Table VII—Partition Coefficients[a,b] for I at 37°

Solvent 2	Solvent 1	Partition Coefficient (*PC*), (±*SD*)
Liquid paraffin	aqueous pH 8.9 solution	2.3(0.6)
Toluene	aqueous pH 8.9 solution	5.2(2.1)
Dimethyl sulfoxide	aqueous pH 8.9 solution	14.4[c]

[a] *PC* = [I] solvent 2/[I] solvent 1. [b] Data are from Ref. 7. [c] Calculated by dividing *PC* from aqueous pH 8.9 solution into liquid paraffin (2.3) by *PC* from dimethyl sulfoxide into liquid paraffin (0.16).

The results in Tables III and IV reveal that the solvent properties of the skin toward I increase in the fall as compared with winter.

The complex nature of the solubility of I does not allow simplistic statements to be made. However, it is very likely that changes in the composition of the emulsified sebum, perhaps even phase changes, led to the observed changes in permeability.

There appears to be very little difference between the skin permeabilities of European breeds of cattle at any particular time, and intrabreed differences are small.

Because the permeability of cattle skin appears to be 10 times greater in early fall as compared with winter and to increase with increasing temperature, it can be predicted that the skin permeability of cattle in the field in mid-summer will be substantially higher than that in mid-winter. Cattle skin is a relatively polar solvent with properties similar to water towards neutral organic molecules. Consequently, water or poorer solvents than water for particular drugs should be selected for topical formulations in preference to better solvents than water in order to maximize the rate of drug penetration.

Studies aimed at characterizing the solvent properties of domestic animal skins are currently underway.

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Determination of Antimicrobial Preservatives in Pharmaceutical Formulations Using Reverse-Phase Liquid Chromatography

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Received November 20, 1981, from *The Upjohn Company, Kalamazoo, MI 49001.* Accepted for publication April 15, 1982. *Present address: Westinghouse Electric Corporation, Bettis Atomic Power Laboratory, West Mifflin, PA 15122.

Abstract □ A specific stability-indicating reverse-phase high-performance liquid chromatographic analytical method has been developed to quantitate the antimicrobial preservatives methylparaben, propylparaben, butylparaben, sorbic acid, and benzoic acid in a series of typical pharmaceutical formulations. The mobile phase of this system is a water–acetonitrile mixture, modified by various acids and buffers. The proportions of water and acetonitrile as well as the type and amounts of modifiers are varied in order to achieve optimum chromatography. This method has been used successfully to quantitate preservatives in solutions, suspensions, creams, lotions, and ointments, and can be readily adapted to routine automated assays, either for routine product evaluation or stability programs.

Keyphrases □ Preservatives, antimicrobial—determination in pharmaceutical formulations using reverse-phase liquid chromatography □ Reverse-phase liquid chromatography—determination of antimicrobial preservatives in pharmaceutical formulations

Antimicrobial preservatives are materials added to formulations to protect the product from microbial contamination. A given preservative material can be used in a wide variety of products and also may be used in combinations with other preservatives. Separate testing methods for each product–preservative combination would not make efficient use of laboratory resources if the tests are to be performed frequently; thus a method which is generally applicable is desirable. However, the analytical methods should be specific to ensure that decomposition products and impurities are not inadvertently measured. Regulatory agencies have also shown interest in specific test methods for preservatives (1, 2). The challenge for the methods developer is to come up with a method that satisfies both criteria of assay efficiency and specificity.

It is currently of interest within the pharmaceutical industry to assure that specific, stability-indicating, and validated testing methods are available for antimicrobial preservatives. This study reports the development of a reverse-phase high-performance liquid chromatographic (HPLC) assay system that, with minor modifications in

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Table I—Data for the Preservative Assays for Pharmaceutical Products Containing Only the Parabens as Preservatives

Product Type	Preservatives	Internal Standard	Mobile Phase	Average Recovery, %	*RSD*, %	Concentration Range Validated
Ointment (5)[a]	Methylbaraben	Calusterone	1	100.3	1.4	0.1–0.3 mg/g
	Butylparaben			100.0	1.4	0.9–2.7 mg/g
Cream A (2)	Methylparaben	Calusterone	1	100.5	1.2	0.5–2.0 mg/g
	Butylparaben			100.1	1.2	2.0–6.0 mg/g
Cream B (1)	Methylparaben	Calusterone	1	98.0	1.2	0.5–2.0 mg/g
	Butylparaben			99.8	1.8	2.0–6.0 mg/g
Cream C (2)	Methylparaben	Calusterone	1	100.9	1.3	2.0–6.0 mg/g
	Butylparaben			101.7	1.5	1.5–4.5 mg/g
Lotion (2)	Methylparaben	Calusterone	1	98.7	0.8	1.0–3.0 mg/g
	Butylparaben			99.7	1.0	1.5–4.5 mg/g
Sterile Suspension D (1)	Methylparaben	Butylparaben	1	98.3	1.0	0.8–3.2 mg/ml
	Propylparaben			102.7	1.5	0.09–0.36 mg/ml
Fluid E (1)	Methylparaben	Butylparaben	1	99.8	1.0	0.5–2.0 mg/ml
Sterile Suspension F (1)	Methylparaben	Butylparaben	1A	99.6	1.8	0.4–1.6 mg/ml
	Propylparaben			99.3	1.3	0.04–0.18 mg/ml
Fluid G (1)	Methylparaben	Ethylparaben	1B	100.2	1.4	0.35–1.5 mg/ml
	Propylparaben			98.1	1.3	0.10–0.5 mg/ml

[a] Number of products in parentheses.

the mobile phase, is capable of quantitating the preservatives methylparaben, propylparaben, butylparaben, sorbic acid, and benzoic acid in a wide range of pharmaceutical formulations.

BACKGROUND

Validation criteria have been developed for the potency assays for pharmaceutically active ingredients (3). This system was used as the starting point for the criteria that would be applied to the preservative assays. However, it was realized that in several important aspects, quantitating preservatives is significantly different from quantitating the active pharmaceutical ingredient, and that consideration of these aspects will influence the criteria used to judge the validity of the test method. If appropriate criteria are established before development work begins, it should be possible for both the developer and the ultimate user of the testing methods to utilize their resources as effectively as possible. These aspects will now be considered in more detail.

Preservatives are neither pharmacologically active, nor are they inert formulation excipients. They are, however, considered essential ingredients because they are active in maintaining product integrity (1).

Efficiency in a Routine Environment—A completely new testing method may be required for a new product, or even for an old drug in a new formulation, to satisfy requirements for potency or impurity quantitation methods. A given preservative material may, however, be used in a wide range of products and types of formulations. To have a different testing method for each different occurrence of the preservative would not be very efficient for laboratory operations but would allow the flexibility of developing a highly precise, accurate assay method. However, the highest laboratory efficiency would be gained if only one automated assay system were needed for all products containing the given preservative, but accuracy and precision would suffer, considering the wide variety of samples to be processed. An automated system is not available for preservatives, and the most reasonable compromise would involve a minimum number of testing methods with assay variabilities between the two extremes, and with only minimal detrimental effects on efficiency. The method reported here is an example of such a system.

Table II—Data for the Preservative Assays for Pharmaceutical Products Containing Sorbic Acid and the Parabens as Preservatives ●

Product Type	Preservatives	Average Recovery, %	*RSD*, %	Concentration Range Validated, mg/ml
Suspension H (2)[b]	Sorbic Acid	100.1	0.5	0.5–2.0
	Methylparaben	100.7	1.0	1.0–4.0
Solution J (2)	Sorbic Acid	98.8	0.8	0.5–2.0
	Methylparaben	99.3	0.7	0.3–1.5
	Propylparaben	99.8	1.1	0.1–0.4
Cream K (1)	Sorbic Acid	99.4	0.5	1.0–4.0

[a] The mobile phase used was mobile phase 2; the internal standard was ethylparaben. [b] Number of products in parentheses.

Preservative Concentrations—Preservatives are generally formulated at concentrations of 1 mg/ml, which can be as much as two orders of magnitude less than the active ingredient. This can put preservatives in the sample at the approximate levels of impurities or degradation products. This can cause problems of interferences from these extra materials leading to poor reproducibility and/or assay bias.

Variation with Time–Degradation Products—The concentration of preservatives may decrease over the shelf-life of a product at a rate faster than that of the drug itself. The analytical testing method must be validated well below the usual preservative concentration to quantitate these amounts, and must be specific for degradation products. (Although in most cases it is not known what contribution, if any, to the preservative capacity of the system could be made by these compounds.) To quantitate these lower levels demands analytical methods that are accurate, linear, and which have acceptable slope and intercept values (ideally one and zero, respectively, within experimental error for plots of amounts added *versus* amounts recovered).

Assay Variability—For a drug in a product, the lowest acceptable concentration is frequently specified by a compendial limit. For preservatives, the lowest acceptable concentration is stated implicitly as the minimum amount which will allow the formulation to pass preservative efficacy tests. Because of the nature of these microbiological tests, this concentration cannot be accurately specified. The analytical method which measures that concentration need not be any more accurate than required by the normal variability of the microbial tests. Since the coefficient of variation of liquid chromatographic analytical methods of the type used here is less than the variability of the microbial tests, liquid chromatographic methods are more than suitable for this purpose.

The approach used in the development of these analytical methods was to start with a water–acetonitrile binary system and add other modifiers as necessary to achieve satisfactory chromatography. However, any mobile phase not otherwise modified was made to contain 2% acetic acid to be in agreement with the mobile phase previously suggested for quantitating parabens[1]. In all cases reported here, satisfactory chromatography could be achieved by adjusting the water–acetonitrile ratio and/or the buffer–pH combinations.

EXPERIMENTAL

The equipment used in these studies included standard HPLC pumping systems[2], injector[3], columns[4], and UV detectors[5]. Sample injections were made by an autosampler system[6], which also actuated the injector valve (4). Solvent[7] flow rates were typically 2 ml/min, and in-

[1] Unpublished communication, Analytical Research Department, Pharmaceutical Products Division, Abbott Laboratories, North Chicago, Ill.
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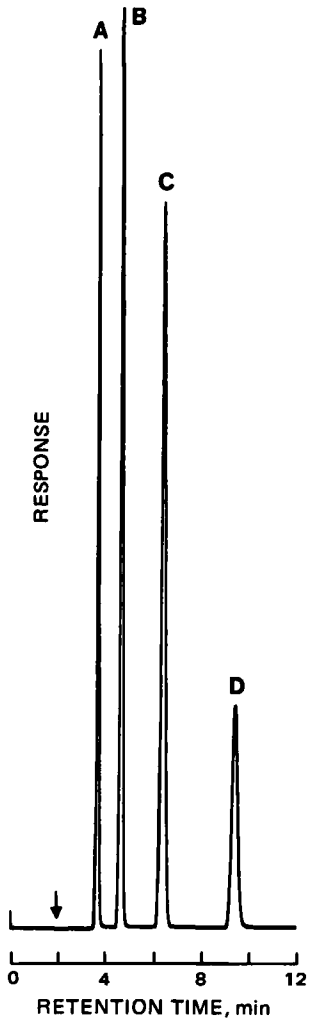

Figure 1—*Chromatogram of a mixture of methylparaben (A), ethylparaben (B), propylparben (C), and butylparaben (D) using mobile phase 1. The solvent front is indicated by the arrow. The mixture was prepared by dissolving 7 mg of methylparaben, 13 mg of ethylparaben, 15 mg of propylparaben, and 7 mg of butylparaben in 100 ml of mobile phase.*

jection volumes were 10 μl. Quantitation was performed by measuring peak heights. The mobile phase compositions referenced later in the text by number are: 1, water–acetonitrile–glacial acetic acid (58:40:2); 1A, water–acetonitrile (70:30) 0.2 *M* ammonium acetate, pH 7.2; 1B, water–acetonitrile (65:35) 0.01 *M* ammonium dihydrogen phosphate, pH 2.2 with concentrated phosphoric acid; and 2, water–acetonitrile (70:30) 0.01 *M* potassium acetate, pH 5.4 with glacial acetic acid.

Recovery Studies—For each product type a sample was prepared containing all ingredients (including active ingredients) in their proper proportion except for the preservatives. Reference standard preservative material was then added to portions of the sample in amounts ranging from 50 to 150% of the normal preservative concentration. These spiked samples were quantitated against the reference standard to determine recovery and linearity for the analytical method. Replicate assay results on several production lots were pooled to determine the relative standard deviation (*RSD*). All analytical methods reported here showed acceptable linearity and have a slope of 1.00 within experimental error for plots of amounts recovered *versus* amount added. Tables I–III list the product type, number of individual products within each type, preservatives, internal standard used, mobile phase, average recovery as a percentage of the amount added, *RSD* calculated for the method, and range of concentrations validated. All recoveries and *RSD*s satisfied the validation criteria.

Sample Preparation for Products Containing Only Parabens (Table I)—*Ointments*—The four ointment products[8] studied make use

[8] Cortaid, Neo-Cortef, Mycitracin, Neo-Oxylone.

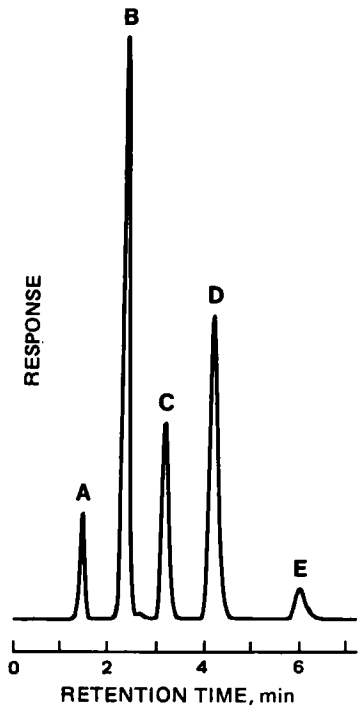

Figure 2—*Chromatogram of a production lot of fluid product J using mobile phase 2. The peaks are: (A) Solvent front and excipients, (B) sorbic acid, (C) methylparaben, (D) ethylparaben, and (E) propylparaben.*

of a typical hydrocarbon ointment base. This base would not dissolve in any solvents that were compatible with the mobile phase. Quantitative recovery was achieved by extraction into dimethylformamide. A 10.0-ml volume of this solvent was added to 1.0 g of ointment and 20.0 ml of the internal standard in a water–acetonitrile solution. The mixture was shaken in a hot water shaker bath at 50° and then centrifuged. The undissolved base congealed at the top and an aliquot of the clear lower layer was assayed.

Creams—The three cream product types[9] (five products) were all variations of typical oil-in-water emulsions. A measure of 0.5 g of each cream was added to 100.0 ml of tetrahydrofuran and 5.0 ml of the internal standard in a water–acetonitrile solution. The samples were shaken for 15 min and then chromatographed.

Lotions—The two lotions[10] were very similar to the cream formulations. The sample preparation was the same as for the cream products.

Sterile Product—Sterile suspension D[11] (1.0 ml) was diluted with 30 ml of water containing the internal standard, mixed, centrifuged to remove the undissolved drug, and chromatographed.

Nonsterile Fluid—The fluid formulation E[12] was an oral antibiotic suspension. One milliliter of this fluid was added to 30.0 ml of an acidified (acetic acid) acetonitrile–water solution of the internal standard to increase solubility and prevent the formation of an emulsion. The sample was then mixed, centrifuged, and chromatographed.

Sample Preparation for Products Containing Sorbic Acid and Parabens (Table II)—One milliliter of fluid samples H and J[13] was added to 30.0 ml of a solution of the internal standard in mobile phase, mixed, and chromatographed. Cream product K[14] (1.0 g) was dissolved in 100 ml of tetrahydrofuran. Five milliliters of this solution was added to 5.0 ml of internal standard in mobile phase, mixed, and chromatographed.

RESULTS AND DISCUSSION

Products Containing Parabens Only—Methyl, propyl, and butyl paraben can occur in these products either singly or in pairs. Ethyl par-

[9] (Cream A) Cortaid, oxylone; (Cream B) Neo-Cortef; (Cream C) Neo-Medrol, medrol.
[10] Cortaid, Neo-Cortef.
[11] Depo-Provera.
[12] Panmycin syrup.
[13] Kaopectate, Kaopectate concentrate.
[14] Florone.

Table III—Data for the Preservative Assays for Pharmaceutical Products Containing Benzoic Acid as the Preservative

Product	Internal Standard	Mobile Phase	Average Recovery, %	*RSD*, %	Concentration Range Validated, mg/ml
Fluid L	Ethylparaben	1B	100.1	0.5	0.5–2.0
Fluid M	Ethylparaben	1B	100.3	0.7	0.5–2.0

aben was not used as a preservative in the formulations studied and, thus, could serve as an internal standard. Figure 1 shows a chromatogram obtained by injecting a solution of these four materials dissolved in mobile phase 1. Excellent resolution was obtained between all four materials. The primary degradation product of all of these compounds was *p*-hydroxybenzoic acid, which eluted with the solvent front under these conditions. The total chromatographic time was ~10 min.

As previously suggested[1], the majority of the products containing only parabens as the preservative could be chromatographed using this mobile phase. The sample preparations were described earlier. The products successfully chromatographed by this method are creams, ointments, lotions, and sterile solutions and suspensions. The products are grouped in Table I by product type, where a product type is a group of products of essentially identical composition except for the active ingredient.

Products Requiring Different Mobile Phases—Two products containing only parabens as preservatives could not be chromatographed successfully by this system: a sterile penicillin suspension (F) and an oral tetracycline suspension (G). In both cases the active ingredient interfered with the preservatives. For the penicillin product it was found that the acidic mobile phase produced decomposition products which could not be adequately separated from the preservatives. By reducing the acetonitrile concentration and buffering mobile phase IA at an apparent pH of 7.2, decomposition could be avoided and all components were resolved. The sample preparation was identical to that for sterile suspension D.

For the oral tetracycline product (fluid G) it was found that the drug eluted as a broad, tailing peak with mobile phase 1. Lowering the pH narrowed the peak and moved it toward the solvent front. At pH 2.3 the tetracycline tail no longer interfered in the quantitation of the methyl paraben. Below pH 2.2, however, no improvement in peak shape was seen. Mobile phase IB was developed with the adjustment to an apparent pH of 2.2 with concentrated phosphoric acid. The sample was prepared by adding 1.0 ml of the fluid to 1.0 ml of a 0.2 *M* $NH_4H_2PO_4$ solution adjusted to pH 1.6. This solution was then diluted with 20.0 ml of an acetonitrile–water solution containing the internal standard. The sample was then mixed and chromatographed.

Recovery data for both products are included in Table I.

Products Containing Sorbic Acid With or Without Parabens—Several products were evaluated which use sorbic acid, either alone or in combination with methylparaben or methyl- and propylparaben. When these samples were chromatographed using mobile phase 1, the sorbic acid and methylparaben coeluted. Since it is well known that the retention times of weak acids are dependent on the pH of the system, it was decided to adjust the pH in an attempt to achieve optimum resolution. An acetic acid–acetate buffer at pH 5.4 was found to give the best chromatography and to separate all formulation excipients as well as *p*-hydroxybenzoic acid. The mobile phase used was mobile phase 2. The apparent pH was adjusted to 5.4 with acetic acid. This system allowed the use of ethylparaben as the internal standard. Products included suspensions (H), solutions (J), and a cream (K).

The results are listed in Table II. All recoveries and *RSD*s satisfy the validation criteria. Figure 2 shows a chromatogram of the solution product (J) and indicates the separation achieved between the three preservatives (sorbic acid, methylparaben, and propylparaben) and the internal standard (ethylparaben). The total chromatographic time was 8 min.

Benzoic Acid—Benzoic acid is used in two fluid products[15] (L and M). Mobile phase 1 was not able to resolve all excipients from the benzoic acid peak. Adopting the same approach as for sorbic acid, it was found that changing the pH could improve peak resolution. Optimum separation was achieved at pH values of ~2.5. Since this value was close to the pH used for mobile phase 1B, that system was tried. Mobile phase 1B did resolve the components sufficiently, and this offered the opportunity to consolidate several products on one analytical system, which would improve laboratory efficiency. The samples were prepared by diluting 1.0 ml of the fluids in 20.0 ml of an acetonitrile–water internal standard solution and chromatographing. The results are shown in Table III.

This paper has reported on a reverse-phase HPLC method which has been used successfully to quantitate antimicrobial preservatives in typical pharmaceutical formulations. The mobile phase used was basically an acetonitrile–water mixture, with various modifiers added as necessary to optimize the chromatography. This method was used successfully on five preservative materials: methylparaben, propylparaben, butylparaben, sorbic acid, and benzoic acid. This system can be easily adapted for automated sample analysis.

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