All of the chromatograms discussed were developed after the mixtures were allowed to stand overnight. Chromatograms from freshly treated mixtures were similar, but there was less decomposition of carbidopa.

There was very little decomposition (Fig. 5A) in the blank that contained only levodopa. The decomposition products of levodopa did separate using this procedure (3). When 3 mg of tyrosine decarboxylase also was added, a dopamine peak (Fig. 5B) appeared, indicating that tyrosine decarboxylase has some activity against levodopa, which may be due to an impurity. Since only ~33% of the levodopa was decarboxylated, tyrosine decarboxylase itself does not appear to be active against levodopa. If it were active, 3 mg (1.14 units) of enzyme should have decarboxylated all of the levodopa. This enzyme is supposed to decarboxylate 1 μmole of tyrosine/min/enzyme unit at 37° and pH 5.5. The solution contained only 2.5 mg (12.68 μmoles) of levodopa. If tyrosine decarboxylase *per se* was active against levodopa, all of the levodopa should have been decarboxylated. The solution was not only heated for 15 min at 37°, but it also was allowed to stand overnight. The possibility of the enzyme being more active against levodopa at a different pH value cannot be ruled out. A pH of 5.5 is the optimum value for tyrosine, as recommended by the manufacturer.

In the presence of pyridoxal 5-phosphate (V), 3 mg of tyrosine decarboxylase decarboxylated ~62% (Fig. 5C) of the levodopa as expected, since pyridoxal 5-phosphate is a coenzyme. When carbidopa was substituted for V, there was no decarboxylation (Fig. 5D), probably due to interaction with V present in the enzyme (none was added). It is possible that carbidopa also may react with the decarboxylase. This study is in progress.

When hydroxylamine was substituted for carbidopa, there was little decarboxylation (Fig. 5E), because hydroxylamine has an inhibitory effect on decarboxylases.

When levodopa was mixed with 3 mg of tyrosine decarboxylase apoenzyme, only ~8% of the levodopa was decarboxylated (Fig. 6A). This enzyme is supposed to be the same as tyrosine decarboxylase except that pyridoxal 5-phosphate must be added to activate the enzyme. The enzyme apparently did have some activity without the addition of V. However, on the addition of V, the decarboxylation increased (Fig. 6B) sharply from 8 to ~62% (as with tyrosine decarboxylase when V was added). When carbidopa also was added to the mixture containing the enzyme and V, the decarboxylation was inhibited (Fig. 6C). In Fig. 6C, the carbidopa peak (not shown) was similar to peak 4 in Fig. 5D. Finally, when hydroxylamine was substituted for carbidopa in this mixture, the decarboxylation again was inhibited (Fig. 6D).

In the presence of hydroxylamine (VI), the peak from V (peak 1 in Fig. 6D) was present since there was no interaction between V and VI. Thus, VI reacts with the enzyme but not with the coenzyme. The peak from V is absent in Fig. 6C due to the interaction of V with carbidopa.

In summary, this study proves that both tyrosine decarboxylase and tyrosine decarboxylase apoenzyme have some activity against levodopa but none against carbidopa. In both enzymes, the activity against levodopa can be increased by adding pyridoxal 5-phosphate. The activity can be inhibited by adding hydroxylamine hydrochloride. It was found that carbidopa and pyridoxal 5-phosphate destroy each other. Preliminary studies indicate that they react on a mole to mole basis. Further investigations are in progress. The finding of the interaction between carbidopa and pyridoxal 5-phosphate is contrary to the conclusions of Porter *et al.* (1).

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Determination of Benzalkonium Chloride by Reversed-Phase High-Pressure Liquid Chromatography

ROGER CAREY MEYER

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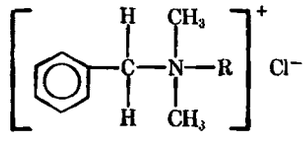
Abstract □ A new, specific, and useful approach for the analysis of benzalkonium chloride is presented. Reversed-phase high-pressure liquid chromatography is used to determine benzalkonium chloride in an ophthalmic system at the preservative level of 0.004%. Since the method separates each homolog, it can be extended to determine the homolog composition. These determinations can be made with an analysis time of 13 min/sample.

Keyphrases □ Benzalkonium chloride—high-pressure liquid chromatographic analysis □ High-pressure liquid chromatography, reversed phase—analysis, benzalkonium chloride □ Preservatives—benzalkonium chloride, reversed-phase high-pressure liquid chromatographic analysis

Various methods have been used for the determination of benzalkonium chloride (I). One common method involves ion-pairing of the material with an acid dye and subsequent extraction into an organic phase. Auerbach (1) described this method using bromphenol blue. Other investigators described similar methods using different organic dye salts (2–4). This type of extraction procedure has been successfully adapted to an automated analysis (5).

Beside dye extraction methods, titration of quaternary ammonium compounds has been employed. These methods include the use of tetraphenylboron (6) and iodate (7). Later methods of analysis have included GLC *via* Hofmann degradation of benzalkonium chloride with subsequent analysis of the formed benzyldimethylamine and the corresponding alkene (8).

The USP (9) lists a titration assay for total alkylbenzyldimethylammonium chlorides based on potassium iodate equivalents. In addition, a method is included to determine the ratio of the R-alkyl components of benzalkonium chloride. The procedure is a complicated microhydrogenation process followed by solvent extraction and GLC. The ratio of alkyl components then is calculated and must meet specific USP requirements. These requirements



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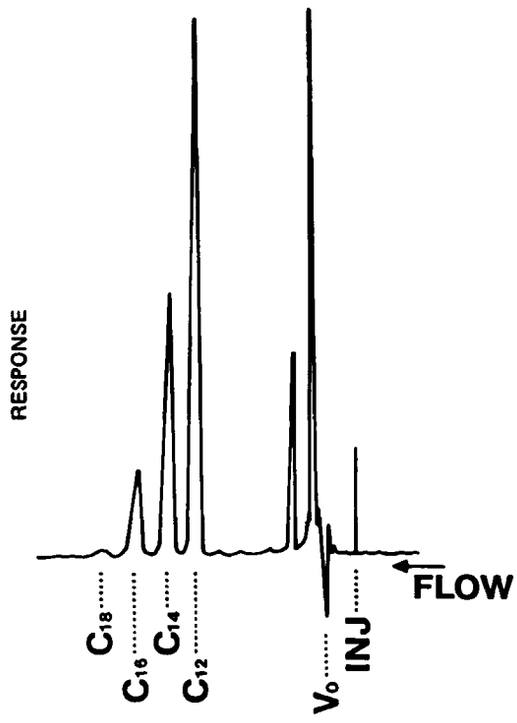

Figure 1—*Chromatogram of benzalkonium chloride.*

state that the C_{12} homolog must comprise at least 40% of the total benzalkonium chloride content and that the C_{14} homolog must be at least 20%. Furthermore, these two homologs together must comprise not less than 70% of the total content.

The procedure presented here determines both parameters (the ratio of the alkyl components and the total alkylbenzyldimethylammonium chlorides) in an easy, one-step operation. This procedure is useful at the antimicrobial preservative level of 0.004% (40 ppm).

EXPERIMENTAL

Apparatus—The high-pressure liquid chromatographic (HPLC) system consisted of a pump[1], an automatic sampler[2], a reversed-phase microcyano column[3], a 254-nm detector[4], and a recorder[5]. Peak integrations were performed with a laboratory data system[6].

Reagents and Solvents—The mobile phase was 60% acetonitrile[7] (UV grade) and 40% 0.1 *M* sodium acetate[8] (ACS reagent grade) with the pH adjusted to 5.0 with acetic acid[9] (analytical reagent grade). Sodium acetate (13.6 g) was mixed with distilled water in a 1000-ml volumetric flask. The acetic acid (~2 ml) was added dropwise until the pH was 5.0 according to a pH meter, and the solution was brought to 1000 ml. Then 800 ml of this solution was mixed with 1200 ml of acetonitrile.

Standards—The benzalkonium chloride reference standard normally used was taken from a stock solution[10] (50%). Appropriate dilutions were made to give a working standard solution of 0.004%. For confirmation, a benzalkonium chloride standard was obtained[11].

Samples—The sample solutions investigated were an experimental, new ophthalmic system preserved with 0.004% benzalkonium chloride.

Assay—After being filtered through a 1-μm filter and degassed with a water aspirator, the mobile solvent was started through the chromatographic system. The chromatographic parameters were a flow rate of 2.0 ml/min, giving a pressure of 1500 psi, a 30-μl loop injector with an analysis time of 13 min, 254-nm detection at 0.01 aufs, and a chart speed of 0.25 cm/min. After a stable baseline was achieved, replicate standards were run to ensure reproducibility[12].

The software statistical package supplied by the manufacturer was utilized with the data system. In the present case, only the C_{12}, C_{14}, and C_{16} homologs were monitored since they comprise the majority of the benzalkonium chloride. However, if required, the entire range of homologs present can be calculated.

Table I—Analysis of a Single Injection of a Benzalkonium Chloride Sample

Homolog	Percent Found	Percent of Label Claim
C_{10}	1.36	0.8
C_{12}	66.09	66.9
C_{14}	23.66	24.0
C_{16}	5.64	5.9
C_{18}	1.36	1.0

Table II—Assay Reproducibility Expressed as Percent Benzalkonium Chloride

Sample[a]	C_{12}	C_{14}	C_{16}	Total
1	51.2	30.9	13.9	96.0
2	52.3	32.0	13.3	97.6
3	53.6	32.2	12.4	98.2
4	54.1	33.3	13.1	100.5
5	54.5	34.0	13.8	102.2
6	53.5	33.4	13.6	100.5
7	52.2	33.1	16.6	101.9
8	52.4	34.1	15.0	101.5
9	53.7	34.8	13.7	102.2
	53.1	33.1	13.9	100.1
10	67.8	22.6	5.4	95.7
11	70.6	24.2	4.3	99.1
12	70.0	21.2	5.4	96.6
13	72.5	23.0	5.3	100.8
14	74.4	23.9	4.8	103.2
15	76.7	24.2	4.3	105.2
16	72.9	22.3	6.6	101.8
17	70.7	22.9	4.1	97.7
18	72.2	23.4	4.3	99.9
	72.0	23.1	4.9	100.0

[a] Samples 1–9 were from a commercial supplier (Ruger Chemical Co.), and Samples 10–18 were from the United States Pharmacopeial Convention.

RESULTS AND DISCUSSION

Benzalkonium chloride, a group of tallow-derived chemicals, is used in pharmaceuticals mainly as an antimicrobial preservative. In chemical terms, benzalkonium chloride is a mixture of alkylbenzyldimethylammonium chlorides, where R represents alkyl groups starting with n-C_8H_{17} and extending through the higher homologs. The C_{12}, C_{14}, and C_{16} homologs comprise the major portion.

The problem of comparing benzalkonium chloride solutions is solved easily by this method. Figure 1 shows a typical chromatogram of benzalkonium chloride. The C_{12} homolog is by far the most prevalent component, with the C_{14}, C_{16}, and C_{18} homologs also easily seen. The sample in Fig. 1 is the standard solution of benzalkonium chloride as supplied by the USP. Table I shows the percentage of each homolog found and the actual label claim. The values obtained are in good agreement with those listed.

At the preservative level of 0.004%, several factors dictate the use of only three peaks when calculating the concentration of benzalkonium chloride. These factors include the increasing noise at these low levels

[1] Model 6000A, Waters Associates, Milford, Mass.
[2] Model 725, Micromeritics, Norcross, Ga.
[3] μBondapak CN (10 μm) (30-cm long × 4-mm i.d. column), Waters Associates, Milford, Mass.
[4] Model 440, Waters Associates, Milford, Mass.
[5] Omniscribe, Houston Instruments, Austin, Tex.
[6] Model 3352B, Hewlett-Packard, Fullerton, Calif.
[7] Burdick & Jackson Laboratories, Muskegon, Mich.
[8] Obtained as the trihydrate, MCB, Cincinnati, Ohio.
[9] Mallinckrodt, St. Louis, Mo.
[10] Ruger Chemical Co., Irvington, N.J.
[11] United States Pharmacopeial Convention, Rockville, Md.

[12] Suitability of the system can be determined by visual inspection of the chromatogram for baseline resolution of the homologs and then by actual determination of the plate counts of the individual homologs. Typical plate counts for the C_{12} homolog are 2000 plates/column, as calculated by $N = 16(v/w)^2$.

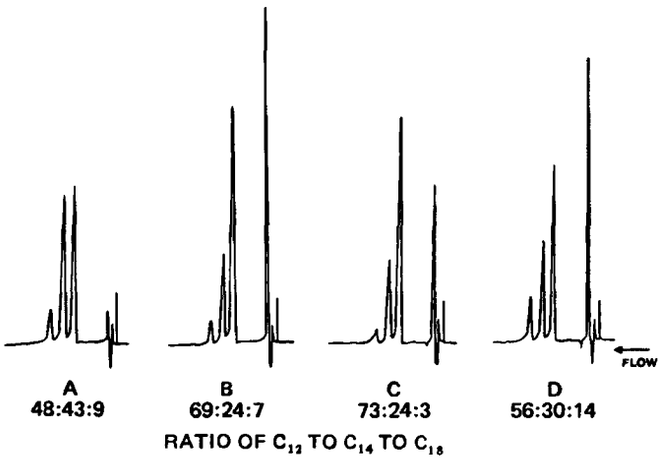

Figure 2—*Homolog variation in samples from commercial suppliers (A–D).*

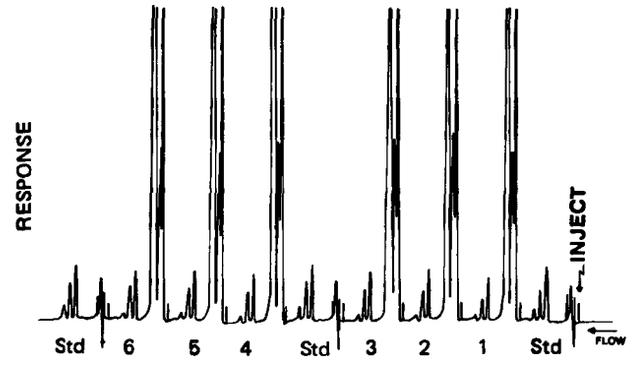

Figure 3—*Experimental ophthalmic system with benzalkonium chloride at 0.400 × 10^{-2}%.*

and the inability of the computer to measure the smaller peaks reproducibly. However, for stability analysis, this limitation should not be a problem since the C_{12}, C_{14}, and C_{16} homologs comprise generally >95% of the total benzalkonium chloride concentration.

Reproducibility of the assay was demonstrated on two benzalkonium chloride samples. The first sample[10] (a 50% solution) was diluted to give nine solutions of 0.005%. The second sample (the 10% solution from the USP[11]) also was diluted to give nine solutions of 0.005%. Table II shows a comparison of the concentrations obtained for the three major peaks of the solutions. A comparison of the total peak areas of both samples shows equivalence of the two solutions to 99.86%. This result is important since the ratios of the major homologs are noticeably different. From the data in Table II, relative standard deviations were obtained for each homolog set. For the commercial sample, these values were ±2.0% for the C_{12} homolog, ±3.7% for the C_{14} homolog, and ±8.7% for the C_{16} homolog. For the USP material, the findings were ±3.6% for the C_{12} homolog, ±4.2% for the C_{14} homolog, and ±16.4% for the C_{16} homolog. As expected, reproducibility for the C_{12} and C_{14} peaks was much better than for the C_{16} peak because of the very small contribution of the C_{16} homolog to the total benzalkonium chloride content. The overall reproducibility for the commercial material gave a relative standard deviation of ±2.3%.

Since considerable variation was noted in the homolog composition of the material as supplied (Table II), benzalkonium chloride from other commercial sources was analyzed by the new procedure. Figure 2 demonstrates the homolog variation that was apparent. Homolog ratios (C_{12}, C_{14}, and C_{16}) on the materials ran from 48:43:9 to 73:24:3. However, even with this range, all samples conformed to the USP requirements as discussed previously.

To ensure day-to-day reproducibility of the procedure, one benzalkonium chloride[10] sample was analyzed on 4 different days (Table III). Relative standard deviations were calculated from the values shown in Table III and were ±1.3% for the C_{12} homolog, ±1.9% for the C_{14} homolog, and ±8.5% for the C_{16} homolog.

Table III—Day-to-Day Assay Variation Expressed as Mean Percent of Total Benzalkonium Chloride

Day	C_{12}	C_{14}	C_{16}	n
1	53.7	34.4	11.9	10
2	52.4	34.2	13.4	4
3	52.2	33.3	14.6	10
4	53.1	33.1	13.9	9

Table IV—Benzalkonium Chloride Content of Experimental, New Ophthalmic Drug System at 4° (Claim Was 0.400 × 10^{-2}%)

Age, days	Percent Found, × 10^{-2}
184	0.400
184	0.397
184	0.404
280	0.397
280	0.404
280	0.403
374	0.440
374	0.423
374	0.417
461	0.395
461	0.370
461	0.382
540	0.319
540	0.388
540	0.394

A calibration graph was obtained for the analysis at the lower benzalkonium chloride concentrations. This graph included points from 0.002 to 0.016% benzalkonium chloride. The calibration graph was linear in the region of interest and passed through zero.

Data then were obtained on a proposed new ophthalmic drug system. The ophthalmic system, which contained dipivefrin, mannitol, and various sodium salts along with benzalkonium chloride, was tested for its benzalkonium chloride content in a stability program. After 540 days, the benzalkonium chloride content was not dramatically different (Table IV).

A typical chromatogram of the ophthalmic system along with standards is illustrated in Fig. 3.

A correlation study was done on this new ophthalmic system. The comparison was between the liquid chromatographic method proposed here and the acid dye extraction method utilizing methyl orange as the ion-pairing reagent. The two methods correlated to 97.2%.

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