solutions, applied on the same plate, are used in a single-point calibration.

For an application of 20 μg of the sample to the plate, the limit of detection for all homologues was about 1% (m/m) (0.2 μg).

3.3. Assay of commercial samples

Several commercial samples of BEC, CTB and CPC were assayed using the described method. In each chromatogram the areas of the spots were divided by the area of the I.S. (TBA). This ratio was calculated for the commercial samples and for the standard solutions. To calculate the percentage of each homologue in a commercial sample, the nearest chromatogram of a standard solution, containing the desired homologue, was used. The concentration of the standard solutions was adapted to the percentage of the homologues present in the samples. Results for different commercial samples of BEC, CTB and CPC are shown in Table 5.

BEC sample 1 was a mixture of four homologues (Fig. 2), with C_{12}-BEC as the principal component. BEC sample 2 contained only C_{14}-BEC, while BEC sample 3 was a mixture of C_{12}- and C_{14}-BEC. The total content in quaternary ammonium homologues varied for BEC samples from 93.5 to 99.9% (m/m), calculated on anhydrous. The residual mass may be explained by the presence of inorganic salts that are not detected by this TLC method. Following the USP and the Ph. Eur. BEC should be a mixture of alkylbenzyldimethylammonium chlorides. BEC sample 2 does not comply with this description. Moreover the USP prescribes that C_{12}-BEC > 40%, C_{14}-BEC > 20% and C_{12}-BEC + C_{14}-BEC > 70%, determined by LC, using peak

Table 5
Composition of commercial samples

Sample	Origin	Homologue compositions (%) on anhydrous[a]				Total homologues (%) on anhydrous	Water (%), Karl Fischer titration
		C_{12}	C_{14}	C_{16}	C_{18}		
(1) BEC	a	61.8 (1.0)	18.5 (5)	6.0 (11)	7.2 (23)	93.5	13.0
(2) BEC	b	ND	99.9 (5)	ND	ND	99.9	1.2
(3) BEC	d	58.6 (4)	31.3 (3)	ND	ND	98.9	9.0
(4) Cetrimide	e	14.0 (5)	71.6 (0.8)	7.9 (7)	ND	93.5	1.1
(5) C_{16}-CTB	a	ND	99.2 (2.6)	ND	ND	99.2	0.0
(6) C_{16}-CTB	f	ND	98.8 (4)	ND	ND	98.8	0.03
(7) C_{16}-CTB	g	ND	97.4 (2.2)	ND	ND	97.4	0.04
(8) C_{16}-CTB	a	ND	ND	96.2 (3)	ND	96.2	0.03
(9) C_{16}-CTB	h	ND	ND	97.4 (2.5)	ND	97.4	0.15
(10) C_{16}-CTB	i	ND	ND	96.6 (5)	ND	96.6	0.6
(11) C_{16}-CTB	j	ND	ND	91.7 (4)	ND	91.7	0.6
(12) C_{16}-CTB	k	ND	ND	83.6 (3)	ND	83.6	0.9
(13) C_{16}-CTB[b]	c	15.0 (5)	59.3 (4)	3.9 (10)	ND	78.2	NA
(14) CPC	h	ND	ND	95.6 (3)	ND	95.6	4.9
(15) CPC	a	ND	ND	95.1 (2.1)	ND	95.1	4.8

ND = Not detected; NA = not applicable.
[a] R.S.D.s (%) are mentioned in parentheses. Number of analyses per sample = 4.
[b] This sample is a 40% (m/v) solution.

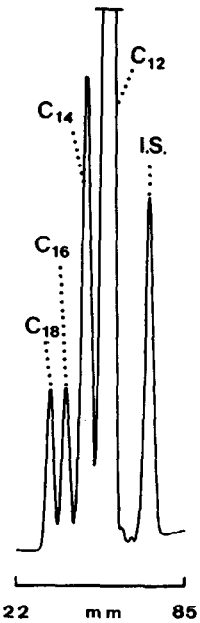


Fig. 2. Typical densitogram of a commercial sample of benzalkonium chloride containing four homologues.

normalisation. BEC samples 1 and 3 comply with these limits. The Ph. Eur. does not prescribe a limit test for the composition.

According to the Ph. Eur. CTB should be a mixture of the bromides of three homologues: mainly C_{14}-CTB with as minor components C_{12}-CTB and C_{16}-CTB. The USP stipulates that cetrimide (CTB) is cetyltrimethylammonium bromide (C_{16}-CTB) [31]. C_{16}-CTB is also called cetrimonium bromide. In fact, the latter two names should be used only for the pure C_{16}-CTB, while cetrimide should be used to indicate a mixture with C_{14}-CTB as the main component. This is clearly stated in the Merck Index [32] and Martindale [33]. As can be seen in Table 5, so-called C_{16}-CTB samples 5 to 7 contain only the C_{14} homologue. C_{16}-CTB samples 8 to 12 contain the correct C_{16} homologue. C_{16}-CTB sample 13 is a mixture and should be called cetrimide (CTB). Most of the cetyltrimethylammonium bromide (C_{16}-CTB) samples are quite pure, except for samples 11 and 12. The low content of sample 13 is probably due to a faulty preparation procedure. CPC samples 14 and 15 contain cetylpyridinium chloride monohydrate. Only the C_{16} homologue is present. R.S.D. values on the results for the main compound were not higher than 5%. For secondary components the R.S.D. values were even higher. Compared to LC methods, the results obtained using this TLC method showed more variation, also because the method uses a colour reaction for detection.

4. Conclusions

The different chain homologues of quaternary ammonium antiseptics can be separated using TLC. The method can be used to determine the composition of commercial samples. The R.S.D. of the assay of the main component is about 5% or better, which is fairly good for a TLC method that uses a colour reaction for detection. Comparison of commercial samples showed that the qualitative composition of benzalkonium chloride samples can differ a lot. Samples of cetylpyridinium chloride mostly contain only the C_{16} homologue. Cetyltrimethylammonium bromide samples mostly contain only one homologue, but the name cetyltrimethylammonium bromide is sometimes erroneously used for samples that contain only the C_{14} homologue instead of the required C_{16} homologue and also for samples which should be called cetrimide. The results indicate the need for limit tests for composition in official texts such as pharmacopoeias. Apart from a recent CE method, no other method has been described that is able to determine the relative amounts of the different chain homologues in CTB.

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