An enzymic technique for the microbiological examination of pharmaceutical gelatin

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The liability of gelatin to contamination with pathogenic micro-organisms has been recognized by the United States Pharmacopeia XIX (1975) and by the British Pharmacopoeia (1973). The former requires the absence of *Escherichia coli* and *Salmonella* from a sample of 10 g of gelatin and imposes a total count limitation of 1000 organisms g^{-1}. The British Pharmacopoeia specifies that *E. coli* be absent from a 1 g sample and *Salmonella* from 10 g.

Methods for the examination of gelatin usually involve enrichment in broth, with or without initial solubilization, at 45° (Leininger, Shelton & Lewis 1971; U.S.P. 1970; B.P., 1973). Membrane filtration is an alternative to the direct enrichment method and is recommended by Bühlmann (1968) and by Miller & Korczynski (1972) as a suitable technique for the testing of soluble material. Membrane filtration has two advantages if used for the testing of gelatin:

1. After filtration, the membrane can be placed in broth and enriched without producing a viscous solution. Rose (1972) states that viscosity complicates the recovery of *Salmonella* from gelatin. Our own observations indicate that increase in viscosity tends to reduce microbial growth.

2. The membrane can be placed directly on an agar plate or nutritive pad. Depending upon the medium used, this procedure can give either a total microbial count or the count of a specific organism such as *Salmonella* or *E. coli*.

Aqueous solutions of gelatin up to concentrations of 10 % prepared by warming at 45°, proved difficult to filter through 0·45 μm pore size membranes. Hence an alternative procedure using enzymic solubilization of gelatin was examined and the weights of gelatin filterable by each of the two methods described were compared.

In each of the procedures described gelatin powder (10 g) was added to 0·1 % peptone water (100 ml) to give a concentration of 10 % w/v.

Method 1, Non-enzymic procedure. The peptone-gelatin suspension was held at 8–10° for 1 h then at 45° for 30 min, or longer if the gelatin failed to dissolve. The resultant viscous solution was diluted 1½ times in peptone water (0·1 %) (45°) containing Tween 80 (0·1 %) and filtered through a membrane (Sartorius 0·45 μm, 47 mm diameter).

Method 2, Enzymic digestion. A sterile solution of a protease ('Proteinase', Nutritional Biochemicals Corporation, Ohio) was added to the peptone-gelatin suspension, and incubated at 37° for between 25 and 45 min, depending upon the rate at which the gelatin sample dissolved. The solution obtained was diluted 1½ times with peptone-Tween solution (37°) and filtered as in Method 1.

Protease 10–20 mg g^{-1} of gelatin, adequately solubilized most gelatin samples. However, some samples required up to 50 mg g^{-1} of gelatin. Thus, 5 mg ml^{-1} is the maximum protease concentration used in the stated method.

Gelatin suitable for use in pharmaceutical preparations was obtained from five suppliers, the 33 samples obtained included 18 different makes or grades. Each sample was solubilized using the two procedures described, and the quantities of gelatin from each that could be filtered through a single membrane were compared. When solubilized by the enzymic procedure, gelatin filtered was greater by weight for all samples than gelatin not enzymatically treated. Although in many cases the 10 g sample of gelatin could not be filtered through one membrane filter it could all be sampled using two filters. This represents an improvement over the non-enzymic procedure where less than 25 % of the gelatin samples could be sampled using two filters.

To ensure the enzymatic procedure had no adverse affect on the recovery of bacteria, control tests were carried out using *Escherichia coli* (NCTC 9002), *Salmonella abony* (NCTC 6017), *Pseudomonas aeruginosa* (NCTC 6750) and *Staphylococcus aureus* (Glaxo Laboratories Strain PRIV).

The organism was added to a 5 mg ml^{-1} solution of the protease in peptone water (0·1 %). The solution was then held at 37° and viable counts made at 15 min intervals for 60 min.

The survival curves indicated that none of the four types of bacteria had changed significantly in numbers during this time.

To demonstrate that these four organisms could be recovered by the enzymic technique 50 bacteria of each type were inoculated into a protease gelatin mixture (5 mg ml^{-1} of enzyme) and the solubilization and filtration procedures were carried out. The membrane filters obtained were either enriched in accordance with the B.P. 1973 method or placed directly on selective agar media: for *S. abony*—bismuth sulphite; for *Ps. aeruginosa*—Pseudosel (Bühlman, 1968); for *S. aureus*—Baird Parker and for *E. coli*—MacConkey (Oxoid No. 2). The organisms were detected by both the B.P. 1973 method and the direct selective agar technique.

This last test was repeated to obtain a quantitative estimation of the recovery of *S. abony* and *E. coli*. The counts on the selective media were compared with control counts obtained by inoculating peptone water (0·1 %).

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Table 1. *Replicate 10 g samples of gelatin tested for* E. coli *contamination using I—B.P. 1973 test, direct enrichment of gelatin; II— enrichment of membrane filter in accordance with B.P. 1973; III—Incubation of membrane filter on MacConkey agar no. 2, IV—U.S.P. test, direct enrichment of gelatin; V—enrichment of membrane filter in accordance with U.S.P.; VI—Incubation of membrane filter on MacConkey agar no. 2.*

Recovery procedure	Number of replicate samples	Samples positive for *E. coli* after 24 h	*Time (h) to obtain 'suspect' positive	*Time (h) to obtain 'confirmed' positive	Mean viable *E. coli* count per 10g of gelatin
I	9	6	48	72	—
II	9	5	48	72	—
III	10	6	24	48	3
IV	15	1	48–72	72–96	—
V	15	6	48	72	—
VI	15	9	24	48	2

* Suspect positive *E. coli*—Considered as colony with *E. coli* characteristics on MacConkey agar no. 2, or, in case of B.P. test, acid and gas production from MacConkey broth (37°) and indole production (37°).
Confirmed positive *E. coli*—Isolates producing acid and gas from MacConkey broth (44°) and producing indole (44°) or isolates confirmed biochemically.

The *t*-test for differences between the pairs of results (10 replicates) showed no significant difference between the control and the test results at the 5 % level, indicating that the enzymatic treatment did not affect the viability of the organisms.

Samples of gelatin (10 g) were tested for *E. coli* contamination using the B.P. 1973 procedure. Equivalent samples of gelatin were solubilized enzymatically and filtered, the membranes were then either enriched according to the B.P. procedure or incubated on MacConkey Agar No. 2. Before placing the membranes on MacConkey agar, they were incubated on a pad soaked in 'Resuscitation Membrane Broth' (Oxoid) for 2 h at 37°.

A similar set of comparative recoveries were carried out based on the U.S.P. XVIII procedure. The U.S.P. enrichments were examined after 24 h and little visible growth occurred when compared with either the lactose broth containing membranes or the B.P. enrichment technique. This may be due to the lower incubation temperature recommended in the U.S.P. of 30–35°, rather than 37°, which will give a gelatin solution of lower viscosity.

The results summarised in Table 1, show that *E. coli* occurring as a natural contaminant of gelatin can be recovered using the enzymatic procedure with the same efficiency as the B.P. procedure, and possibly more efficiently than the U.S.P. procedure. If the membranes are placed directly onto MacConkey agar No. 2 a quantitative estimation of *E. coli* is obtained and suspect and confirmed positive results for *E. coli* can be obtained 24 h sooner than by enrichment techniques.

The enzymic procedure offers membrane filtration as a practical alternative method for the examination of high grade gelatin suitable for inclusion in pharmaceutical preparations.

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