for bonding atoms contribute to the bond so that the geometric mean of the fraction products characterizes the new physical environment. Thus, a bond index is born. The bond indexes $[(1/\delta_A^v)(1/\delta_B^v)]^{1/2}$ are summed to form the molecular connectivity index, $^1\chi^v$.

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Analysis of Drug Contamination from Parabens in Theophylline Olamine

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Abstract □ Contaminants in a commercial enema sample of theophylline olamine were found to be derived from parabens and ethanolamine. These contaminants, whose presence was characterized by the loss of preservatives and solubilizing agent, were isolated directly from the drug sample and identified. A high-performance liquid chromatographic (HPLC) system was developed to separate completely and to measure quantitatively theophylline, the two impurities, 4-hydroxybenzoic acid and *N*-(2-hydroxyethyl)-4-hydroxybenzamide, and the remaining parabens. The material balance obtained from the results of quantitative HPLC indicated the formation of these impurities at the expense of parabens. TLC, IR, and UV spectrophotometry and NMR and mass spectrometric analyses were used for identification or for comparisons of the new compound and the known *N*-(2-hydroxyethyl)benzamide.

Keyphrases □ Theophylline olamine—contaminants, identification and quantitation, high-performance liquid chromatographic analysis □ Contaminants—identification and quantitation, theophylline olamine enema solutions, high-performance liquid chromatographic analysis □ Paraben preservatives—contamination in theophylline olamine enema solutions, high-performance liquid chromatographic analysis, identification and quantitation □ High-performance liquid chromatography—contamination from paraben preservatives in theophylline olamine enema solutions

During a survey of xanthine derivative drugs conducted by the National Center for Drug Analysis, assay of some enema solutions containing theophylline olamine was attempted with a high-performance liquid chromatographic (HPLC) method that had been used successfully for theophylline elixirs. The analysis showed that, in addition to theophylline and parabens, the sample contained two impurities. This paper discusses the identification and quantitation of these two substances.

BACKGROUND

The approach followed in this laboratory study was dictated primarily by the small amounts of sample initially available. It involved, first, the original detection of the impurities 4-hydroxybenzoic acid (I) and *N*-(2-hydroxyethyl)-4-hydroxybenzamide (II) by HPLC and TLC, with characterization of their aromatic nature by UV spectrophotometry; and, second, identification by isolation of the impurities from the drug sample and direct comparison with standards by mixed melting-point determinations and IR and NMR analyses. Identities were confirmed by HPLC and TLC comparisons of the original drug solution with commercial and synthesized standards and by a wide collection of spectral data. Finally, the theophylline peak was displaced from the narrow region between the impurity peaks by adjusting the mobile phase to enable quantitative HPLC analysis.

The question of isomerism in the conventional syntheses used to prepare the amides arose because two materials whose elemental analyses were consistent for *N*-(2-hydroxyethyl)benzamide (III) showed different properties. The problem was resolved by purification that showed the materials to be identical; moreover, the mass fragmentations were consistent with the structures given for II and its structural analog, III.

This work demonstrates that parabens can interact chemically with amines used as complexing agents in the compounding of theophylline

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drugs. As a result, foreign substances are introduced into the drug sample, the amount of active drug in the form of the absorbable soluble amine complex is reduced, and the bactericidal and fungicidal properties of the paraben preservative are reduced because of its consumption.

EXPERIMENTAL

Materials—Distilled-in-glass chloroform[1], acetone[1], methanol[1], and acetonitrile[1] and reagent grade sodium bicarbonate, hydrochloric acid, acetic acid, ethanol, silver nitrate, and magnesium sulfate were used. Deionized water was used in preparing mobile phases and in extraction. Benzene[2] was 99 mole % pure, and ether[3] was anhydrous analytical reagent grade. The reactants, primary standard benzoic acid[3], organic reagent 4-hydroxybenzoic acid[3], reagent grade methyl and propyl *p*-hydroxybenzoate[4], and purified grade ethanolamine[4], were used as received.

Theophylline monohydrate[3] (USP grade), phenol[3] (analytical reagent grade), and ferric chloride[2] (certified ACS grade) were used. The spray reagents, ninhydrin[5] [0.2% in methanol–isopropanol (30:70)] and bromcresol green[6] (0.05% in isopropanol), were used as detection agents for TLC. Base-range[7] and short-range[8] pH paper, silica gel columns[9] (2.5 cm), and TLC plates[10] were used.

Instruments and Parameters—Grating IR spectrophotometers[11], a UV/near-IR spectrophotometer[12], a 60-MHz NMR spectrometer[13], a high-performance liquid chromatograph[14], and a double-focusing mass spectrometer[15] interfaced to a data system[16] were employed. Melting points were taken in a capillary melting-point apparatus[17].

Direct insertion by probe was used for mass spectrometry under the following conditions: acceleration voltage, 300 v; source temperature, 200°; electron energy, 70 ev; emission current, 1 mamp; scan rate (SR 22), 4 sec/mass decade, exponential; and multiplier, 16-stage dynode at 2.6 kv (10^6 gain, minimum).

Instrument parameters for HPLC included a UV detector with a fixed wavelength of 254 nm and a microparticulate reversed-phase column[18] (30 cm × 3.9 mm i.d.). The mobile phase was 0.1% acetic acid in 10% acetonitrile. A flow rate of 2.0 ml/min and an automatic injector with a 20-μl loop were used. Retention times were converted to capacity factors.

TLC—Substances were spotted as methanol solutions. The drug sample was spotted directly on silica gel plates (5 × 7.5 cm) or was acidified toward litmus with hydrochloric acid and extracted once or twice with equal volumes of chloroform before spotting. The chromatograms were developed with a mixed solvent (equal volumes of chloroform and acetone) by the equilibrated, ascending technique in a tank lined with filter paper.

Spots were placed 1 cm from the base of the plate, and the solvent was developed just to the top of the plate. Most spots were located by short-wave UV irradiation, but ethanolamine spots were developed either by spraying the plate with ninhydrin and placing it in an oven at 100° for 3 min or by spraying with bromcresol green.

Detection of Impurities by HPLC and TLC–UV Characterization—The mobile phase consisted of 0.1% acetic acid in 10% acetonitrile. A sample of the drug solution diluted with 10 volumes of absolute methanol was injected directly for HPLC analysis. To remove the parabens, another sample was made acidic to litmus with 6 *N* HCl and washed three times with equal volumes of chloroform prior to HPLC analysis. These aqueous layers also were analyzed by TLC. Materials represented by the outside halves of the two peaks of the impurities were collected during elution in the HPLC of the drug sample that previously had been washed free of parabens. These eluents were examined by TLC and then by UV spectrophotometry.

An authentic theophylline monohydrate sample was dissolved in a solvent consisting of 0.1% acetic acid in 10% acetonitrile and also was examined by UV spectrophotometry for comparison.

Extraction of Drug Sample Impurities—*Separation of Impurities from Theophylline and Parabens and Isolation of I*—A 10-ml portion of the basic (pH 8.8) enema solution was adjusted to pH 5.0 with short-range paper by dropwise addition of 6 *N* HCl and was extracted by shaking for 5 min with 60 ml of chloroform. The extraction was repeated three times to provide complete separation of theophylline and parabens from the aqueous solution as ascertained by TLC. The aqueous solution then was extracted twice with 40-ml portions of ether and saved for subsequent isolation of II.

The combined ether extracts, containing any free I, were dried over anhydrous magnesium sulfate and filtered. To obtain the product, the ether was removed from the solution on a steam bath under nitrogen; the residue was kept on the steam bath under nitrogen for 5 min to ensure complete solvent removal. The material was recrystallized from acetonitrile, dried at 55°/3 mm for 1 hr, and examined by IR and NMR analyses and melting-point determination.

Isolation of II—A 50-ml portion of the drug sample was treated as already described, and the aqueous solution remaining after chloroform and ether extraction was evaporated to dryness on a steam bath under a strong nitrogen flush. The solid residue was extracted twice with 15 ml of boiling acetonitrile to remove II. The undissolved portion of the residue remained fluid during the hot extraction but solidified immediately upon cooling, permitting solvent decantation. The undissolved residue was recrystallized from absolute ethanol and identified by comparison with a synthesized sample of ethanolamine hydrochloride, whose preparation will be described.

The acetonitrile solutions were combined and filtered, and a portion of the filtrate was tested by TLC for the presence of II and residual ethanolamine. The remaining filtrate was evaporated on a steam bath under a nitrogen flush to remove the solvent, leaving a semisolid residue. Amide II was detected on TLC plates by fluorescence diminution, and ethanolamine at the origin of the chromatogram was detected by pH-sensitive bromcresol green spray or by ninhydrin spray followed by a 3–4-min exposure to 100° heat.

A 2.5-cm silica gel cartridge[9] was preconditioned by passing 40 ml of chloroform through it. The semisolid residue from the acetonitrile evaporation was dissolved in the minimum amount (~4–5 drops) of absolute methanol, transferred with a capillary tube to the top of the column, and washed onto the cartridge three times with minimum portions of chloroform.

Chloroform (100 ml) was passed through the column to remove any impurities present in the forerun, and II then was eluted with chloroform–acetone (1:1). For product isolation and identification, the first few milliliters of eluate were evaporated to dryness by warming under a flush of nitrogen. The residue was used for subsequent identification of II.

In addition to characterizing the impurities directly in the drug sample through extraction, HPLC evidence was obtained by spiking the drug sample with additional amounts of reagent grade I and synthesized II.

Preparation of Ethanolamine Hydrochloride—An aqueous solution of ethanolamine containing 10% molar excess of hydrochloric acid was evaporated almost to dryness by boiling and completely to dryness by azeotropic removal of water with benzene. The white crystals obtained upon cooling were recrystallized from a small amount of absolute ethanol under a nitrogen atmosphere to preclude moisture, but they were not particularly deliquescent. The crystallized material was dried at 55°/3–4 mm for 3 hr, mp 83.0–83.8°.

Anal.—Calc. for C_2H_8ClNO: C, 24.68; H, 8.27; N, 14.36. Found: C, 24.81; H, 8.35; N, 14.29.

Synthesis of II from Methylparaben and Ethanolamine—Methyl 4-hydroxybenzoate (15.2 g, 0.10 mole) and 6.11 g of ethanolamine (0.10 mole) were placed in a round-bottom flask fitted with a vacuum adapter to a small 15-ml receiver. The system was flushed with nitrogen, and the reaction was carried out under a nitrogen atmosphere. The flask was immersed in an oil bath at 103–107° for 10.5 hr, with the clear melt being agitated by a magnetic stirring bar. Only a few drops of liquid collected in the receiver, but a hard, sticky semisolid formed in the reaction mixture upon cooling. The solid was triturated for 10 min with 60 ml of chloroform.

This process was repeated four times, during which the semisolid became tacky and hard. The solid was triturated twice with 40 ml of dry ether, which left a granular white solid. Crystallization from a small amount of absolute ethanol gave 6.00 g (33%) of white solid (II), mp 145–151°. Recrystallization two more times and drying at 100°/5 mm for 2 hr gave a sample, mp 155.3–156.3°, suitable for analysis. IR spectral

[1] Burdick & Jackson Laboratories, Muskegon, Mich., and Taylor Chemical Co., St. Louis, MO 63144.
[2] Fisher Scientific Co., Fair Lawn, NJ 07410.
[3] Mallinckrodt Chemical Works, St. Louis, MO 63147.
[4] Sigma Chemical Co., St. Louis, MO 63118.
[5] Quantum Industries, Fairfield, NJ 07006.
[6] EM reagent, Curtin Matheson Scientific, Maryland Heights, MO 63043.
[7] Accutint indicator paper, Anachemia Chemicals, Champlain, NY 12919.
[8] Short-range Alkacid, Fisher Scientific Co., Fair Lawn, NJ 07410.
[9] Sep-Pak silica cartridge, Waters Associates, Milford, MA 01757.
[10] EM 60 F-254 silica gel plates, EM Laboratories, Elmsford, NY 10523.
[11] Models 337 and 621, Perkin-Elmer Corp., Norwalk, CT 06856.
[12] Model ACTA MIV, Beckman Instruments, Fullerton, CA 92634.
[13] Varian Instrument Division, Palo Alto, CA 94303.
[14] Model 3500B, Spectra-Physics, Santa Clara, CA 95051.
[15] MAT 311, Varian Associates, Instrument Group, Palo Alto, CA 94303.
[16] 620 L 100, Varian Associates, Instrument Group, Palo Alto, CA 94303.
[17] Thomas–Hoover, Arthur H. Thomas Co., Philadelphia, PA 19105.
[18] μBondapak C_{18}, Waters Associates, Milford, MA 01757.

analysis showed prominent bands at 3370, 3240, 1610, 1570, 1510, 1320, 1290, 1240, 1185, 1080, 1065, 860, and 710 cm^{-1}. The product was insoluble in chloroform and ether and also could be crystallized from water, acetonitrile, and acetone. A sample also was submitted for mass spectrometric and elemental analyses.

Anal.—Calc. for $C_9H_{11}NO_3$: C, 59.66; H, 6.12; N, 7.72. Found: C, 59.80; H, 6.44; N, 7.52.

Preparation of II by Reaction between I and Ethanolamine—A very low yield of product was obtained in this procedure. A mixture of 13.8 g of I (0.10 mole) and 6.11 g of ethanolamine (0.10 mole) was heated gradually to 200° according to the procedure of Wenker (1). Vacuum distillation, after a small forerun, gave ~10 ml of a colorless liquid having a phenol odor, bp 77–79°/11 mm. This distillate had a boiling point corresponding to that of phenol but gave a negative test for phenol with 1% ferric chloride solution and with ferric chloride–urea reagent (2), and it gave a poor IR spectral match with pure phenol.

A developed TLC plate containing the material indicated the presence of ethanolamine when sprayed with bromcresol green. Approximately 4 drops of the distillate were dissolved in chloroform and placed on the top of a silica gel cartridge that had been conditioned previously with 40 ml of chloroform. When ethyl acetate was passed through the cartridge, pure phenol eluted within the first 2 ml and was identified by TLC after comparison with phenol spotted alongside the distillate (R_f 0.68). When the chromatogram was sprayed with bromcresol green, no blue spot for ethanolamine appeared. The material purified by chromatography gave a deep-purple color with 1% ferric chloride solution and with ferric chloride–urea reagent, as expected for phenol. The IR spectrum also matched that of an authentic phenol sample.

A small amount of the desired product was obtained from the sticky, semisolid residue (2.3 g) left after the distillation. This residue was triturated twice with 10 ml of chloroform and once with 10 ml of anhydrous ether, leaving 2.13 g (11.8%) of light-brown granular solid. After two recrystallizations from absolute ethanol, the product melted at 155.2–156.2°. It was shown to be II by mixed melting point with the analytically pure sample of this substance prepared as already described and by comparison of the IR spectrum with that of the pure compound. TLC of the two synthesized samples and the isolated amide II showed identical R_f values.

Synthesis of III from Benzoic Acid and Ethanolamine—Compound III was synthesized by the procedure of Wenker (1). In the present study, a reaction on a 0.1 *M* scale gave a forerun of ~1 g of sublimate upon distillation. This white solid was recrystallized twice from 95% ethanol (mp 143.5–144.5°) and dried at 55°/3 mm for 1.5 hr. Results of elemental analysis were consistent with the structure for β-hydroxyethylammonium benzoate.

Anal.—Calc. for $C_9H_{13}NO_3$: C, 59.00; H, 7.15; N, 7.65. Found: C, 59.12; H, 7.41; N, 7.57.

The identity of this substance was confirmed as follows. Known β-hydroxyethylammonium benzoate was obtained as a white solid, which crystallized on cooling from a hot acetone solution of equimolar amounts of benzoic acid and ethanolamine. No melting-point depression was observed from a mixture of the known and unknown materials.

Continued distillation yielded ~8 g of an oily product, which failed to give a dry crystalline product on attempted crystallization from common solvents; however, a crystalline product was obtained in two ways. First, in a crystal growth technique, a small amount of the oil was placed in an erlenmeyer flask with a small overlayer of dry ether; the flask was stoppered and stored in the freezer compartment of a refrigerator. Soft, fibrous, single needles, 3 cm long, grew out of the mass overnight, leaving at the origin only a very small amount of liquid unconverted to crystals. The product was collected quickly, washed with a small amount of dry ether, dried in air, and crushed to a white powder, R_f 0.30, mp 63.5–65.8° [lit. (3) mp 66–67°].

The product (III) was dried over phosphorus pentoxide for 1 hr and then at 55°/4 mm for 1.5 hr. It then was submitted for mass spectrometric and elemental analyses.

Anal.—Calc. for $C_9H_{11}NO_2$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.20; H, 6.93; N, 8.31.

Some crystals were used to seed the crude distillate. On standing several hours, the distillate crystallized to a semisolid laced with needles. However, the needles were thixotropic, and the crystals became fluid and were adsorbed when collection on filter paper by filtration or by pressing on tile was attempted.

In the second purification procedure, a double silica gel cartridge (two silica gel cartridges[9] connected in tandem with an inserted small piece of glass tubing) was preconditioned with chloroform as already described. A portion of the crude distillate (119 mg) was dissolved in 0.2 ml of chloroform, applied to the top of the column, and washed into the column by two applications of a few drops of chloroform. Chloroform was used for development and elution. The first 10 ml of eluate contained only small amounts of impurities and was discarded.

The amide product (III) eluted completely in the next 70 ml. The solvent was removed from this fraction by warming under nitrogen, leaving a heavy oil. When scratched with a stirring rod, this oil formed a hard white solid, mp 63.8–64.5°. This product was free of ethanolamine, as determined by TLC and bromcresol green spray. The products isolated by the crystal growth and silica gel cartridge techniques were shown to be identical by mixed melting-point and IR analyses (3300, 3140, 1600, 1520, 1440, 1370, 1320, 1290, 1200, 1150, 1060, 1040, 870, 800, 760, and 690 cm^{-1}).

Quantitative Study by HPLC—*Mobile Phase*—Impurities II and I and theophylline were determined by HPLC, using a mobile phase of 0.5% acetic acid in 2.8% aqueous acetonitrile. Methylparaben was determined with 0.5% acetic acid in 10% aqueous acetonitrile, and propylparaben was determined with 0.5% acetic acid in 25% aqueous acetonitrile.

Sample and Standard Solutions—A stock solution of II (synthesized from paraben) was prepared by dissolving 22.1 mg of the recrystallized compound in 250.0 ml of methanol (88.4 μg/ml). This stock solution was diluted to 79.6, 44.2, and 26.5 μg/ml with methanol. A sample solution was prepared by diluting 5.0 ml of enema solution to 50.0 ml with methanol.

A solution of I was prepared by dissolving 60.0 mg in 200.0 ml of methanol (300 μg/ml) and was used directly. Another solution of I was prepared by dissolving 42.6 mg in 200.0 ml of methanol (213 μg/ml), and an aliquot of this solution was diluted to 102 μg/ml with methanol. A sample solution was prepared by diluting 5.0 ml of enema solution to 50.0 ml with methanol.

The stock methylparaben solution was prepared by dissolving 20.0 mg of methylparaben in 100.0 ml of methanol (200 μg/ml), and aliquots were diluted to 160, 80, 60, and 44 μg/ml with methanol. A sample solution was prepared by diluting 6.0 ml of enema solution to 50.0 ml with methanol.

The stock propylparaben solution was prepared by dissolving 20.0 mg of propylparaben in 100.0 ml of methanol (200 μg/ml). This stock solution was diluted to 128, 100, and 50 μg/ml with methanol. A sample solution was prepared by diluting 17.0 ml of enema solution to 50.0 ml with methanol.

A stock solution of theophylline was prepared by dissolving 153 mg of anhydrous theophylline in 100.0 ml of methanol (1.530 mg/ml), and aliquots were diluted to 1.150, 0.770, and 0.380 mg/ml with methanol. A sample solution was prepared by diluting 3.0 ml of enema solution to 50.0 ml with methanol.

HPLC Method—After the approximate concentration of each component of the sample solution was determined, standard solutions were prepared to range from ~50 to 150% of these concentrations. Each sample and standard solution was injected at least three times, and peak heights were measured manually. Each sample component was calculated by:

$$C_c = H_s/H_{std}C_sDF \qquad \text{(Eq. 1)}$$

where C_c and C_s are the concentrations (milligrams per milliliter) of each component of the sample solution and of the corresponding standard, respectively; H_s and H_{std} are the peak heights of the sample and standard solutions, respectively, such that the standard concentration is chosen to yield a standard peak height within 5% of the sample peak height; and DF is the sample dilution factor.

The full-scale absorbance settings were: theophylline and I, 0.64; II and methylparaben, 0.16; and propylparaben, 0.08. The recorder chart was operated at 30.48 cm/hr.

Ethanolamine—Ethanolamine was determined by the official NF method (4).

RESULTS AND DISCUSSION

The chromatogram obtained from the HPLC (0.1% acetic acid in 10% acetonitrile) determination of the diluted enema solution or the drug solution applied directly on the C_{18} reversed-phase column showed a cluster of peaks reflecting capacity factors of 0.87 for II, the first impurity to elute, 1.62 for theophylline, and 2.25 for I, the second impurity to elute. The peak of the major impurity, I, was almost three times as large as that of the minor impurity, II, and the theophylline peak was the largest of the three. The parabens eluted much later than this cluster.

Acidification of the drug sample and subsequent extraction with chloroform completely removed the parabens and concentrated the im-

Table I—R_f Values for Isolated and Synthesized Compounds [a]

Compound	R_f
Theophylline	0.22
I	0.27
II	0.17
III [b]	0.30
Methylparaben	0.70
Propylparaben	0.74
Ethanolamine	0.00 [c]

[a] Silica gel plates and solvent system composed of equal volumes of chloroform and acetone. [b] *N*-(2-Hydroxyethyl)benzamide. [c] Developed with bromcresol green spray or ninhydrin spray followed by heating at 100° for 3 min.

purities at the expense of theophylline. The completeness of this process was reflected in chromatograms of the extracted samples by the absence of peaks for parabens and the modification of peak heights in the cluster. Relative heights of the peaks for the impurities remained essentially unaltered and suggested repeated extractions of this type as a first step in the isolation of the impurities from the drug sample. The UV spectra of selected fractions eluted during HPLC showed a λ_{max} of 252 nm for II and a λ_{max} of 256 nm for I, indicating the aromatic nature of the compounds. The λ_{max} of theophylline in the mobile phase was 272 nm, which suggested that the impurities were not simple derivatives of that substance. Methylparaben itself showed an absorption at 255 nm.

TLC confirmed that two impurities were present in the drug sample. The R_f values for the smaller impurity (0.17), theophylline (0.22), and the larger impurity (0.27) (Table I) indicated a reverse order of migration for the substances on 7.5-cm silica gel TLC plates compared with that on C_{18} reversed-phase HPLC, as expected. The R_f values from 20-cm TLC plates were essentially the same as those from 7.5-cm plates.

After the acidified drug solution was washed repeatedly with chloroform, no theophylline could be detected by TLC. The first impurity (I) was isolated by ether extraction and was obtained as a white solid after removal of the ether solvent. Two such treatments involving chloroform and ether extractions of acidified drug sample gave 8.0 and 7.4 mg of this impurity, representing 4.8 and 4.4%, respectively, relative to the weight of the therapeutic component, theophylline. After recrystallization, the isolated impurity melted at 205.5–206.0°.

Anal.—Calc. for $C_7H_6O_3$: C, 60.86; H, 4.38; mol. wt. 138. Found: C, 60.91; H, 4.27; mol. wt. 142.

After analysis indicated that the extracted product was I, identification was confirmed by using an authentic sample of I in IR and NMR comparisons and by mixed melting-point determination.

Because limited sample was available and the impurities were present in a very dilute state, subsequent extractions leading to identification of II, which was present in an even smaller amount, seemed difficult without a reference standard. UV analysis of the isolated HPLC eluate suggested that II could have an aromatic structure similar to that of the parabens. In the search for a reference standard, various xanthines and uric acids were examined, as were heated reaction mixtures from methylparaben with theophylline and with ethanolamine. The latter reaction provided the desired source. Initial HPLC of the crude product indicated that the major product of the reaction was identical with the minor impurity (II) in the commercial sample. TLC also supported this identity.

After I was extracted from the aqueous residue, II was isolated by evaporation of the residue to dryness and preliminary extraction with acetonitrile to remove II from the insoluble ethanolamine hydrochloride. This procedure left 175 mg of residue which, after crystallization from absolute ethanol, yielded 122 mg of ethanolamine hydrochloride, mp 83.0–83.5°. This material appeared to be a dry, not readily deliquescent material, whereas the literature described the substance as deliquescent (5) and gave two melting points, 75–77° (5) and 100° (6). Therefore, the recrystallized substance was identified further as ethanolamine hydrochloride by comparing its IR and NMR spectra with those of synthesized ethanolamine hydrochloride. A mixture of known ethanolamine hydrochloride and the unknown gave no depression in melting point.

The acetonitrile extraction removed most of the amide impurity from the ethanolamine salt, and TLC examination of the extract by fluorescence diminution showed only II and no I or theophylline. Moreover, only a small amount of ethanolamine was detectable compared with a similar examination of the residue before the acetonitrile treatment. The ethanolamine showed little or no migration from the origin (Table I) and was detected as a blue spot with bromcresol green or as a red spot with ninhydrin followed by heat. This low migration allowed purification of II on a short silica gel column.

The residue remaining after acetonitrile evaporation was applied to the column; passage of a large volume of chloroform removed traces of impurities from II, which showed little migration with this eluent. When the solvent was changed to chloroform–acetone (1:1), II eluted immediately; elution was complete within the next 7 ml without release of any ethanolamine. An additional 40 ml of the solvent failed to elute any ethanolamine, attesting to the effectiveness of the method. Solvent removal from the 7-ml fraction of mixed solvent left 9.33 mg of dry white powder, mp 154.0–156.0°. The impurity was identified as II by IR and NMR comparisons with the analytically pure, synthesized compound. A mixture of the known and unknown compounds gave no depression in melting point.

Although I and II were detected initially by HPLC and TLC, these procedures allowed identification of the impurities by direct extraction from the drug sample.

In terms of the active ingredient, ~7.7 mg of I [4.6% by weight relative to the weight (166 mg) of theophylline expected by label declaration] and ~1.9 mg or 1.1% of II were obtained by extraction of 10 ml of the drug sample. Heavy losses were expected from the multiple extractions used in purification. Refinement of the HPLC technique, which allowed quantitative analysis and reflected chemical changes that resulted in formation of the contaminant, will be described.

The impurities in the drug sample were identified directly by extraction and the use of reference standards. Confirmatory identification was obtained from HPLC by spiking drug samples with the reference standards, resulting in enlargements of the peaks representing the impurities without any new peaks.

The problems associated with the isolation and identification of II were the lack of a preparation method for it and the high solubility of such amides in water, a property shared by ethanolamine, a possible contaminant of these amides. Ethanolamine can be removed by silica gel columns, onto which the ethanolamine is tenaciously adsorbed. A method (1) was described for the preparation of the structural analog, amide III, directly from I and the amine, but a more effective approach seemed to be a reaction between an ester and the amine. Moreover, the reaction between paraben esters and ethanolamine warranted study as a source for generation of II in the drug sample. The reaction gave a product that was identical with one impurity in the drug sample and whose identity was confirmed by elemental analysis.

Reported methods for preparation of III are not without discrepancies, but they were resolved. Prepared as already described, the compound was initially a water-soluble liquid, in accordance with the description of Wenker (1), who reported that the compound could not be obtained in crystalline form. However, Fränkel and Cornelius (3) reported that the compound was a crystalline solid when prepared by the Schotten–Baumann reaction followed by hydrolysis of the dibenzoyl derivative of ethanolamine. The structure of these two products rested on the reported elemental analysis for nitrogen.

In this laboratory, a reaction was attempted between I and ethanolamine by gradually heating the mixture to 200° under the conditions used by Wenker to prepare the liquid parent compound. The reaction gave phenol primarily, which resulted from the ease of decarboxylation attributed to resonance effects of the 4-hydroxy group. However, some II was generated and was identical with the product obtained by reaction of methylparaben and ethanolamine and with the impurity contained in the drug sample. The reaction of Wenker was repeated and yielded a liquid that could not be obtained as a crystalline solid by the usual procedures but was obtained in crystalline form by a crystal growth technique under dry ether or by use of a silica gel cartridge. The melting point of these crystals was essentially the same as that previously obtained (3), indicating that this amide and that of Wenker were identical. It is unlikely that I formed first in the drug sample and then partially reacted with ethanolamine to generate II. Even when these reactants were heated gradually to 200°, as already described, phenol was formed primarily by decarboxylation.

In view of the slight differences in reports describing the synthesis of the parent amide, both II and III were analyzed by mass spectrometry. A rather general similarity of fragmentation pattern was shown by these substances (Table II). The spectra for II and III showed molecular ions at *m/z* 181 and 165, respectively, and base peaks, as expected, at *m/z* 121 and 105, respectively. The odd *m/z* ratio of the molecular ion confirmed the odd number of nitrogen atoms in the compounds. Postulated structures for some major ions are given in Table II.

The NMR spectrum of II (methanol-d_4 solvent) showed essentially two doublets for the *ortho* and *meta* aromatic hydrogens centered at 6.75 and 6.90 ppm, splitting each other with a coupling constant of $J = 4.2$ Hz. Both I and methylparaben also had very similar pairs of doublets for

Table II—Postulated Structures of Some Major Ions Generated in Mass Spectrometric Fragmentation of $RCONHCH_2CH_2OH$

Fragment Ion	R = HOC_6H_4[a]		R = C_6H_5[b]	
	m/z	Intensity[c]	m/z	Intensity[c]
$RCONHCH_2CH_2OH^{+\cdot}$ ($M^{+\cdot}$)	181	3.28	165	2.24
$RCONHCH{=}CH_2^{+\cdot}$ ($M - H_2O$)	163	20.45	147	13.63
$RCONHCH_2^+$	150	5.29	134	8.96
$R(HO)C{=}NH_2^+$	138	12.50	122	20.01
$RC{\equiv}O^+$	121	100.00	105	100.00
R^+	93	32.19	77	66.68

[a] Probe heated at 125° to volatilize sample; background subtracted. [b] Probe heated at 80° to volatilize sample; background subtracted. [c] Ratio against base peak, percent.

coupling between these two types of aromatic protons. Both III and II showed similarly shaped multiplets for the methylene protons, 3.48–3.78 ppm. The aromatic regions for these compounds were quite different, and aromatic absorptions for III with complicated multiplets centered at 7.26 and 7.82 ppm were identical with those in the spectrum of *N*-(2-hydroxyethyl)thiobenzamide previously reported (7).

Other investigators (8) recently reported a chemically related contamination resulting from a reaction between ethylenediamine complexing agent and a suppository base.

The high efficiency of the reversed-phase column produced a wide range of capacity factors, which necessitated the use of three mobile phases to separate the five components of the enema solution. Resolution to baseline was achieved for the analysis of each component. Acetic acid was added to the mobile phases to reduce peak tailing.

The original conditions (10% acetonitrile, Table III) used for detection of the impurities in the drug sample placed the peak for theophylline in a narrow region between the peaks for the impurities, leading to considerable overlap and precluding quantitative determination of the impurities. Fortunately, the capacity factor of theophylline on the lipophilic column was much more sensitive to the polar nature of the mobile phase than were the impurities. In general, the capacity factors increased as the percentage of acetonitrile declined. However, from 10.0 to 2.8% acetonitrile, the differential capacity factor $k'(I)-k'(T)$ for I relative to theophylline went through an inversion (Table III) from 0.2 to −4.8, *i.e.*, the separation factor [relative retention, $\alpha = k'(I)/k'(T)$] became fractional. This general trend was reflected by collection of data with a mobile phase containing no acetonitrile (Table III). The problem was resolved by removing the theophylline peak from its location between the peaks for the impurities, allowing quantitation of the impurities by use of 2.8% acetonitrile as the mobile phase. However, since retentions for the parabens were excessively long with this mobile phase, 10 and 25% acetonitrile were used for quantitation of methyl- and propylparabens, respectively.

Before the use of peak heights to measure sample concentrations, it was determined that injection volumes were reproducible to better than 1%. Standard calibration curves for each enema component demonstrated linearity of peak heights with concentrations between 50 and 150% of the actual component concentration in the enema sample. The mean peak height for at least three injections was used in all calculations. The concentration of each component of the enema solution is listed in Table IV.

These analytical data indicate that only 96 mg of total paraben remained from a label claim of 360 mg of total paraben/100 ml of enema solution. The residual parabens along with impurities accounted for 106% of the label claim for total parabens on a molar basis, indicating that 73.3% of the parabens had chemically decomposed. Thus, the fungistatic and bacteriostatic properties of the solution were seriously lowered. In addition, 6% (0.26 mg/ml) of the label quantity of ethanolamine was converted to II, decreasing the ethanolamine concentration to 23.6% of the labeled quantity of theophylline olamine; the official limits are 22–28% of label.

Table III—Capacity Factors in Quantitative HPLC of Enema Solution

Acetonitrile[a], %	Amide Impurity $k'(II)$	Theophylline $k'(T)$	4-Hydroxybenzoic Acid $k'(I)$	Methylparaben	Propylparaben
0	10.4	33.0	12.1	—	—
2.8	3.4	10.0	5.2	—	—
10.0	1.0	2.8	3.0	12.5	—
25.0	—	—	—	—	16.6

[a] Each mobile phase contained 0.5% acetic acid.

Table IV—HPLC Analysis of Enema Solution

Component	Label Claim, mg/ml	Found, mg/ml	Percent of Label Claim
Methylparaben	3.00	0.67	22.3
Propylparaben	0.60	0.29	48.3
Theophylline	12.61	12.96	103.0
Ethanolamine	4.28	3.92	91.6
I	—	1.95	—
II	—	0.77	—

By salt formation with xanthine drugs, *e.g.*, theophylline and theobromine, amines such as ethanolamine and ethylenediamine effectively solubilize these drugs. However, because they are rather reactive substances, esters such as parabens are chemically vulnerable, particularly toward attack by amines. These reactions of the complexing agent may reduce the amount of drug in solution and consume the preservative. Loss of preservative may result in drug biodegradation or in a health hazard through bacterial or fungal contamination of the dosage form; such loss also may create a source of chemical contamination by generation of foreign substances. Improper treatment, such as excessive temperatures and extremely high or low pH during formulation, packaging, and storage, may increase reaction rates and lead to such paraben reactions as acylation and hydrolysis. Hydrolysis of methylparaben in basic and acidic solutions (pH 6–8) was previously studied (9). Autoclaving at pH 9 (close to the measured pH of the sample used in this study) for 30 min left only 58% of the initial methylparaben concentration. The hydrolysis rates of methylparaben at 70, 80, and 85° were greater when the solutions were basic, as were the enema solutions investigated.

In this study, relative to theophylline, 4.6% by weight of II and 11.7% of I were found in the drug sample with a corresponding 73.3% loss of parabens in the solution. The corresponding amide from salicylic acid, *N*-(2-hydroxyethyl)-3-hydroxybenzamide, was described previously (10), but to the knowledge of the authors, II is unknown and its physiological effects are unexplored.

It should be noted that paraben losses may occur by direct chemical decomposition as well as by microbial degradation or complex formation with macromolecules. Despite their phenolic moiety, structures such as paraben esters are not resistant to all microbes and, like other phenols with electron-withdrawing substituents (*e.g.*, picric acid), may give complex formation with macromolecules. These factors may result in loss of the preservative effects of parabens in drug samples. This study shows that the ester functional group of parabens also may give rise to loss of preservative action by direct chemical decomposition. Interactions similar to those between parabens and ethanolamine described here also should apply to ethylenediamine complexing agent.

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