Table IV—Statistical Comparison [a] of Drugs Tested in Gravid and Nongravid CF-1 Mice

Test Group	LD_{50}, mg/kg	Slope	Slope Ratio	Slope Ratio Function	Potency Ratio	Potency Ratio Function
Morphine sulfate			1.11	1.20	1.08	1.22
Nongravid mice	480 (428–538)	1.17				
Gravid mice	520 (488–603)	1.30				
Cobalt chloride			1.04	1.60	1.11	1.26
Nongravid mice	157 (138–179)	1.33				
Gravid mice	174 (144–210)	1.28				
Phenytoin sodium			1.09	1.33	1.11	1.19
Nongravid mice	273 (250–298)	1.16				
Gravid mice	246 (214–283)	1.26				

[a] Values calculated by the method of Litchfield and Wilcoxon (2).

overnight in cages containing one male. On the next morning (Day 0), all females were observed for the presence of coagulated intravaginal semen, which was the criterion used for determining pregnancy. Then all those deemed pregnant were weighed and placed in individual cages. All of the nonpregnant mice were returned to the aggregate cages for later use.

On Day 9, gravid mice were randomly assigned to one of the appropriate dosage groups, and the respective solutions were administered subcutaneously; the same number of animals were used as for the nongravid groups. After 24 hr, fatalities were recorded. All gravid mice then were cervically dislocated and laparotomized to confirm pregnancy by the presence of fetal swellings.

RESULTS AND DISCUSSION

The teratogenic effects of morphine sulfate, cobalt chloride, and phenytoin sodium were reported previously (1, 3, 4). The determination of dosage schedules in these studies was made under the assumption that the LD_{50} of each drug did not vary between gravid and nongravid mice.

The data in Tables I–III were subjected to probit analysis according to the method of Litchfield and Wilcoxon (2). Because the slope ratios were less than the slope ratio functions, it can be concluded, with 95% confidence, that the dose–response curves did not deviate significantly from parallelism (Table IV). Furthermore, because the potency ratios were calculated to be less than the potency ratio functions, it can be concluded, with 95% confidence, that the LD_{50} of the respective drugs did not change significantly on Day 9 of gestation in CF-1 mice.

The range of the conclusions in this study is limited by the strain of mouse and the peculiarities of the drugs. However, because the drugs from three different classes did not vary in gravid and nongravid mice with respect to their LD_{50} curves, these results possibly can be extrapolated to other drugs. Hence, one LD_{50} curve obtained from nongravid mice may serve as a valid reference to determine the dose of an agent to be tested for teratogenicity.

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Rapid Colorimetric Analysis of Chlorhexidine in Pharmaceutical Preparations

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Received April 30, 1979, from the *Analytical Research and Development Department, P.O.S. Laboratories, 68240 Kaysersberg, France.* Accepted for publication August 21, 1979.

Abstract □ A colorimetric determination of chlorhexidine is described. The method is based on the formation of a yellow complex between the drug and bromcresol green. The absorption peak of this complex, extracted by chloroform, is at 410 nm, and linear response is obtained from 2.5 to 30 μg of chlorhexidine/ml. The accuracy and reproducibility of this rapid method make it useful for chlorhexidine determination in the manufacturing control of pharmaceutical mixtures.

Keyphrases □ Chlorhexidine—analysis, colorimetric determination in commercial dosage forms □ Antimicrobial agents—chlorhexidine, colorimetric determination in commercial dosage forms □ Colorimetry—analysis of chlorhexidine in commercial dosage forms

Chlorhexidine (I), an antimicrobial agent, is a common ingredient in antiseptic preparations. The presence of chlorhexidine as a preservative in other pharmaceutical dosage forms (eye drops) and cosmetics necessitated a rapid, economical, and sensitive determination.

BACKGROUND

The primary method available previously was a colorimetric procedure (1), but several components in pharmaceutical formulations interfere in the determination. Another colorimetric assay using *N*-(1-naphthyl)-ethylenediamine was reported for the determination of chlorhexidine in biological fluids (2), but it is time consuming and has background interference. A direct spectrophotometric assay of chlorhexidine using bromthymol blue was reported recently (3). Chlorhexidine is estimated based on differences in the absorbance before and after complexation with the acid dye. These techniques yield rather large errors.

Polarographic estimation (4) and an automated liquid chromatographic determination (5) also were reported recently. Chlorhexidine also has been determined successfully by GLC after hydrolysis to *p*-chloroaniline and suitable derivatization to *p*-chloroiodobenzene (6, 7), but this technique is time consuming and unsuitable for multiple analyses.

This paper describes a rapid colorimetric method that is based on the solvent extraction of a dye complex and is suitable for chlorhexidine analysis in pharmaceutical mixtures and cosmetics.

EXPERIMENTAL

Instrumentation—A double-beam spectrophotometer[1] and a shaker[2]

[1] Perkin-Elmer model 554.
[2] Lab-Line.

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Table I—Chlorhexidine Digluconate Absorbance–Concentration Relationship

Concentration, μg/ml	Absorbance at 410 nm (Average ± *SD*, $n = 4$)	*RSD*, %
2.5	0.0380 ± 0.0005	1.3
5	0.0740 ± 0.0021	3.2
10	0.192 ± 0.001	0.7
15	0.1880 ± 0.0066	3.5
20	0.225 ± 0.012	5.5
25	0.285 ± 0.006	2.1
30	0.343 ± 0.002	0.6

Table II—Assay of Solutions of Known Chlorhexidine Digluconate Concentrations

Amount, mg	Concentration, μg/ml	Amount Found, mg ($\bar{X} \pm SD$, $n = 4$)	Amount Found, %
5	10	5.3 ± 0.2	106
10	10	10.4 ± 0.4	104
40	10	39.2 ± 2.1	98
50	10	50 ± 1.7	100

Table III—Assay of Commercially Available Chlorhexidine Digluconate Formulations

Formulation	Amount Claimed, mg/100 ml	Amount Found, mg/100 ml	Average Label Claim, %
Eye drops[a]	5	5.3	106
		5.2	104
		4.9	98
Viscous solutions[b]	5	5.3	106
		5.15	103
		5.24	105
Shampoos[c]	20	19.4	97

[a] Contains niacinamide, inosine monophosphate, and sodium glycerophosphate. [b] Contains thimerosal, hydroxyethylcellulose, sodium phosphate, and potassium phosphate. [c] Contains saponins, hydroxyethylcellulose, and bronidox.

Table IV—Within-Day Reproducibility of Color Extraction of Replicate 10-μg/ml Samples of Chlorhexidine Digluconate

Sample	Absorbance at 410 nm
1	0.128
2	0.129
3	0.128
4	0.121
5	0.123
6	0.123
7	0.124
8	0.126
9	0.128
10	0.132
11	0.127
12	0.127
Average	0.126
SD	0.003
RSD, %	2.37

were used. To prevent adsorption of the drug–dye complex onto the glass, screw-top plastic tubes were required (8).

Reagents—A pH 5.70 buffer was prepared by mixing 100 ml of 0.1 *M* citric acid with 140 ml of 0.2 *M* anhydrous Na_2HPO_4. The bromcresol green solution was prepared by dissolving 30 mg of bromcresol green[3] in 100 ml of the pH 5.70 buffer and filtering. The solution was stable for 1 week. A 1:100 dilution had an absorbance of 0.154 ± 0.003 at 617 nm.

Chlorhexidine digluconate[4], 5 mg, was dissolved in 100 ml of distilled water. This stock solution was diluted to give 2.5–30-μg/ml working standards, which were prepared fresh for each run.

Procedure for Chlorhexidine Standard Solutions—One milliliter of standard solution was transferred to a screw-top plastic tube, and 3 ml of the bromcresol green reagent and 2.5 ml of chloroform were added. The tubes were stoppered, shaken for 3 min, and centrifuged at 3000 rpm for 3 min. The aqueous supernate was discarded. The chloroform layer was transferred carefully with an automatic pipet[5] to a 1-cm light path cell, and the absorbance was measured against a blank (1 ml of distilled water instead of the chlorhexidine solution) at 410 nm.

[3] Pechiney Ugine-Kuhlmann.
[4] ICI Chemicals.

Table V—Between-Day Reproducibility of Samples Containing 10 μg of Chlorhexidine Digluconate/ml

Sample	Day 1	Day 2	Day 3
1	0.128	0.120	0.122
2	0.129	0.126	0.123
3	0.128	0.129	0.123
4	0.121	0.128	0.122
5	0.123	0.132	0.124
6	0.123	0.128	0.120
7	0.124	0.132	0.122
8	0.126	0.134	0.120
9	0.128	0.131	0.139
10	0.132	0.132	0.120
Average	0.126	0.127	0.123
SD	0.003	0.006	0.005
RSD, %	2.4	4.7	4.0

Procedure for Chlorhexidine Viscous Solutions, Shampoos, and Lotions—The chlorhexidine base was extracted from ~10–25 mg of chlorhexidine digluconate-containing samples by adding 50 μl of 10 *N* NaOH and shaking the solution for 15 min with 5 ml of chloroform. After centrifugation for 3 min at 3000 rpm, 2.5 ml of the chloroform layer was transferred to a screw-top plastic tube, 3 ml of bromcresol green reagent was added, and the procedure was followed as described for the standard solutions beginning with: "The tubes were stoppered . . . ."

RESULTS AND DISCUSSION

Color Stability—The yellow color was monitored with a recording spectrophotometer and was stable for 8 hr.

Linearity—The absorption peak of the bromcresol green–chlorhexidine complex was at ~410 nm. At this wavelength, the reaction followed Beer's law to an absorbance of 1.0, and linear response was obtained for 2.5–30 μg of chlorhexidine/ml (Table I).

The linear regression line was $A_{410} = (11.1C + 17.1)\ 10^{-3}$, where A_{410} is the absorbance at 410 nm and C is the concentration expressed in micrograms per milliliter. The calculated molar absorptivity, ϵ, was 11,302 ± 270. The correlation coefficient was 0.998.

Accuracy—The mean recoveries of known chlorhexidine amounts added to the formulations are shown in Table II. The precision was calculated by a literature method (9) as ~3.8% and was similar for viscous solutions and formulated products.

Table III shows the method to be fairly precise for chlorhexidine in eye drops, contact lens cleaning solutions, and shampoos, so the method can be used for manufacturing controls. No interference was observed with formulations of alcoholic solutions, eye drops, viscous solutions, and gels.

Detection Limits—The detection limit, as determined on 20 samples at the 95% confidence level, was 2.5 μg of chlorhexidine digluconate/ml.

Reproducibility—To show the within-batch variation, the chlorhexidine digluconate content of a 10-μg/ml standard solution was determined 12 times (Table IV). To show the between-batch variation, duplicate determinations were carried out on several days. The results were highly reproducible (Table V). Interanalyst variations of five chemists were determined at five levels by the proposed method, and rectilinear results were obtained (Table VI).

Method Limitations—Attempts to extend the method to chlorhexidine creams were unsuccessful due to coextraction of the formulation excipients that complex with bromcresol green. Emulsions also can be formed with the solvent and the chlorhexidine-containing creams.

This colorimetric determination of chlorhexidine salts offers improved precision, convenience, and speed over the previously reported methods. The high color stability offers an advantage over the colorimetric procedure of Holbrook (1). These considerations make the method suitable for the automated analysis of chlorhexidine salt formulations.

[5] Gilson.

Table VI—Results (Mean ± *SD*) of Intralaboratory Collaborative Study on Chlorhexidine Digluconate ($n = 4$)

Concentration, μg/ml	Chemist A	Chemist B	Chemist C	Chemist D	Chemist E
3.5	0.057 ± 0.001	0.050 ± 0.001	0.044 ± 0.002	0.045 ± 0.002	0.036 ± 0.002
8.5	0.118 ± 0.005	0.115 ± 0.002	0.107 ± 0.002	0.109 ± 0.001	0.096 ± 0.001
16	0.236 ± 0.003	0.216 ± 0.002	0.208 ± 0.004	0.220 ± 0.003	0.215 ± 0.003
23	0.313 ± 0.005	0.299 ± 0.017	0.300 ± 0.010	0.320 ± 0.002	0.306 ± 0.001
28	0.392 ± 0.008	0.367 ± 0.008	0.376 ± 0.013	0.420 ± 0.012	0.380 ± 0.005
Correlation coefficient	0.997	0.974	0.997	0.996	0.998

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Brain-to-Blood and Saliva-to-Blood Mepivacaine Ratios in Rats

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Received June 18, 1979, from the *Department of Biological Sciences, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104.* Accepted for publication August 24, 1979.

Abstract □ Mepivacaine hydrochloride, 25 and 50 mg/kg sc (with sacrifice at 15 min) produced higher ($p < 0.005$) drug levels in neonate (24–36-hr-old) rat brain and blood than in adult rat brain and blood; however, there was no significant difference in the brain-to-blood ratio of the drug between neonates and adults at either dose level. Intraarterial infusion of mepivacaine hydrochloride (20 μg/min) in adult rats resulted in measurable (GLC) mepivacaine base levels in pilocarpine-induced parotid salivary secretions collected throughout 30- and 45-min infusion periods. The saliva-to-blood ratios (±*SEM*) of mepivacaine base were 0.64 ± 0.13 after a 30-min infusion and 2.13 ± 0.48 after a 45-min infusion.

Keyphrases □ Mepivacaine—brain-to-blood and saliva-to-blood ratios, rats □ Anesthetics—mepivacaine, brain-to-blood and saliva-to-blood ratios, rats □ Models, animal—mepivacaine, brain-to-blood and saliva-to-blood ratios, rats

Several clinical studies demonstrated behavioral deficiencies and toxic manifestations in newborn infants whose mothers received local anesthesia during labor (1–8). Mepivacaine, widely used for such anesthesia, has been implicated in neonatal bradycardia, depression, apnea, convulsions, and death (1–4, 9, 10).

Because critical determinations of drug levels in the blood and tissues of healthy infants rarely are feasible, an animal model was employed to explore quantitative aspects of mepivacaine-related neural toxicity in neonates. Brain-to-blood ratios of mepivacaine base were determined for both neonate (24–36-hr-old) and adult rats after subcutaneous administration (25 and 50 mg/kg) of mepivacaine hydrochloride. Mepivacaine levels in adult rat whole blood and parotid saliva were compared to determine whether the salivary mepivacaine level could serve as an index of the blood drug concentration.

EXPERIMENTAL

Female Sprague–Dawley rats were supplied with food and water *ad libitum*. One group consisted of nonpregnant adult rats (250–300 g). Another group contained newborn rats (6–9 g) which remained with their mothers and nursed freely until drug or saline administration 24–36 hr postpartum.

Adult rats were prepared for saliva collection by anesthetization with urethan (1.2 mg/kg ip) followed by tracheotomy and cannulation of the parotid ducts according to a literature method (11). Tapered polyethylene tubing (PE 50) was inserted into each duct with the aid of a binocular dissecting microscope. Pilocarpine hydrochloride[1], 0.25 mg/ml, was infused at 0.2 ml/min into the right brachial artery to stimulate parotid salivary secretion. Mepivacaine hydrochloride[2], 1 mg/ml, was infused intraarterially (0.2 ml/min) concomitantly with the pilocarpine solution. Saliva was collected continuously throughout either a 30- or 45-min pilocarpine–mepivacaine infusion period, immediately after which 2–4 ml of blood was withdrawn *via* cardiac puncture into a heparinized vacutainer.

In another study, nonpregnant adult female rats were injected subcutaneously with 25 or 50 mg of mepivacaine hydrochloride/kg in 1.0 ml of saline; after 15 min, blood was collected *via* cardiac puncture into a heparinized vacutainer. The animals then were decapitated, and the brain was removed.

Two neonates were selected randomly from each of 15 litters and were injected subcutaneously with mepivacaine hydrochloride; one littermate received 25 mg/kg and the other received 50 mg/kg in 0.1 ml of saline. Animals were sacrificed 15 min after drug administration. Procedures for blood collection from neonates combined two techniques (12, 13). Brain tissue was removed after the spinal cord was severed at the neck.

Newborn and adult rat tissue and fluid samples also were collected from saline-injected animals, spiked with mepivacaine, and extracted for standard curves constructed each day for each tissue type. Mepiva-

[1] Aldrich Chemical Co., Milwaukee, Wis.

[2] Courtesy of Sterling-Winthrop Research Institute, Rensselaer, N.Y.

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