Table II—Recovery of Acetaminophen Added to Placebo Elixir Vehicle

Acetaminophen added, mg.	101.1	101.6	102.0	100.6	101.1	100.6
Acetaminophen found, mg.	100.4	102.8	101.2	99.2	100.4	98.8
Percent recovered	99.3	101.2	99.2	98.6	99.3	98.2

of sodium nitrite from 10 to 1.25% and the elimination of sulfamic acid as a reagent. Excess nitrous acid was minimized because its gaseous decomposition products tended to disrupt the flow patterns. The concentration used was sufficient to nitrate the drug; however, the excess was dissipated readily in the time-delay coil. It was shown previously (1) that excess nitrous acid does not interfere at the analytical wavelength for the anion of 2-nitro-4-acetamidophenol. Glass tubing was used in the apparatus in place of the customary plastic lines, because the latter reacted with nitrous acid.

Acetaminophen tablets NF[6], declaring 325 mg./tablet, and two of its combination product tablets were assayed. One of these[7] declared 150 mg. of acetaminophen with phenacetin, phenyltoloxamine dihydrogen citrate, and phenylpropanolamine hydrochloride; the other[8] was similar except that phenyltoloxamine was omitted. Recoveries of added acetaminophen in six replicate assays of each formulation by the automated method were 99.3 ± 1.49% for acetaminophen tablets, 101.6 ± 1.47% for the combination product containing phenyltoloxamine, and 103.5 ± 1.30% for the other tablet. The reproducibility of this method with three standards is illustrated in Fig. 2 by typical recorder tracings.

[6] Accu-Med Division, Warner-Lambert Co.
[7] Sinutab tablets, Warner-Chilcott Laboratories.
[8] Sinutab II, Warner-Chilcott Laboratories.

SUMMARY AND CONCLUSIONS

A method for colorimetric determination of acetaminophen as its 2-nitro derivative (1) was extended to elixir formulations of the drug, and the assay of tablets by this reaction was adapted to an automated apparatus. In both cases, recovery data on added acetaminophen were excellent, and the procedures were shown to be applicable to commercial formulations of the drug.

REFERENCE

(1) L. Chafetz, R. E. Daly, H. Schriftman, and J. J. Lomner, *J. Pharm. Sci.*, **60**, 463(1971).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 7, 1971, from the *Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950*

Accepted for publication February 9, 1972.

The authors thank Dr. B. M. Scheinthal for preparation of the figures.

▲ To whom inquiries should be directed.

Rapid GLC Quantitation of Salicylic Acid in Multicomponent Codeine and Propoxyphene Analgesic Formulations

J. R. WATSON▲, FUMI MATSUI, P. M. J. McCONNELL, and R. C. LAWRENCE

Abstract □ A rapid GLC procedure for the precise estimation of salicylic acid in codeine- and propoxyphene-type capsule and tablet analgesic formulations is presented. The sample material is treated with diazomethane prepared in tetrahydrofuran solution, and the salicylic acid is eluted as its methyl ester. Methyl *o*-methoxybenzoate serves as the internal standard. Peak areas are quantitated by means of an electronic digital integrator of wide input signal range capacity. The results obtained by applying the method to the analysis of 25 commercial preparations were in excellent agreement with those given by the trap column spectrophotometric procedure, except at salicylic acid levels exceeding about 15%. The proposed GLC technique precludes some drawbacks of the column method and is superior in many respects.

Keyphrases □ Salicylic acid in analgesic formulations—GLC analysis □ Codeine–aspirin formulations—GLC analysis of salicylic acid □ Propoxyphene–aspirin formulations—GLC analysis of salicylic acid □ Aspirin and codeine or propoxyphene formulations—GLC analysis of salicylic acid □ GLC—analysis, salicylic acid in analgesic formulations

The superior qualities of aspirin as an antipyretic and general analgesic have resulted in the proliferation of a wide variety of commercial products in which aspirin is formulated with antihistaminic, sedative, tranquilizer, and other analgesic therapeutic agents. Despite this multiplicity of preparations, the long-standing and well-documented problem of aspirin stability in commercial formulations is of continuing concern to manufacturing firms and pharmacopeial commissions. For example, it has become apparent that aspirin–propoxyphene capsule preparations tend to be much less stable than the more common aspirin formulations; realization of this fact is reflected in the 3.0% free salicylic acid limit allowed for such preparations in the NF XIII (1). Recently, the USP XVIII (2) raised the salicylic acid limit from 0.15 to 0.30% in compressed aspirin tablets and from 0.75 to 3.0% in coated or buffered tablets.

Over the years, numerous studies have been directed to the elucidation of the mechanism and to the factors governing this degradation. The collective conclusion is that, given the clear prerequisite of sorption of moisture (3, 4), the aspirin molecule appears to be at the mercy of its chemical environment, although other factors no doubt come into play. Interreactions between aspirin and other active components such as codeine (5), phenyl-

ephrine (6), acetaminophen (7, 8), and antipyrine (9) to give salicylic acid and acetylated products are well known. Under normal conditions, certain excipient materials (10–13), antacids (14, 15), and packaging materials (16) also have been reported to induce and catalyze appreciable hydrolytic cleavage of aspirin.

Whatever the factors associated with the formulation design and manufacturing process that dictate the ultimate stability of aspirin in the finished product, the generation of high levels of salicylic acid in aspirin dosage forms can result in a significant loss of pharmacological activity and can present a potential hazard to the consumer.

The official procedures for the determination of salicylic acid in single- and multicomponent aspirin products involve the use of a ferric chloride–urea trap column and subsequent spectrophotometric measurement of the eluate, as described some years ago by Levine and Weber (17, 18). Although the method is accurate and precise, it is tedious and can be quite time consuming for two reasons. The recommended weight of sample is predicated on a salicylic acid content at about, or not much higher than, the allowable limit for the particular preparation, so that this step is really a trial-and-error one, particularly with aged samples. In some other cases, chloroform trituration of the sample results in an overestimation of the salicylic acid content, probably due to an aspirin–excipient time-dependent interaction giving rise to some species which contributes to the absorbance. In these instances, the analyst must revert to a different extraction medium (19). Shane and Stillman (20) recently reported a fluorometric determination of salicylic acid in buffered aspirin products. Other published techniques for the analysis of salicylic acid in aspirin preparations were reviewed by Kelly (21).

The GLC method described here utilizes electronic peak integration for the rapid quantitation of salicylic acid in codeine- and propoxyphene-type multicomponent analgesic formulations and is a modification of the methylation procedure previously developed in this laboratory for the rapid, simultaneous determination of salicylic acid and aspirin in single-component aspirin tablets (22).

The aim of the present work is to assess the feasibility and merits of the GLC procedure described here *vis-à-vis* the trap column method for the precise estimation of the salicylic acid content in aspirin–codeine and aspirin–propoxyphene multicomponent tablets and capsules.

EXPERIMENTAL

GLC System—The gas chromatograph[1], equipped with a flame-ionization detector unit and a linear temperature programmer, was fitted with a 6% OV-17[2] on Gas Chrom Q[3] (100–120 mesh) coiled glass column [1.8 m. (6 ft.) × 3 mm. o.d.] preconditioned at 275° for 30 hr. Several 5-μl. portions of Silyl 8[2] column conditioner were injected directly into the gas chromatograph at column temperatures between 170 and 200° during the early stages of the conditioning phase. The support was coated by means of a Hi-Eff[3] fluidizer maintained at a temperature of 150° for 2 hr., with a suitable flow to ensure gentle yet thorough drying of the packing material. Injection port and detector block temperatures were maintained at 220 and 230°, respectively. The gas flows were: nitrogen, 30 ml./min.; hydrogen, 30 ml./min.; and air, 315 ml./min.

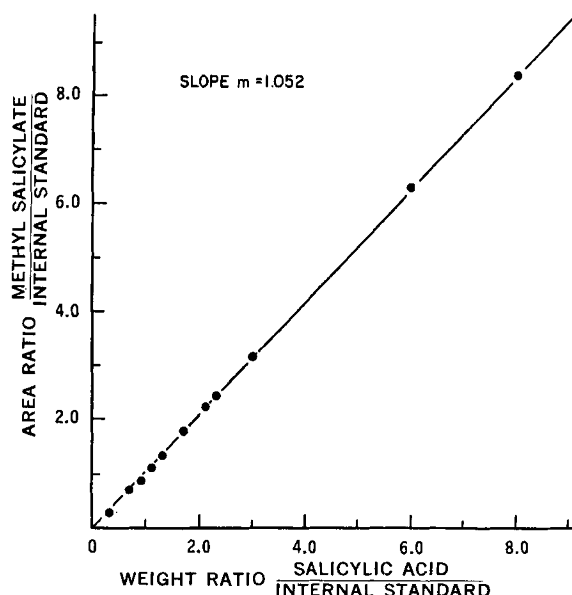

Figure 1—*Salicylic acid–internal standard calibration curve.*

The detector analog signal was supplied to a continuous balance 1-mv. recorder[4], with a chart speed of 0.64 cm. (0.25 in.)/min., connected to a fully automatic printout electronic digital integrator[5], with a set input signal range of 0–100 mv., thus permitting a constant attenuation setting of 1 × 4.

Preparation of Diazomethane—The materials and procedure used were the same as those reported previously (22) but are repeated here for the sake of convenience. The materials used were: (*a*) tetrahydrofuran[6], reagent grade; (*b*) 2-(2-ethoxyethoxy)ethanol[7], Baker grade; and (*c*) *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Baker grade.

The apparatus consisted of a 50-ml. distilling flask with a side arm bent so as to fit through a cork stopper into a 50-ml. receiving conical flask. Through a second hole in the stopper was inserted a U-shaped outlet tube passing into and below the surface of the tetrahydrofuran (4 ml.) contained in a second unstoppered conical flask. Both receiving flasks were cooled in an ice–salt mixture. The solution of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (4.3 g.) in tetrahydrofuran (25 ml.) was added dropwise over 60–75 min. through a 60-ml. long-stemmed dropping funnel, adjusted so that the tip was just above the surface of the magnetically stirred solution in the distilling flask [KOH, 1.2 g.; 2-(2-ethoxyethoxy)ethanol, 7 ml.; tetrahydrofuran, 2 ml.; and H_2O, 2 ml.] heated in a water bath at 80–90°. Distillation of diazomethane was carried out *in situ* with the recommended safety measures.

Preparation of Salicylic Acid–Internal Standard Calibration Curve—*Materials*—The following were used: (*a*) salicylic acid[8], reagent grade; and (*b*) methyl *o*-methoxybenzoate[9] (internal standard), highest purity.

Solutions—The following were used: (*a*) diazomethane in tetrahydrofuran, prepared as already described; (*b*) salicylic acid in distilled tetrahydrofuran, accurately weighed to contain about 5 mg./ml.; and (*c*) internal standard in distilled tetrahydrofuran, accurately weighed to contain about 5 mg./ml.

Preparation of Mixtures, Methylation, and GLC—The exact volume of solution required to give 5.00 mg. of internal standard was dispensed into each of nine separate flasks from a 5-ml. microburet (graduated in 0.01 ml.) with a 24-gauge, 1.9-cm. (0.75-in.) hypodermic needle delivery tip. The exact volumes of salicylic acid solution calculated to give weight ratios of salicylic acid–internal

[1] Varian Aerograph Series 200, model 204-B.
[2] Pierce Chemical Co.
[3] Applied Science Laboratories.
[4] Minneapolis-Honeywell, Electronik 15 strip-chart recorder.
[5] Kent, model Chromalog 2.
[6] British Drug House.
[7] Carbitol.
[8] Anachemia.
[9] Eastman.

Table I—Salicylic Acid Content in Propoxyphene-Type Analgesic Formulations

Sample Number and Dosage Form	Manufacturer	Active Ingredients[a]	Percent Salicylic Acid Found—GLC Method	Percent Salicylic Acid Found—Trap Column Method
I Capsules	A	1, 2, 3, 4	0.42 0.45	0.41 0.40
II Film-coated tablets	B	1, 2, 3, 4	1.45 1.28	1.52 1.61
III Capsules	C	1, 2, 3, 4	5.86 5.93	5.62 5.11
IV Capsules	A	1, 2, 3, 4	0.21 0.22	0.16 0.17
V Capsules	A	1, 4	0.17 0.20	0.13 0.12
VI Capsules	A	1, 4	0.53 0.55	0.57 0.63
VII Capsules	A	1, 2, 3, 4	3.43 3.09	3.70 3.72
VIII Capsules	C	1, 2, 3, 4	1.74 1.80	1.73 1.70
IX Capsules	A	1, 2, 3, 4	12.11 12.13	11.92 10.79
X Capsules	A	1, 2, 3, 4	15.82 15.89	14.30 14.09
XI Capsules	A	1, 2, 3, 4	30.64 30.90	27.01 26.90
XII Capsules	D	1, 2, 3, 4	10.27 10.28	9.59 9.10
XIII Film-coated tablets	B	1, 4	0.65 0.58	0.63 0.63
XIV Film-coated tablets	B	1, 2, 3, 4	2.19 2.20	2.44 2.42

[a] 1 = aspirin, 2 = phenacetin, 3 = caffeine, and 4 = propoxyphene hydrochloride.

standard of 0.30, 0.70, 0.90, 1.10, 1.30, 1.70, 2.10, 2.30, and 3.00 were then dispensed into respective flasks. Each mixture was treated in turn with diazomethane to a permanent yellow color, and 1 μl. of the methylated solution was injected (within 2 min.) into the gas chromatograph by means of a Hamilton microsyringe. The column temperature was kept isothermal at 135° for 8 min. and then programmed at a rate of 2°/min. until complete elution of the internal standard. The area ratios of methyl salicylate to internal standard were plotted against the weight ratios of salicylic acid to internal standard, and the line of best fit was determined.

Analysis of Commercial Products by GLC—*Sampling*—Aspirin–propoxyphene and aspirin–codeine analgesic formulations were sampled as follows: 10 capsules were emptied and their contents were weighed and then thoroughly mixed. In the case of the tablets, 10 were selected at random, weighed, finely powdered, and passed through a 60-mesh sieve.

Analysis—A quantity of the powder equivalent to about 100 mg. of aspirin was accurately weighed into a suitable flask. Immediately after the addition of the exact volume of solution equivalent to 5.00 mg. of internal standard, the sample was treated with diazomethane and shaken; then 1 μl. of the clear supernate esterified mixture was quickly (within 2 min.) injected into the gas chromatograph using the column temperature program conditions already described. After elution and quantitation of the two peaks of interest (methyl salicylate and internal standard), the column temperature was raised to 275° for 15 min. to remove the other components (not quantitated) in the mixture; the system was then readied for the next run. Each lot number was analyzed in duplicate. The amount of salicylic acid, x (in milligrams), in the sample aliquot was computed from the relationship ($5y/m = x$), using an m value of 1.052 and y as the experimental peak area ratio of methyl salicylate to internal standard. The percentage salicylic acid values were calculated on the basis of the label claim of aspirin.

Analysis of Commercial Products by Trap Column Procedure—The procedure used for all samples was that indicated for propoxyphene hydrochloride with aspirin capsules in the NF XIII. Sample weight modifications were made when warranted. To minimize the possibility of overestimation resulting from impurities in the siliceous earth, the blanks were passed through the column prior to spectrophotometric measurement at 306 nm.

RESULTS

The salicylic acid–internal standard calibration curve is shown in Fig. 1 for weight ratios in the range of 0–8.0:1. For the sake of convenience and to preclude the inaccuracies attending small quantity weighings (less than 15 mg.), it was expedient to prepare the salicylic acid–internal standard mixtures by dispensing calculated volumes of stock solution of each substance from a microburet prior to the methylation step. The weight ratio–area ratio relationship was linear, of the type $y = mx$, and held true for weight ratios of up to at least 8.0:1 which, on the basis of the recommended 5-mg. weight of internal standard, would correspond to about 40% salicylic acid in a sample aliquot equivalent to 100 mg. of aspirin. Each of the 11 points on the line represents the average of duplicate injections. The best slope, m, was computed from the least-squares formula to give a value of 1.052, with a coefficient of variation of ±2.09%. Continued use of the OV-17 column over 3 months under various temperature conditions did not effect a change in this relative response factor any greater than the mean slope error.

The GLC procedure was applied to the salicylic acid quantitation in 14 commercial propoxyphene–aspirin-type analgesic formulations, in both capsule and tablet form, from four manufacturers. In Table I the results are compared for precision and accuracy with those obtained using the official siliceous earth trap column method. Other active ingredients in the preparations are number coded and appear in the third column. The results were in excellent agreement for all of the preparations except X and XI, where the salicylic acid content exceeded 15%. The discrepancies observed in these cases may be due to incomplete chloroform extraction and to the errors accompanying the increased number of manipulations required in the official procedure when such high levels of salicylic acid occur in the formulation.

The data obtained with the codeine-type analgesic formulations given in Table II further underline the feasibility of the GLC procedure as a rapid means of evaluating the stability of these widely prescribed preparations. The percentage salicylic acid values (based on aspirin label claim) showed no significant variations between the

Table II—Salicylic Acid Content in Codeine-Type Analgesic Formulations

Sample Number and Dosage Form	Manufacturer	Active Ingredients[a]	Percent Salicylic Acid Found—GLC Method	Percent Salicylic Acid Found—Trap Column Method
I Compressed tablets	E	1, 2, 3, 5	0.28 0.31	0.23 0.23
II Compressed tablets	E	1, 2, 3, 5	34.53 34.35	36.73 37.63
III Compressed tablets	B	1, 2, 3, 5	1.25 1.29	1.27 1.31
IV Compressed tablets	B	1, 2, 3, 5	6.74 6.35	6.83 7.05
V Compressed tablets	B	1, 3, 5	0.16 0.16	0.10 0.09
VI Compressed tablets	E	1, 2, 3, 5	0.11 0.13	0.09 0.08
VII Compressed tablets	F	1, 2, 3, 5	1.17 1.07	1.21 1.24
VIII Enteric-coated tablets	B	1, 5	1.06 0.99	1.10 1.11
IX Enteric-coated tablets	B	1, 5	0.58 0.59	0.49 0.49
X Compressed tablets	G	1, 3, 5	0.41 0.36	0.32 0.32
XI Buffered tablets	G	1, 3, 5	0.59 0.61	0.58 0.58

[a] 1 = aspirin, 2 = phenacetin, 3 = caffeine (as free base or citrate salt), and 5 = codeine phosphate.

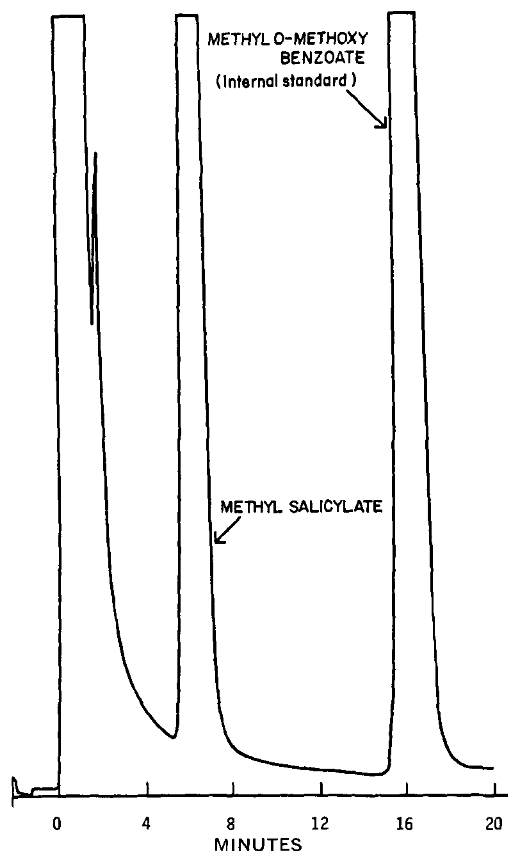

Figure 2—*Partial gas chromatogram of a mixture containing methyl salicylate and internal standard.*

two methods, regardless of whether the aspirin was compounded with one, two, or three additional active ingredients. The one exception was Sample II, where a difference of about 2% was observed, but anomalies of this sort are not unexpected in such highly decomposed tablets because a number of conceivable component interreactions could lead to the formation of several UV and GLC interfering end-products. In such instances, the tablets have a pronounced discolored appearance. The high free salicylic acid solutions were diluted further and reinjected, but no double peaks or shoulders that would interfere with the quantitation were observed.

Simple dissolution of Sample X in chloroform, as required in the NF XIII monograph for aspirin–phenacetin–caffeine tablets, gave a salicylic acid value significantly higher than that obtained by the GLC procedure. Changing the contact time from 3 to 40 min. resulted in an increase of from 1.02 to 3.68%, which suggested that some sort of surface-catalyzed transformation of aspirin was occurring. The difficulty was surmounted and good agreement between the two methods was achieved by applying Guttman and Salomon's (19) method, involving prior trituration of the powdered aliquot with citric acid. The important point to consider with this preparation is that, since it is not labeled as being buffered, one would normally apply the usual chloroform extraction which would clearly tend to yield spuriously high values. In the GLC procedure, this difficulty appears to be obviated by the low contact time permitted by the ease of methylation of salicylic acid with diazomethane and the high solubility of the methyl ester in tetrahydrofuran.

DISCUSSION

Since tetrahydrofuran is particularly prone to potentially hazardous peroxide formation, the commercially available grades are normally stabilized by the addition of small quantities of antioxidant substances such as butylated hydroxyanisole. On a 6% OV-17 GLC column, this compound elutes on the tail of the internal standard peak, thereby prohibiting its accurate quantitation. For this reason, it was necessary to distill the tetrahydrofuran prior to the preparation of the stock solutions required for the calibration work and for the analysis of the dosage forms.

The fast, complete, and essentially clean reaction of diazomethane with salicylic acid (23), coupled with the very real possibility of appreciable hydrolysis of aspirin in the highly solubilizing tetrahydrofuran environment, was a strong factor favoring the desirability of a minimum solute–solvent contact time. Consequently, for optimum accuracy and precision, it was advisable not to dispense the internal standard solution until just prior to methylation and injection. In practice, the time lapse from the addition of the internal standard solution to the weighed sample aliquot to injection of the methylated solution into the gas chromatograph was kept at 2 min. This was the minimum time required for the mixtures to settle and for complete esterification. Injection of methylated salicylic acid–internal standard mixtures at intervals of 2, 5, 10, 15, and 20 min. showed no significant changes in area ratios.

In a previous communication (22) on the simultaneous analysis of salicylic acid and aspirin in single-component aspirin tablets, the methyl esters were eluted from a 5% OV-210 column. However, when analgesic formulations containing codeine phosphate are methylated with diazomethane and chromatographed on this type of column, an extraneous peak, which interfered with the electronic integration of the methyl salicylate peak, was observed. The component responsible for this signal was identified as trimethylphosphate by comparing its retention times (at various temperatures from 90 to 120°) to that of the peak given by a standard trimethylphosphate[10] solution. In the present work, a different stationary phase, capable of separating the methyl salicylate from secondary peaks, was required. The phenylmethyl silicone OV-17 was selected because of its high thermal stability and its proven usefulness in the analysis of pharmaceuticals. With a 6% loading of this phase on a Gas Chrom Q support, the analysis time was only 18 min. and no significant bleedoff was encountered in the temperature range of interest (135–160°), even with single-channel temperature programming. The slow program rate of 2°/min., after the initial isothermal phase at 135°, gave good separation of the peaks of interest (Fig. 2), constant retention times, and no interference from aspirin, phenacetin, caffeine, propoxyphene, codeine, or excipient materials. To maintain low bleedoff and high column efficiency, several microliters of a silylating column conditioner were injected periodically at temperatures between 170 and 200°. This also served to prolong the life of the column considerably. A constant attenuation of 1×4 was selected to permit a sufficient number of counts for the reliable quantitation of the methyl salicylate peak for the low levels of salicylic acid generally encountered in the samples. Because of the high input signal range capacity of the integrator, no modifications in sample weight were required, even in the case of extensively degraded products. This is one obvious advantage of the GLC method over the official procedure.

CONCLUSION

The proposed GLC technique for the quantitation of salicylic acid in codeine- and propoxyphene-type multicomponent analgesic formulations is rapid and precise and, aside from the calibration, is a one-step operation requiring a minimum of expertise. It eliminates the tedium and the trial-and-error steps of the existing trap column procedure and appears to be more suitable for medium- or large-scale investigational work. It should prove useful in future in-depth stability studies of aspirin–propoxyphene formulations, which would permit a valid assessment of the degradation problem in terms of the formulation design and manufacturing process.

REFERENCES

(1) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, p. 607.

(2) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 55.

(3) L. J. Leeson and A. M. Mattocks, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 329(1958).

(4) J. W. Conine, *J. Pharm. Sci.*, **54**, 1580(1965).

(5) A. L. Jacobs, A. E. Dilatush, S. Weinstein, and J. J. Windheuser, *ibid.*, **55**, 893(1966).

(6) A. E. Troup and H. Mitchner, *ibid.*, **53**, 375(1964).

[10] K & K Labs.

(7) K. T. Koshy, A. E. Troup, R. N. Duvall, R. C. Conwell, and L. L. Shankle, *ibid.*, **56**, 1117(1967).
(8) E. Kalatzis, *ibid.*, **59**, 193(1970).
(9) R. Yamamoto and T. Takahashi, *Ann. Rep. Shionogi Res. Lab.*, **4**, 79(1954).
(10) D. Ribeiro, D. Stevenson, J. Samyn, G. Milosovich, and A. M. Mattocks, *J. Amer. Pharm. Ass., Sci. Ed.*, **44**, 226(1955).
(11) S. Lee, H. G. DeKay, and G. S. Banker, *J. Pharm. Sci.*, **54**, 1153(1965).
(12) S. S. Kornblum and M. A. Zoglio, *ibid.*, **56**, 1569(1967).
(13) F. Jaminet and G. Louis, *Pharm. Acta Helv.*, **43**, 153(1968).
(14) M. R. Nazareth and C. L. Huyck, *J. Pharm. Sci.*, **50**, 608 (1961).
(15) F. J. Bandelin and W. Malesh, *J. Amer. Pharm. Ass., Pract. Ed.*, **19**, 152(1958).
(16) T. Canback, *Sv. Farm. Tidskr.*, **47**, 621(1943).
(17) J. D. Weber and J. Levine, *J. Pharm. Sci.*, **55**, 78(1966).
(18) J. Levine and J. D. Weber, *ibid.*, **57**, 631(1968).
(19) D. E. Guttman and G. W. Salomon, *ibid.*, **58**, 120(1969).
(20) N. Shane and R. Stillman, *ibid.*, **60**, 114(1971).
(21) C. A. Kelly, *ibid.*, **59**, 1053(1970).
(22) J. R. Watson, P. Crescuolo, and F. Matsui, *ibid.*, **60**, 454 (1971).
(23) E. Seoane and A. Carnicer, *Microchem. J.*, **12**, 291(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 29, 1971, from the *Research Laboratories, Food and Drug Directorate, Tunney's Pasture, Ottawa, Ontario, KIA OL2, Canada.*
Accepted for publication February 9, 1972.
▲ To whom inquiries should be directed.

Determination of *p*-Hydroxyphenoxyacetic Acid in Phenoxymethyl Penicillin Fermentations

JAN BIRNER

Abstract □ A method based on TLC separation and subsequent colorimetric determination was developed for the determination of *p*-hydroxyphenoxyacetic acid in phenoxymethyl penicillin (penicillin V) fermentation samples. The *p*-hydroxyphenoxyacetic acid is extracted from penicillin culture fluid into amyl acetate. A portion of the extract is dried and then dissolved in acetone. An aliquot is chromatographed on a silica gel thin-layer plate by ethyl acetate–chloroform–acetic acid (9:1:0.15 v/v). The zones are located by spraying with an aqueous mixture of equal volumes of 1% ferric chloride and 1% potassium ferricyanide. The zones are transferred into glass tubes, treated by sodium nitrite in dilute sulfuric acid, and neutralized by ammonia. The absorbance of the orange supernate, which is proportional to the concentration of *p*-hydroxyphenoxyacetic acid, is measured spectrophotometrically at 478 nm.

Keyphrases □ *p*-Hydroxyphenoxyacetic acid—from phenoxymethyl penicillin fermentations, TLC separation, colorimetric determination □ Phenoxymethyl penicillin fermentations—determination of *p*-hydroxyphenoxyacetic acid, TLC separation, colorimetric determination □ TLC—separation of *p*-hydroxyphenoxyacetic acid from phenoxymethyl penicillin fermentations □ Colorimetry—determination of *p*-hydroxyphenoxyacetic acid separated from phenoxymethyl penicillin fermentations

The presence of *p*-hydroxyphenoxymethyl penicillin was first detected in phenoxymethyl penicillin fermentation samples by de Flines *et al.* (1), and the authors assumed that this compound is formed either by: (*a*) direct hydroxylation of penicillin by the mold or (*b*) by oxidation of the precursor which is then used to form *p*-hydroxyphenoxymethyl penicillin. A method for quantitative determination of this compound in fermentation samples was developed (2). A colorimetric method for the determination of *O*-hydroxyphenylacetic acid in benzylpenicillin (penicillin G) was developed by Pan in 1955 (3). When this method was applied to phenoxymethyl penicillin fermentation samples, it gave positive results, indicating that phenolic compounds were present in the broth. An attempt to apply this method for the determination of *p*-hydroxyphenoxyacetic acid was unsuccessful due to interference by other phenolic or similar substances present in fermentation media.

When *p*-hydroxyphenoxyacetic acid is treated with a small amount of sodium nitrite in dilute sulfuric acid, heated for 15 min., and then neutralized by ammonia, an orange compound is produced with maximum absorbance at 478 nm. When a dried amyl acetate extract from a penicillin fermentation sample is similarly treated, the formed compound has a yellow color and higher absorbance at 420 nm. than at 478 nm. It is thus evident that for the determination of *p*-hydroxyphenoxyacetic acid in extracts, this compound first must be separated from interfering substances. This is achieved by extracting the acidified culture fluid, saturated with sodium chloride, with amyl acetate. A portion of the amyl acetate extract is dried by an air current, and the dry residue is dissolved in acetone. An aliquot is chromatographed on silica gel by ethyl acetate–chloroform–acetic acid (9:1:0.15 v/v). After spraying with a freshly prepared mixture of ferric chloride and potassium ferricyanide, the zones appear as blue spots of the same R_f value as *p*-hydroxyphenoxyacetic acid controls. The zones are transferred into glass tubes, treated with sodium nitrite in dilute sulfuric acid, and then neutralized by ammonia. After separation from silica by centrifuging, the orange phenolic nitroso compound is measured spectrophotometrically.

EXPERIMENTAL

Reagents, Solutions—The following were used: sodium chloride A.R.; amyl acetate A.R.; sulfuric acid, 12 *N*, A.R.; sulfuric acid, 1 *N*; sodium nitrite, 5% w/v; ammonia (0.880 specific gravity)–water (1:2); silica gel according to Stahl (mean particle size 10–40 μ,