Journal of Chromatography, 156 (1978) 211–217

CHROM. 10,922

DETECTION OF PRESERVATIVES IN PHARMACEUTICAL AND COSMETIC PRODUCTS USING AGAR GEL ELECTROPHORESIS

K. E. MOORE and R. J. STRETTON

Microbiology Unit, Department of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire LE11 3TU (Great Britain)

(First received September 19th, 1977; revised manuscript received January 30th, 1978)

SUMMARY

A method is described using high voltage agar gel electrophoresis for the separation and microbiological detection of preservatives in pharmaceutical and cosmetic products. The positions of the preservatives were detected as zones of inhibition of a test organism within the nutrient agar gel and can be characterised by their migration, shape and presence of a halo. A wide range of preservatives commonly used in pharmaceutical and cosmetic preparations could be detected in low concentrations. The method however was not suitable for certain volatile preservatives and many primarily antifungal compounds could only be detected above their use concentration.

INTRODUCTION

A number of chemical procedures have been used to detect and identify preservatives but the main emphasis has been on compounds used in foods and beverages. A large bibliography of this work has recently been assembled[1]. Some of the techniques have also been used to detect and identify preservatives present in pharmaceutical and cosmetic preparations.

Sheppard and Wilson[2] used partition chromatography followed by UV spectroscopy to detect and assay preservatives in cosmetics. They concluded that the method could be broadly applied to assay many preservatives but that it is unreliable for identifying preservatives and recommended thin-layer chromatography (TLC) for this purpose. TLC has been used to identify preservatives in pharmaceuticals[3], cosmetics[4,5] and specifically in eye drops[6].

Gas–liquid chromatography has been used to detect in pharmaceutical preparations selected preservatives, *e.g.*, bisphenols[7,8], parabens[9] and a range of preservatives[10].

Microbiological methods have been used to detect added preservatives in foods and beverages, the preservatives being detected by the inhibition of spore germination[11–13], fermentation[14,15] and by macroscopic growth[16,17]. These microbiological methods are non-specific and can only give an indication of the presence and nature of added preservatives.

The study investigates the suitability of agar gel electrophoresis for the separation and microbiological detection of preservatives in pharmaceutical and cosmetic products.

MATERIALS AND METHODS

The modified method of Lightbown and De Rossi[18] was used. Electrophoretic separation was performed in a layer of nutrient agar, inoculated with *c.* 10^8 *Bacillus subtilis* spores per ml, for the detection of antibacterial preservatives, and *c.* 10^8 *Aspergillus niger* spores per ml, for the detection of antifungal compounds. The nutrient agar employed for *Bacillus subtilis* contained dextrose (0.1%, w/v); Lemco beef extract (Oxoid) (0.15%, w/v); yeast extract (Oxoid) (0.3%, w/v); Casitone (Difco) (0.08%, w/v); Agar No. 3 (Oxoid) (1.2%, w/v). The pH was adjusted to 6.8 after sterilization. The nutrient agar for *Aspergillus niger* contained malt extract (Oxoid) (3.0%, w/v); Mycological peptone (Oxoid) (0.5%, w/v); Agar No. 3 (Oxoid) (1.5%, w/v). The pH was adjusted to 5.0 after sterilization.

The apparatus consisted of a water-cooled levelled aluminium plate carrying a sheet of plate glass. With the aid of an aluminium frame, previously sealed to the glass with a little molten agar, 125 ml of sterile, molten (50°), seeded nutrient agar was poured onto the glass. When the agar was set, the frame was removed leaving a level slab of agar of 47 × 13 cm and *c.* 0.2 cm thick. The buffer tanks containing platinum electrodes were connected to the agar by means of gauze wicks. The electrode reservoirs contained nutrient broth (750 ml per tank) of the same pH and composition but without added agar, as the nutrient agar. The whole apparatus was enclosed in a box with a Perspex lid.

Samples of 10 μl of the preservative solutions and the pharmaceutical and cosmetic products were applied. Where creams, gels and shampoos were tested, sufficient sterile distilled water was added to the products to reduce their viscosity and thus to enable the preparation to be applied to the agar gel. The points of application were marked on the gel by previously punching a hole in the agar with a pasteur pipette.

A constant voltage of 800 V was applied to the electrodes for 2 h, this gave a current of *c.* 80 mA. Throughout the run the plate was cooled by passing tap water through the cooling block.

Following electrophoresis the wicks were removed, the frame replaced and a second piece of plate glass placed as a lid on top of the frame. The plate was then incubated at 30° overnight for *Bacillus subtilis* and 48–72 h for *Aspergillus niger*.

The following preservatives were tested (unless otherwise stated they were obtained from Koch-Light, Colnbrook, Great Britain). Chlorhexidine diacetate and gluconate (ICI); alkylbenzylmethylammonium chloride (benzalkonium chloride); dodecylbenzylmethylammonium chloride; cetylpyridinium chloride; cetyltrimethylammonium bromide (domiphen bromide); pyrithione zinc (zinc omadine, Kingsley and Keith, Croydon, Great Britain); 2-bromo-2-nitropropane-1,3-diol (bronopol, The Boots Company Limited, Nottingham, Great Britain), 5-bromo-5-nitro-1,3-dioxan (bronidox, Henkel Limited, London, Great Britain); trichlorohydroxydiphenyl ether (Irgasan D.P. 300, Ciba-Geigy, Basle, Switzerland); trichlorocarbanilide; hexachlorophene; phenylmercuric acetate and nitrate; sodium ethylmercurithiosalicylate

(thiomersal); potassium hydroxyquinoline sulphate; phenol; *m*-cresol; chlorocresol; chlorbutol (Hopkin and Williams, Chadwick Heath, Great Britain); phenyl ethanol; benzyl alcohol; sodium benzoate; potassium sorbate; propyl- and methyl-*p*-hydroxybenzoate (propyl and methyl paraben); LFD preservative, a commercial mixture (70:30, w/w) of chloracetamide and sodium benzoate (Henkel).

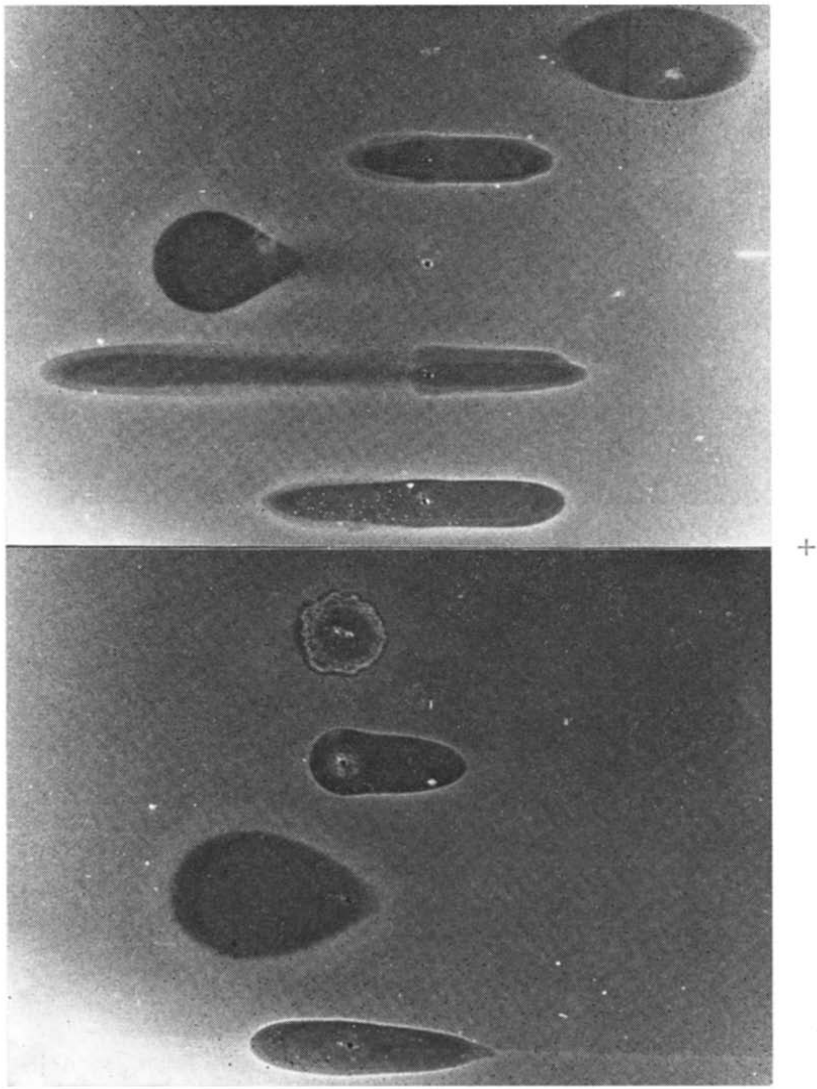

Fig. 1. Electrophoretic separation of preservatives. Of each sample 10 μl was applied. Voltage, 800 V at *c.* 80 mA for 2 h. The preservatives applied starting from the top were as follows. Phenylmercuric acetate (100 μg/ml), chlorhexidine HCl (400 μg/ml), bronopol (500 μg/ml), benzalkonium chloride (500 μg/ml), benzethonium chloride (500 μg/ml), trichlorocarbanilide (10 mg/ml), hexachlorophene (1 mg/ml), Irgasan DP 300 (1 mg/ml), chlorhexidine acetate (400 μg/ml).

RESULTS AND DISCUSSION

The positions of the preservatives were detected as zones of inhibition. Fig. 1 shows the electrophoretic patterns of a range of preservatives. Drawings of other preservatives that gave a zone of inhibition are shown in Fig. 2. Whilst many preservatives could be distinguished from their position alone, the shape and presence of a halo also served to characterise the preservative. Table I groups preservatives with zones of inhibition of similar shape and with similar mobilities. Preservatives within a group are difficult or impossible to distinguish from one another but can be easily distinguished from preservatives of any other group. Thus bronopol and Bronidox in group e appear to be identical but are clearly different from, *e.g.*, hexachlorophane (group h) or the members of group a.

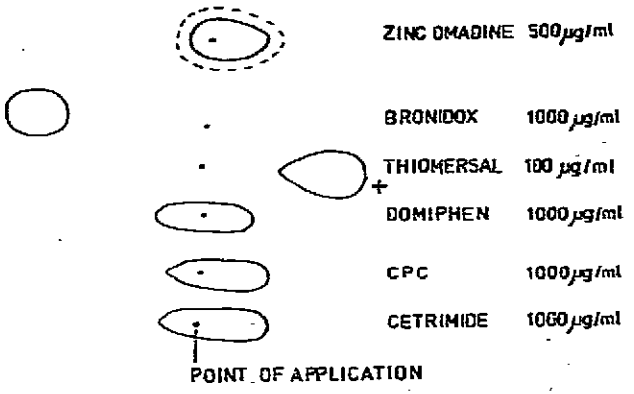

Fig. 2. Electrophoretic separation of preservatives; Of each sample 10 µl was applied. Voltage, 800 V at *c.* 80 mA for 2 h.

TABLE I

GROUPS OF PRESERVATIVES THAT ARE EASILY DISTINGUISHABLE FROM ONE ANOTHER

Group	*Preservatives(s)*
a	Chlorhexidine diacetate and gluconate; Cetylpyridinium chloride; Cetyltrimethylammonium bromide; Domiphen bromide;
b	Alkylbenzylmethylammonium chloride
c	Benzethonium chloride
d	Zinc omadine
e	Bronopol; Bronidox
f	Irgasan DP 300
g	Trichlorocarbanilide
h	Hexachlorophane
i	Phenylmercuric acetate and nitrate; Thiomersal
j	Potassium hydroxyquinoline sulphate

Many of the quaternary ammonium compounds, especially at concentrations approaching the minimum detection level, were difficult to differentiate. However alkylbenzylmethylammonium chloride had a characteristic double zone of inhibition, which is probably due to the C_{12} and C_{14} homologues present, also the zone of inhibition of benzethonium chloride was more elongated than the other quaternaries.

When the preservative was present at high concentrations it was frequently possible to distinguish certain preservatives within a group. By referring to a standard solution run alongside the test preparation it was possible to distinguish, on the basis of zone shape and presence of a halo, chlorhexidine salts and domiphen bromide from the other members of this group.

Table II lists the minimum detection levels; the results of the analysis of a range of pharmaceutical and cosmetic preparations for the presence of preservatives are shown in Table III.

The technique can be used to separate and detect low concentrations of a wide range of preservatives that are commonly used in pharmaceutical and cosmetic

TABLE II

APPROXIMATE MINIMUM DETECTION LEVELS

Preservative	*Approximate minimum detection level (μg/ml)*
Test organism *Bacillus subtilis*	
Chlorhexidine diacetate	10
Chlorhexidine gluconate	10
Alkylbenzylmethyl ammonium chloride	25
Cetylpyridinium chloride	50
Cetyltrimethylammonium bromide	50
Domiphen bromide	50
Benzethonium chloride	50
Zinc omadine	10
Bronopol	50
Bronidox	100
Irgasan DP 300	200
Trichlorocarbanilide	50
Hexachlorophane	5
Phenylmercuric acetate	1
Phenylmercuric nitrate	1
Thiomersal	5
Potassium hydroxyquinoline sulphate	Not determined
Phenyl ethanol	Not detected
Chlorocresol	Not detected
Phenol	Not detected
Cresol	Not detected
Chlorbutol	Not detected
Butanol	Not detected
Test organism *Aspergillus niger*	
Chloracetamide	2,000
Sodium benzoate	Not detected
Potassium sorbate	Not detected
Methyl paraben	10,000
Propyl paraben	2,000

TABLE III

DETECTION OF PRESERVATIVES IN A RANGE OF PHARMACEUTICAL AND COSMETIC PRODUCTS

Nature of Product	*Preservative(s) known to be present*	*Preservative(s) detected*
Nasal solution	Not known	None
Nasal solution	0.1% Benzalkonium chloride 0.4% Phenyl ethanol	Dodecylbenzylmethyl-ammonium chloride
Nasal solution	0.001% Thiomersal	Thiomersal
Nasal solution	0.001% Benzalkonium chloride	None
Nasal solution	Not known	None
Nasal solution	Not known	Unidentified quaternary ammonium compound
Ophthalmic solution	0.001% Thiomersal	None
Ophthalmic concentrate	0.3% Benzalkonium chloride	Benzalkonium chloride
Topical cream	0.5% Cetyltrimethyl ammonium chloride	Unidentified quaternary ammonium compound
Topical cream	0.018% Chlorhexidine diacetate	Chlorhexidine
Topical cream	0.005% Chlorhexidine diacetate	None
Topical gel	0.01% Cetalkonium chloride	Unidentified quaternary ammonium compound
Respiratory solution	Not known	Benzalkonium chloride
Aural drop	1.0% Chlorbutol 0.1% Potassium hydroxyquinoline sulphate	Potassium hydroxy-quinoline sulphate
Antiseptic "paint"	0.5% Chlorbutol 0.5% Chlorhexidine	Chlorhexidine
Vitamin solution	Sodium benzoate Methyl paraben	None
Shampoo	0.02% Bronopol	Bronopol or Bronidox
Shampoo	0.05% Bronidox	Bronopol or Bronidox
Shampoo	0.35% Chloracetamide 0.15% Sodium benzoate	Choracetamide

preparations. It must, however, be emphasized that confirmatory chemical tests should be carried out to identify such preservatives detected. The electrophoresis results will give an indication of the nature of the preservative present. In certain preparations the presence of other compounds with antimicrobial activity can mask the presence of the preservative (*e.g.*, antibiotics and surface active agents) but this problem can often be obviated by the use of resistant test organisms.

The volatile preservatives phenol, cresol, chlorocresol, chlorbutol, phenyl ethanol and benzyl alcohol were lost from the plate and so could not be detected. In general the method was very good for the detection of preservatives with antibacterial activity, but the results with antifungal compounds were less satisfactory, in many cases the minimal detection level being higher than the in-use concentration.

An additional advantage of the method was that a bioassay of the preservative could be carried out in the presence of compounds which interfered with a normal bioassay, *e.g.*, when a fluorescein solution (1%, w/v) was preserved with bronopol (0.1%, w/v); the bronopol could be separated from the fluorescein by electrophoresis and bioassayed on the same agar plate.

The sensitivity of the technique is comparable with reported chemical methods. Wilson[4] reported the lower detection level of seven preservatives after a simple ex-

traction procedure using TLC. Between 0.1 and 0.5 μg of each preservative could be detected. The above technique without an extraction procedure will similarly achieve this high sensitivity, furthermore if necessary a simple extraction procedure would undoubtedly reduce the lower detection levels for many preservatives.

In Great Britain the use of hexachlorophane has been restricted[19]. This method would provide a simple technique to ensure that this order is complied with. In a single stage, hexachlorophane could be detected and assayed without any extraction procedure. With the world-wide tightening of standards by regulatory authorities it is possible that other preservatives could be banned, *e.g.*, the toxicity of organomercurials is well known. Also it has recently been shown that benzalkonium chloride, a commonly used preservative in ophthalmic solutions, causes significant damage to rabbit corneas[20]. Should such legislation be introduced this method could be used as a simple technique to assist in monitoring such fraudulently added preservatives.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. J. D. Adams for typing the manuscript and Mr. E. Miller for the photography.

REFERENCES

1 M. E. Endean, *Sci. Tech. Survs. Br. Fd. Mfg. Ind. Res. Ass.*, 90 (1976).
2 E. P. Sheppard and C. H. Wilson, *J. Ass. Offic. Agr. Chem.*, 58 (1975) 937.
3 M. Nonnet, *Sci. Tech. Pharm.*, 1 (1972) 475.
4 C. H. Wilson, *J. Soc. Cosmet. Chem.*, 26 (1975) 75.
5 M. B. Graber, I. I. Domsky and M. E. Ginn, *J. Amer. Oil Chem. Soc.*, 46 (1969) 529.
6 K. Ludwikowska and A. Kowalska, *Farm. Pol.*, 31 (1975) 37.
7 P. J. Porcaro, *Anal. Chem.*, 36 (1964) 1664.
8 R. W. Daisley and C. J. Olliff, *J. Pharm. Pharmacol.*, 22 (1970) 202.
9 S. S. Donato, *J. Pharm. Sci.*, 54 (1965) 917.
10 S. Iguchi, M. Yamamoto and T. Aoyama, *Yakugaku Zasshi*, 83 (1963) 721.
11 F. Baum and C. Wardsack, *Ernährungs Forschung*, 6 (1961) 584.
12 F. Baum and H. Lamm, *Ernährungs Forschung*, 8 (1963) 355.
13 S. Hruby, P. Maresova and L. Prokop, *Čslká Hyg.*, 10 (1965) 10.
14 D. A. A. Mossel, *Food. Mf.*, 31 (1956) 190.
15 W. Pilnik and M. Piek-Faddegon, *Mitt. Geb. Lebensmittelunters. Hyg.*, 63 (1972) 461.
16 D. A. A. Mossel, H. Zwart and H. S. de Bruin, *Analyst (London)*, 85 (1960) 410.
17 J. M. Debever and J. P. Voets, *Meded. Fac. Landbouwwet*, 39 (1974) 1505.
18 J. W. Lightbown and P. de Rossi, *Analyst (London)*, 90 (1965) 89.
19 Anon. The medicines (hexachlorophane prohibition) order 1973; *Statutory Instrument*, 1120 (1973). H.M.S.O.
20 A. R. Gasset, Y. Ishii, H. E. Kaufman and T. Miller, *Amer. J. Ophthalmol.*, 78 (1974) 98.