CHROM. 4777

IDENTIFICATION AND QUANTITATIVE ANALYSIS OF *p*-HYDROXYBENZOIC ACID AND ITS ESTERS USING REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

R. RANGONE AND C. AMBROSIO
Control Laboratory, Gruppo Lepetit, Torre Annunziata Plant, Naples (Italy)

(Received March 31st, 1970)

SUMMARY

The *p*-hydroxybenzoic acid and its methyl, ethyl, propyl, butyl and benzyl esters are separated on thin layers of silanized silica gel using borate buffer (pH 11) as mobile phase, adding a given volume of organic solvent as required. The separation of the various compounds is complete and an accurate quantitative determination of the *p*-hydroxybenzoic acid and its esters is possible using a simple technique of elution and spectrophotometric determination.

INTRODUCTION

The increasing use of *p*-hydroxybenzoic acid esters as food and drug preservatives has led to the development of many analytical techniques for identifying and assaying these compounds. In recent years, thin-layer chromatography (TLC) in particular has often been used with good results[1–20].

In this report we shall briefly describe a reversed-phase TLC method based on the use of silanized silica gel as stationary phase. Coupled with a simple elution and spectrophotometric determination technique, it has proved highly effective in separating and determining *p*-hydroxybenzoic acid and its principal esters.

EXPERIMENTAL

Preparation of the chromatographic plates

About 30 g of HF_{254} (Merck) silanized silica gel were suspended in 60 ml of a (2 : 1) water–methanol mixture by shaking vigorously for a few minutes in order to obtain a homogeneous suspension. The latter was then distributed on the plates using a Desaga spreader so as to obtain a uniform layer of 0.25 mm thickness. The plates were dried in air for about 3 h and stored over silica gel in a desiccator. They should not be used for at least 24 h after preparation.

Preparation of the chromatography solutions

Methanol solutions of pure commercial products (Merck, BDH, Carlo Erba)

were used as reference solutions for *p*-hydroxybenzoic acid and its esters. The methods described in a previous report [21] were used to extract the preservatives from pharmaceutical specialities and to prepare the sample solutions.

Application of the solutions to the plates

To identify the substances, the sample and reference solutions were applied as spots in parallel on the same plate using a micropipette and taking care to pipette no more than 5 μl each time, and to deposit from 2 to 10 μg of each preservative on the plate. For the quantitative determination, the solutions to be analyzed were applied in bands on 5 × 20 cm plates, pipetting from 0.025 to 0.1 ml of solution so that from 20 to 100 μg of each substance are applied to each plate. The solutions were applied 2 cm from the starting edge leaving a margin of approx. 0.5 cm on both sides of the plate.

Chromatographic development

Chromatographic development was carried out using borate buffer, pH 11 (502 ml of 0.2 *M* H_3BO_3 solution diluted to 1000 ml with 0.2 *M* NaOH solution) as mobile phase, adding a given volume of organic solvent as required. The following buffer–solvent combinations have proved most suitable for separation of the various preservatives: buffer–methyl alcohol (90 : 10), buffer–ethylcellosolve (90 : 10), buffer–ethyl acetate (saturated solution at 25°), buffer–ethyl ether (saturated solution at 15°), buffer–tetrahydrofuran (95 : 5), buffer–dioxane (90 : 10).

The plates are developed in glass chromatography chambers lined with filter paper. Before putting the plates in them, the chromatographic chambers were saturated with the solvent vapours for about 1 h. The plates are allowed to develop until the solvent front has risen about 14 cm from the point where the substance was deposited (90 to 120 min). When double development is desired, the plate is dried at room temperature for about 1 h and then returned to the chromatographic chamber. Chromatographic development is performed at about 25° except when the mixture contains ethyl ether in which case the temperature of the chromatographic chamber should be 15°.

Detection of the compounds

After the plates have been developed they are dried at room temperature. The spots or bands of the various compounds were visualized under a 250-mμ UV light source.

Quantitative analysis

After detection, the band of each substance is isolated by cutting away the sides of the layer of adsorbent with a spatula, and then performing the quantitative determination by removing the silica gel and extracting it with methyl alcohol. A silica gel collection method using a vacuum described by several authors[22–25] has proved useful for this purpose. We have adopted the simple device shown in Fig. 1 which permits the easy collection of the silica gel and quick elution of the substances under vacuum. The device consists basically of a small glass funnel inserted, with a special plastic (polyvinylchloride) fitting, into a Pyrex glass volumetric flask (Fig. 1A). Near the lower end of the funnel stem is a perforated sintered glass filter plate with

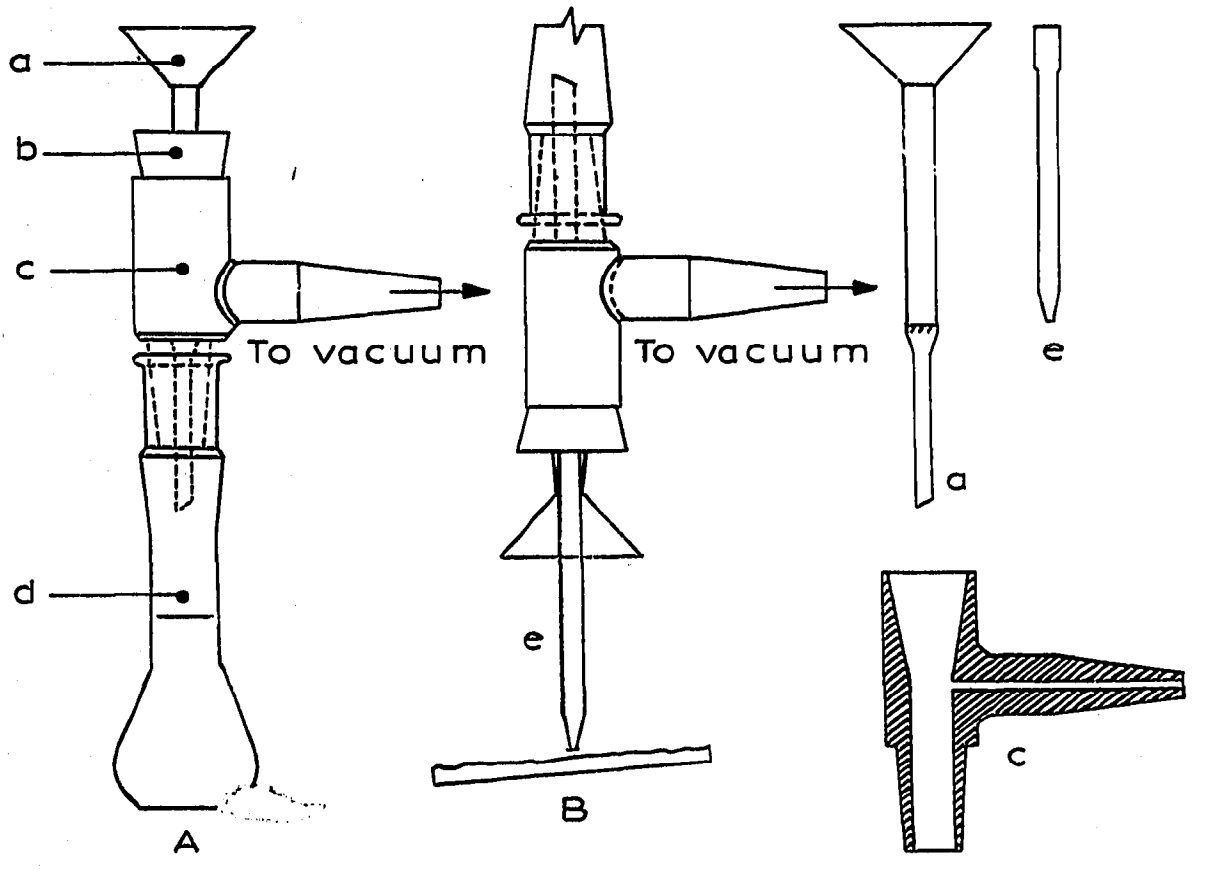

Fig. 1. (1:2 scale). A = system for elution of substances; B = system for suction of silica gel. a = Pyrex glass funnel with perforated sintered glass filter plate, b = rubber stopper, c = polyvinylchloride fitting with side inlet for vacuum, d = volumetric flask, e = glass tube with tygon ring.

gauze or fiber glass pressed onto it; the system can be connected to the vacuum pump by means of the side attachment on the plastic fitting. The silica gel is collected by inserting a small glass tube with a pointed end in the funnel using a tygon ring. The device is turned upside down, and the silica gel is sucked in by applying a low vacuum (Fig. 1B). When the silica gel has been collected, a few tenths of ml of methyl alcohol are sucked through to wash the inlet tube (taking care that no solvent emerges from the bottom end of the funnel stem), and the device is quickly uprighted. The vacuum is broken, the inlet tube is removed, 1 or 2 ml of methyl alcohol are pipetted into the funnel, the vacuum is again applied, and the solvent is collected in the volumetric flask. Methyl alcohol is added a couple more times until elution of the substance to be determined is complete (generally after 4–5 ml of methyl alcohol have passed through the funnel, elution is complete).

The eluent in the volumetric flask is diluted to volume with methyl alcohol and divided into two portions. One was acidified and the other was made alkaline by adding 2% of 1 *N* HCl and 1 *N* NaOH, respectively. Light absorption is measured at 297 mμ using the alkaline solution as test solution and the acidified solution as blank following the differential spectrophotometric method described in our previous report[21].

E I

LUES OBTAINED ON HF_{254} SILANIZED SILICA GEL FOR THE VARIOUS COMPOUNDS STUDIED

und	R_F *values*[a]					
	Mixture A pH 11 buffer–methyl alcohol (90:10)	*Mixture B pH 11 buffer–ethyl cellosolve (90:10)*	*Mixture C pH 11 buffer saturated with ethyl acetate at 25°*	*Mixture D pH 11 buffer saturated with ethyl ether at 15°*	*Mixture E pH 11 buffer–tetra-hydrofuran (95:5)*	*Mixture F pH 11 buffer–dioxane (90:10)*
l-*p*-hydroxybenzoate	0.04	0.08	0.03	0.02	0.03	0.08
p-hydroxybenzoate	0.06	0.11	0.06	0.05	0.08	0.13
l-*p*-hydroxybenzoate	0.15	0.22	0.14	0.15	0.14	0.20
p-hydroxybenzoate	0.23	0.32	0.25	0.25	0.24	0.28
l-*p*-hydroxybenzoate	0.30	0.38	0.34	0.35	0.33	0.35
lroxybenzoic acid	0.60	0.70	0.69	0.75	0.57	0.59

[a] Each R_F is the mean value of six separate chromatographic runs.

RESULTS AND DISCUSSION

Table I shows the R_F values obtained on developing the chromatograms with the various buffer–solvent mixtures.

Of the mobile phases studied, mixtures C, D, E, and F gave the best separation of the various compounds, especially of benzyl- and butyl-*p*-hydroxybenzoates. However, mixtures C and D are rather difficult to use because the solvents are volatile and the solutions are saturated; good chromatographic development and reproducibility of data are closely controlled by temperature stability in the chromatographic chamber. Mixtures E and F are more practical, and we normally use them for the analysis of preservatives in pharmaceutical preparations.

In quantitative zone chromatography, the separation may not be good enough

TABLE II

R_F' VALUES CALCULATED AFTER SECOND DEVELOPMENT ON HF_{254} SILANIZED SILICA GEL

Compound	R_F' *value*[a]	
	Mixture E pH 11 buffer–tetrahydrofuran (95:5)	*Mixture F pH 11 buffer–dioxane (90:10)*
Benzyl-*p*-hydroxybenzoate	0.06	0.17
Butyl-*p*-hydroxybenzoate	0.14	0.25
Propyl-*p*-hydroxybenzoate	0.24	0.38
Ethyl-*p*-hydroxybenzoate	0.36	0.49
Methyl-*p*-hydroxybenzoate	0.47	0.58
p-Hydroxybenzoic acid	0.68	0.75

[a] Each R_F' is the mean value of six separate chromatograms.

for elution and quantitative determination of the components of the mixture when the amounts of some of the compounds to be separated are much larger than others. In this case, the mobility of the substances and the chromatographic separation can be greatly enhanced by performing a second development with the same solvent mixture, especially when low R_F paraseptics are involved.

Table II shows the R_F' values obtained with mixtures E and F after a second development. In this case the R_F' values represent the proportion between the distance of the spots of substance from the point of application after the second development and the distance of the solvent front from the point of application (the solvent run was the same in the first and second development).

The quantitative analysis of the eluents by differential spectrophotometry eliminates background absorption due to the extraction of the silica gel with methanol. The amount of the test compounds may be calculated from the spectrophotometric results using a calibration straight line plotted from results of chromatographic analyses performed on solutions containing known amounts of preservatives.

Greater precision in quantitative analyses may be obtained by using the internal standard method. In this case a known amount of a compound with a different R_F from those of the test compounds is added to the test solution before chromatography (since the six preservatives discussed here are not generally used together in the same pharmaceutical speciality, one which is known to be absent in the test sample may be used as an internal standard). After chromatographic development and spectrophotometric determination of all the compounds, it is

TABLE III

QUANTITATIVE CHROMATOGRAPHIC ANALYSIS OF METHANOL SOLUTIONS CONTAINING DIFFERENT CONCENTRATIONS OF METHYL AND PROPYL-*p*-HYDROXYBENZOATE

Chromatography was performed on 5 × 20 cm H_{254} silanized silica gel plates using a pH 11 buffer–dioxane mixture (90:10) as mobile phase; 0.05 ml of sample solution was pipetted as a band on the plates. Method A = determinations performed using the calibration straight line. Method B = determinations performed by adding butyl-*p*-hydroxybenzoate as internal standard.

Solution No.	*Theoretical (mg/ml)*	*Found*[a]			
		Method A		*Method B*	
		mg/ml	*% difference*	*mg/ml*	*% difference*
(a) Methyl-p-hydroxybenzoate					
1	0.424	0.426	+0.5	0.424	0.0
2	0.824	0.807	−2.1	0.836	+1.5
3	1.227	1.196	−2.5	1.260	+2.7
4	1.595	1.552	−2.7	1.585	−0.6
5	2.006	1.992	−0.7	2.046	+2.0
(b) Propyl-p-hydroxybenzoate					
1	2.008	2.045	+1.8	2.053	+2.2
2	1.621	1.580	−2.5	1.613	−0.5
3	1.206	1.164	−3.5	1.211	+0.4
4	0.804	0.781	−2.9	0.807	+0.4
5	0.419	0.420	+0.2	0.416	−0.7

[a] Mean of three chromatographic determinations performed under the same conditions.

easy to calculate the amount of each single compound in the test solution using the formula:

$$\frac{P \cdot Ec \cdot F}{Est} = \text{mg of compound contained in the sample solution}$$

where P is the amount of internal standard added, expressed in mg; Ec and Est are the differential absorption of the eluents of the test compound and of the compound used as internal standard respectively; F is a correction factor calculated from the ratio between the differential absorptions of equal amounts of the internal standard and test compounds (experimentally; the F factor is determined by repeated quantitative chromatographic analyses of mixtures containing known amounts of the two compounds).

Tables III (a) and (b) show the results of a quantitative chromatographic determination performed on solutions with different concentrations of methyl-*p*-hydroxybenzoate and propyl-*p*-hydroxybenzoate using the calibration straight line method and the internal standard method.

The data given in Table III show that the quantitative analysis, especially using the internal standard method, is quite accurate; the internal standard method is also more precise than the calibration straight line method because the quantitative determination is not affected by pipetting errors which may occur when the solutions are applied to the chromatographic plates. A repeatibility test consisting of 15 chromatographic determinations on a methyl- and propyl-*p*-hydroxybenzoate mixture showed that the coefficient of variation for both paraseptics is about 3% for the calibrated straight line method and about 1.5% for the internal standard method.

REFERENCES

1 H. GÄNSHIRT AND K. MORIANZ, *Arch. Pharm.*, 293 (1960) 1065.
2 T. SALO AND K. SALMINEN, *Z. Lebensm. Untersuch. Forsch.*, 124 (1964) 448.
3 J. W. COPIUS-PEEREBOOM AND H. W. BEEKES, *J. Chromatog.*, 14 (1964) 417.
4 I. CALVARANO, *Essenze Deriv. Agrumari*, 34 (1964) 137.
5 M. COVELLO AND O. SCHETTINO, *Riv. Ital. Sostanze Grasse*, 41 (1964) 75.
6 M. COVELLO AND O. SCHETTINO, *Riv. Ital. Sostanze Grasse*, 41 (1964) 337.
7 A. AMATI AND A. FORMAGLINI, *Riv. Viticolt. Enol.*, 18 (1965) 387.
8 R. PINZON, A. MIRIMANOFF AND I. KAPÉTANIDIS, *Pharm. Acta Helv.*, 40 (1965) 141.
9 J. D. BENMAMAN AND D. L. SORBY, *J. Chromatog.*, 20 (1965) 607.
10 G. I. DICKES, *J. Assoc. Publ. Analysts*, 3 (1965) 73.
11 E. LUDWIG AND U. FREIMUTH, *Nahrung*, 9 (1965) 751.
12 S. J. PINELLA, A. D. FALCO AND G. SCHWARTZMAN, *J. Assoc. Offic. Anal. Chemists*, 49 (1966) 829.
13 C. LEVORATO AND R. MANTOVAN, *Boll. Chim. Farm.*, 105 (1966) 450.
14 J. A. W. GOSSELÉ AND S. SREBRNIK-FRISZMAN, *J. Chromatog.*, 23 (1966) 305.
15 H. WOIDICH, H. GNAUER AND E. GALINOVSKY, *Z. Lebensm. Untersuch. Forsch.*, 133 (1967) 317.
16 A. GRÜNE AND V. NOBBE, *Riechstoffe Aromen*, 17 (1967) 501.
17 H. SCHRIFTMAN, *J. Pharm. Sci.*, 57 (1968) 1760.
18 J. SCHMID, *Pharm. Zentralh.*, 107 (1968) 653.
19 K. NAGASAWA, H. YOSHIDOME AND R. TAKESHITA, *J. Chromatog.*, 43 (1969) 473.
20 H.-C. CHIANG, *J. Chromatog.*, 44 (1969) 201.
21 R. RANGONE, *Farmaco (Ed. Prat.)*, 21 (1966) 275.
22 F. J. RITTER AND G. M. MEYER, *Nature*, 193 (1962) 941.
23 B. GOLDRICK AND J. HIRCH, *J. Lipid Res.*, 4 (1963) 482.
24 B. M. MULHERN, *J. Chromatog.*, 34 (1968) 556.
25 J. H. NAGEL AND J. C. DITTMER, *J. Chromatog.*, 42 (1969) 121.