Table III—Relative Retention Times [a] of Etodolac Congeners

Compound	Substituents R_1	R_2	R_3	R_4	Relative Retention Time
Etodolac	H	H	C_2H_5	C_2H_5	1.00
I	H	H	n-C_3H_7	H	0.80
II	H	H	phenyl	H	0.94
III	H	CH_3	CH_3	H	0.62
IV	OH	H	CH_3	H	0.44

[a] Chromosorb column; mobile phase: 38% acetonitrile–phosphate buffer; etodolac retention time, 5.0 min.

Table IV—Stability of Etodolac in Serum [a]

Temperature	Recovery (%) on day 1	3	7
20°	100.5 ± 0.5	97.6 ± 0.4	100.2 ± 0.5
4°	98.6 ± 0.5	97.4 ± 0.5	100.4 ± 0.6

[a] Pooled serum spiked with 60 μg/ml of etodolac.

chlorothiazide, glyburide, or diazepam were administered, no peaks were seen in the HPLC scans. Phenylbutazone and dicumarol produced strong interfering peaks with either column. With indomethacin, acetominophen, and salicylate, peaks separate from etodolac were noted, but due to tailing, these peaks partially overlapped the etodolac peak when injected on column A. A better separation was achieved on column B. Because concomitant administration of salicylate or acetominophen is likely to occur clinically, pooled rat serum spiked with etodolac was supplemented with 50–500 μg/ml of salicylate or 20–250 μg/ml of acetominophen, carried through the HPLC procedure, and analyzed on column B. No interference could be demonstrated, nor was there any adverse effect on the recovery of etodolac. In the presence of coadministered drugs, column B is preferred.

Stability—When etodolac was added to pooled serum and kept for up to 7 days at room temperature (20°) or in the refrigerator (4°), no significant loss of etodolac was detected at either temperature (Table IV).

Because of day-to-day variations in recovery, the stability of the test-tube standards and the extracted spiked control serum standards was also investigated. The peak height response of the test-tube standards (n = 12) declined from 100.0 ± 1.3 to 91.5 ± 1.2% in 1 hr. In contrast, the extracts from spiked control serum remained unchanged: 100.0 ± 1.3 *versus* 100.7 ± 2.2%. For this reason, the test-tube standard is used only to adjust mobile phase concentrations when the method is being set up. Quantitation is based on the stable extracts from spiked control sera, and no recovery factor is needed.

Application of the method—Initial studies in humans have indicated that activity is achieved with 100-mg etodolac doses given twice daily. At these doses the peak concentrations of etodolac varied between 4.0 and 14.3 μg/ml, and the daily minima between 0.3 and 3.5 μg/ml. Thus, the HPLC procedure possesses the specificity and sensitivity required to monitor etodolac concentrations in humans.

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Analysis of Chlorobutanol in Ophthalmic Ointments and Aqueous Solutions by Reverse-Phase High-Performance Liquid Chromatography

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Abstract □ A reverse-phase high-performance liquid chromatographic assay for chlorobutanol was developed and found suitable for the routine analysis of ophthalmic ointments and aqueous solutions. The method utilized a column packed with 10-μm octadecylsilane with a mobile phase of methanol–water (50:50). Peak detection was by UV absorption at 210 nm. In this chromatographic system, chlorobutanol had a capacity factor (K') of 4.1. Standard curves obtained in the presence of ointment vehicle containing an aminoglycoside were linear, intercepted at zero, and averaged 99.4% recovery. Degradation studies indicated that the method was stability indicating. The analytical results for a complete experimental ophthalmic ointment and an aqueous ophthalmic diluent are presented. This high-performance liquid chromatographic method of analysis represents an alternative to GC procedures for determining chlorobutanol.

Keyphrases □ Chlorobutanol—analysis in opthalmic ointments and aqueous solutions by reverse-phase high-performance liquid chromatography □ High-performance liquid chromatography—reverse-phase, analysis of chlorobutanol in ophthalmic ointments and aqueous solutions □ Ophthalmic ointments—aqueous solutions, chlorobutanol, analysis by reverse-phase high-performance liquid chromatography □ GC—alternative method for analysis of chlorobutanol in opthalmic ointments and aqueous solutions, reverse-phase high-performance liquid chromatography

Chlorobutanol (1,1,1-trichloro-2-methyl-2-propanol) is a commonly used preservative in ophthalmic medications. It is readily degraded by base as shown in Scheme I to form acetone, carbon monoxide, and chloride ion (1). Chlorobutanol is also highly volatile, and loss by evaporation from ophthalmic solutions was reported through porous plastic bottles and closures (2).

BACKGROUND

A variety of analytical methods were developed for the analysis of chlorobutanol. Originally, analysis consisted of decomposing chlorobutanol by heating with base and then analyzing for chloride ion. This was done titrametrically (3, 4) iodometrically (5), gravimetrically (6), and amperometrically (7). Degradation using a known amount of base and determination of excess base by acid titration and degradation followed by an iodometric determination of acetone (8) also were used. These

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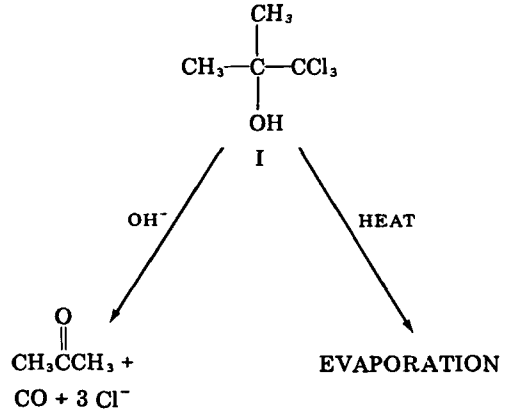

Scheme I—Potential routes for the loss of chlorobutanol (I) from ophthalmic formulations.

methods are not specific, because they are unable to differentiate from excipient chloride sources such as electrolytes or other organic compounds which also contain chlorine. In addition, tedious sample preparation such as steam distillation is often required.

Two colorimetric procedures were developed. The Fujiwara alkali–pyridine colorimetric reaction for chloroform has been used successfully to analyze for chlorobutanol (9, 10). By using 3-substituted pyridines, increased sensitivity to chlorobutanol was reported (11). Reaction of hydroxylamine and chlorobutanol in basic solution forms a product which produces a colored complex with ferric ion (12). Both colorimetric reactions lack specificity, and colors are produced with a variety of organic moieties.

Instrumental methods of analysis include polarography, which is sensitive but requires separation of chlorobutanol from excipients by steam distillation (13), and NMR spectrometry (14), which is difficult to justify for a routine analysis. IR spectrophotometry has also been used for samples eluted through a diatomaceous earth column to remove excipients (15, 16). Because of the high volatility of chlorobutanol, the most successful method of analysis has been gas chromatography (GC). Flame ionization detection is satisfactory with a large number of different stationary phases (2, 15, 16). In one reported GC method, electron capture detection was used (17).

The official USP analytical method for chlorobutanol is a GC procedure utilizing flame ionization detection, a 5% polyethylene glycol 20M stationary phase, and a benzaldehyde internal standard (18). However, attempts to analyze chlorobutanol in an experimental ointment formulation containing an aminoglycoside using a similar GC procedure yielded results which slowly increased according to the length of time samples were allowed to stand at room temperature before analysis. Evidence indicated that the benzaldehyde was slowly reacting with the vehicle, and the resulting variance in internal standard concentration was causing variance in the results.

A high-performance liquid chromatographic (HPLC) method of analysis for chlorobutanol is discussed, which uses an octadecylsilane column with UV peak detection at 210 nm. This reversed-phase system was found to be satisfactory for the routine analysis of chlorobutanol in ophthalmic ointments and aqueous solutions. Data is included to demonstrate that the procedure gives a linear response and reproducible recoveries from an ointment vehicle. The actual analyses of a complete bulk experimental ointment formulation and an aqueous ophthalmic diluent are presented. The results for the aqueous diluent are compared with the results obtained from a GC method using a cyclohexanol internal standard instead of benzaldehyde.

Table I—GC Analysis of a Chlorobutanol Vehicle Standard Using a Benzaldehyde Internal Standard

Time Left Standing Before Analysis, min	Ratio of Chlorobutanol Peak Height to Benzaldehyde Peak Height	Recovery, %[a]
0	1.13	100.9
10	1.17	104.9
21	1.19	106.1
31	1.22	109.4
40	1.26	112.7
50	1.29	115.4
59	1.35	120.6
69	1.37	122.3
78	1.40	125.0

[a] The vehicle standard contained 0.57 mg of chlorobutanol/ml and ~4.0 g of ointment vehicle which contained an aminoglycoside.

Table II—HPLC Analysis of Chlorobutanol Vehicle Standards Containing 4.0 g of Ointment Vehicle

Chlorobutanol Concentration, mg/ml	Chlorobutanol Peak Height, cm	Chlorobutanol Concentration Found[a], mg/ml	Recovery, %
0.281	8.70	0.281	100.0
0.281	8.70	0.281	100.0
0.328	10.20	0.330	100.5
0.328	10.20	0.330	100.5
0.375	11.50	0.372	99.1
0.375	11.45	0.370	98.7
0.422	12.90	0.417	98.8
0.422	12.90	0.417	98.8
0.469	14.45	0.467	99.6
0.469	14.45	0.467	99.6

[a] A standard containing 0.375 mg of chlorobutanol/ml was extracted and used to calculate these concentrations.

EXPERIMENTAL

Reagents and Solvents—Chlorobutanol hemihydrate[1], USP grade, was used without further purification. Its absolute purity on an anhydrous basis was established by titration of total chloride (3), and all subsequent calculations were corrected using this factor. Methanol[2] and hexane[2] were reagent grade.

Gas Chromatography—The gas chromatograph[3] was equipped with a flame ionization detector and a 91.4-cm × 6.4-mm glass column packed with 5% polyethylene glycol 20M[4] coated on 100–120 mesh diatomaceous earth[5]. The temperatures of the injector, column, and detector were 140, 80, and 250°, respectively. The gas flow rates of helium, hydrogen, and compressed air were 20, 20, and 350 ml/min, respectively.

Preparation of GC Ointment Samples—A sample of ointment equivalent to ~25 mg of chlorobutanol was weighed into a 60-ml separatory funnel. After dissolving in hexane (35 ml), the cloudy solution was extracted with three 7-ml portions of methanol. Each time the lower (methanol) layer was drained into a 50-ml volumetric flask. A 1.0-ml al-

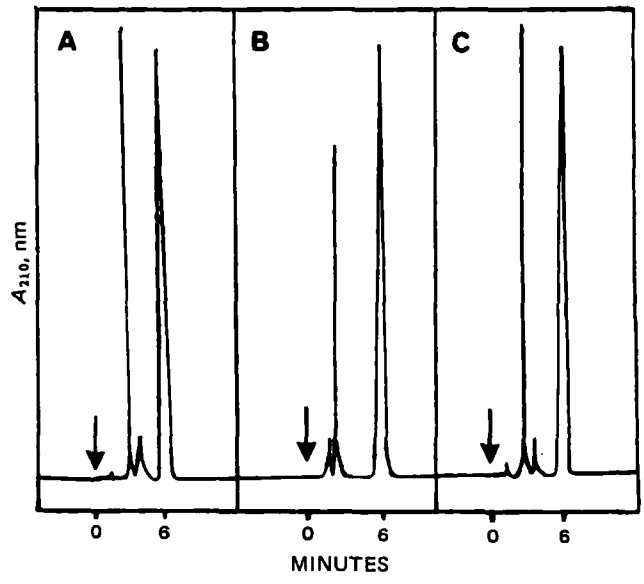

Figure 1—*Typical chromatograms for chlorobutanol analysis. Key: (A) a methanol standard; (B) sample extracted from an ophthalmic ointment; (C) a diluted sample of an aqueous ophthalmic diluent.*

[1] Stauffer Chemical Co.
[2] 'Baker Analyzed' Reagent, Baker Chemical Co.
[3] Model 402, Hewlett-Packard.
[4] Carbowax 20M, Supelco.
[5] Gas Chrom Q, Supelco.

Table III—HPLC Analysis of Complete Ophthalmic Samples

Sample (Age)	Chlorobutanol Analysis, Percent Label[a]
Experimental bulk ointment sample (14 days)	111.8, 112.1, 112.4, 111.2, 111.2
Experimental bulk ointment sample (15 days)	109.0, 111.7, 112.6, 112.5, 112.7
Aqueous diluent (5 months)	107.8, 107.8, 107.8, 108.3, 107.8, 107.5, 107.9, 107.9, 107.9

[a] The chlorobutanol concentrations were labeled 5.0 mg/g; however, since a 15% excess was added, the theoretical content is 115% of label.

iquot of a 7-mg/ml methanolic benzaldehyde solution was added, and the mixture was diluted to volume with methanol. A 1.0-μl injection of this solution was made directly into the gas chromatograph.

High-Performance Liquid Chromatography—A liquid chromatograph, equipped with a high-pressure pump[6], a variable-wavelength UV detector[7], an autoinjector[8], and a strip-chart recorder[9] was used with a 25-cm × 4.0-mm i.d. column packed with 10-μm octadecylsilane[10]. A 10-cm × 2.0-mm i.d. precolumn packed with 47-μm octadecylsilane[11] was used to protect the analytical column from the absorption of ointment vehicle.

Mobile Phase—Methanol (500 ml) and water (500 ml) were mixed and filtered through a 0.47-μm filter[12] before use.

Analysis Conditions—The mobile phase was pumped through the column at a flow rate of 1.8 ml/min. UV detection was at 210 nm, and the injection size was 100 μl. A sample was injected every 6 min. The plate count for the column, determined at a chart speed of 5.08 cm/min with a 0.37-mg/ml chlorobutanol solution, was 3000 plates/m[13]. After completion of the analysis, methanol was pumped through the column prior to storage.

Preparation of Standard Curves—The following were prepared using a methanolic 2.35 mg/ml of chlorobutanol stock solution:

Chlorobutanol Standard Curve—Five aliquots, giving final concentrations in the range of 0.28–0.48 mg of chlorobutanol/ml, were added to different 125-ml separatory funnels. Hexane (50 ml) was added and the methanolic layer adjusted to 15 ml with methanol–water (75:25). After shaking, the lower (methanolic) layer was drained into a 50-ml volumetric flask. The mixture was extracted twice more with 15 ml of methanol–water (75:25), and the lower layers were collected each time. The extracts in the volumetric flask were diluted to volume with methanol–water (75:25), and the resulting solution was injected directly into the HPLC.

Chlorobutanol Vehicle Standard Curve—The same series of aliquots used for the standard curve was added to different 125-ml separatory funnels which contained 4.0 g of ointment vehicle. The samples were dissolved in hexane and extracted with a methanol–water (75:25) solution as described above.

Preparation of Ophthalmic Ointment Samples—A sample of ointment equivalent to ~20 mg of chlorobutanol was weighed into a 125-ml separatory funnel. After dissolving in hexane (50 ml) the sample was extracted as described above. A standard was prepared by extracting 8.0 ml of a methanolic 2.5 mg of chlorobutanol/ml stock solution in a similar manner.

Preparation of Ophthalmic Aqueous Solutions—The sample was diluted with methanol to a final concentration of 0.5 mg of chlorobutanol/ml. A chlorobutanol standard of similar concentration was prepared in methanol.

RESULTS AND DISCUSSION

The chlorobutanol analysis of an experimental ointment formulation containing an aminoglycoside by gas chromatography was found to change if the samples were allowed to stand before being analyzed. In an attempt to isolate the problem, a vehicle standard containing 0.57 mg of chlorobutanol/ml was prepared and injected into a gas chromatograph every 10 min. The results presented in Table I show an apparent increase in chlorobutanol concentration with time at room temperature. This is probably due to a slow removal of the benzaldehyde internal standard caused either by oxidation to benzoic acid or reaction with the aminoglycoside present. A methanol chlorobutanol standard similarly injected showed no increase in concentration with time.

Since long automated runs were clearly impossible using this GC procedure, a reverse-phase HPLC system was developed. Using the HPLC conditions described under *Experimental*, chlorobutanol eluted at ~6 min, which corresponds to a capacity factor (K') of 4.1. Typical chromatograms are given in Fig. 1.

Table IV—HPLC Analysis of Forcibly Degraded Chlorobutanol Vehicle Standards Containing 4.0 g of Ointment Vehicle

Method of Degradation[a]	Theoretical Chlorobutanol Concentration, mg/ml	Chlorobutanol Peak Height, cm	Chlorobutanol Concentration Found, mg/ml	Recovery, %[b]
Heat alone	0.3773	2.20	0.0722	19.1
Heat + Acid	0.3773	5.00	0.1641	43.5
Heat + Base	0.3773	4.60	0.1509	40.0
Heat + Peroxide	0.3773	2.85	0.0935	24.8

[a] All samples were heated at 110° overnight. [b] No interfering HPLC peaks were observed.

Five chlorobutanol standards prepared in methanol over the concentration range of 0.28–0.48 mg/ml were extracted with hexane, as if they were ointment samples, and analyzed. A least-squares regression analysis of these results yielded a coefficient of determination (R^2) of 0.9983, a y-intercept of −0.24 cm, a slope of 29.54 cm/mg/ml, and a standard error of 0.093. Five vehicle standards containing ~4.0 g of ointment vehicle were also prepared over approximately the same concentration range and analyzed (Table II). A coefficient of determination (R^2) of 0.9990, a y-intercept of 0.19 cm, a slope of 30.28 cm/mg/ml, and a standard error of 0.070 were calculated. These statistics indicate that the HPLC analysis gives a linear response, and a single point standard may be used. The recoveries from 10 vehicle standards containing ointment vehicle averaged 99.4 ± 0.2%[14].

Two complete ophthalmic formulations were analyzed for chlorobutanol content (Table III). One was an experimental ophthalmic ointment which contained an aminoglycoside, mineral oil, and petrolatum as excipients. The other formulation was an aqueous ophthalmic diluent[15] which contained boric acid and polysorbate 80.

The ointment was analyzed on 2 consecutive days to check assay reproducibility. Fourteen days after manufacture, the chlorobutanol analysis of five samples taken from the bulk ointment averaged 111.7 ± 0.5%[14]. On the next day, five new samples were analyzed. The results were 111.7 ± 1.6%[14]. Fifteen percent excess chlorobutanol had been added, so the theoretical content was 115% of label. These data indicate that the HPLC assay has good day-to-day reproducibility with a standard deviation for each set of samples in the range acceptable for the analysis of a preservative.

Nine samples of the aqueous ophthalmic diluent were diluted with methanol and analyzed for chlorobutanol. Theoretical content was 115% of label. The results averaged 107.9 ± 0.2%[14]. These samples were also later analyzed using a GC method similar to the one described under *Experimental*, but utilizing a cyclohexanol internal standard instead of benzaldehyde and a 3% polyethylene glycol 20M stationary phase. By this procedure, the chlorobutanol content averaged 107.4% label, which is in good agreement with the HPLC results.

Four vehicle standard samples of chlorobutanol containing ~4.0 g of ointment vehicle were forcibly degraded to ensure that chlorobutanol or excipient degradation products did not interfere with the HPLC assay. To the first sample several drops of concentrated hydrochloric acid was added, to the second several drops of concentrated ammonium hydroxide was added, to the third several drops of 30% hydrogen peroxide was added, and to the fourth nothing was added. The vials were tightly capped and heated at 110° overnight. These samples correspond to the potential degradation pathways catalyzed by acid, base, oxidation, and heat.

[6] Model 110 A, Altex Scientific Co.
[7] Model SF 770, Schoeffel Instruments.
[8] WISP 710 B, Waters Associates.
[9] Omniscribe Model A 5111-1, Houston Instruments.
[10] ODS-10 (10 μm), Bio-Rad Laboratories.
[11] Bondapak C_{18}/Corasil (47 μm), Waters Associates.
[12] Fluoropore, Millipore Corp.
[13] Theoretical plates per meter (N) = 16 $(T/T_w)^2(100/L)$, where T is the retention time of chlorobutanol, T_w is the peak width of chlorobutanol measured along the baseline, and L is the column length in centimeters (19).
[14] Mean ± SD.
[15] Echodide Diluent, Alcon Laboratories.

Degradation was observed (Table IV), but no interfering HPLC peaks were present. The HPLC method thus appears to be stability indicating.

Analysis of chlorobutanol by HPLC using UV detection at 210 nm is a reasonable alternative to the GC methods of analysis. Several years ago, the HPLC analysis of 'non-UV absorbers,' such as chlorobutanol, was thought to be difficult if not impossible (20); however, modern UV detectors can readily operate at the lower wavelengths (*i.e.*, 200–220 nm), and the quantitation of 'non-UV absorbers' has now become routine.

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Adjuvant Effects of Glyceryl Esters of Acetoacetic Acid on Rectal Absorption of Insulin and Inulin in Rabbits

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Abstract □ The promoting effect of glyceryl esters of acetoacetic acid on the rectal absorption of insulin and inulin was studied. A decrease in the serum glucose level was observed in rabbits following the administration of an insulin suppository containing glyceryl-1,3-diacetoacetate (adjuvant II) or 1,2-isopropylideneglycerine-3-acetoacetate (adjuvant IV). The promoting effects of adjuvants II and IV on the rectal absorption of insulin and inulin were suppressed by the addition of calcium and magnesium to the suppository. This indicates that adjuvant interaction with the calcium and magnesium ion located in the rectal membrane is involved in the enhanced absorption of insulin and inulin. Adjuvant release from the suppository formulation in addition to adjuvant lipid solubility were found to be other important factors for enhanced absorption of insulin and inulin.

Keyphrases □ Insulin—adjuvant effects of glyceryl esters of acetoacetic acid on rectal absorption, rabbits, inulin □ Inulin—adjuvant effects of glyceryl esters of acetoacetic acid on rectal absorption, rabbits, insulin □ Glyceryl esters—acetoacetic acid, adjuvant effects on rectal absorption of insulin and inulin, rabbits

In a previous paper (1), the effect of enamine derivatives of DL-phenylglycine on the rectal absorption of insulin was reported. It was suggested that rectal absorption of insulin was enhanced due to an interaction between the enamine derivatives and the calcium ions in the membrane. This interaction caused a temporary change in the integrity of the membrane allowing the insulin to pass more easily through the barrier. The active forms of the phenylglycine enamines are considered to be predominantly anionic. The interaction of these adjuvants with calcium and magnesium may be through the carboxylate moiety and/or the enamine moiety.

It has been reported (2) that intravenous administration of acetoacetic acid enhanced the distribution of chloropropamide and sulfadimetoxide to the red blood cells, indicating some change in erythrocyte membrane permeability. In the present paper, the glyceryl esters of acetoacetic acid were examined as adjuvants for promoting the rectal absorption of insulin and inulin. The ability of these nonionic compounds to be released from the suppository formulations, to permeate the rectal membrane, and to interact with divalent metal ions was examined.

EXPERIMENTAL

Materials—Glyceryl esters of acetoacetic acid were routinely synthesized by adding acetoacetic acid to glycerol or 1,2-isopropylideneglycerol in the presence of potassium acetoacetate (a catalyst) at

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