A Method for Extracting and Purifying Streptomycin Suitable for Large-scale Production

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SUMMARY: A method is described for extracting streptomycin from culture filtrates by adsorption on charcoal at pH 6–8, elution with 1·2 % (v/v) aqueous phosphoric acid, readsorption of the eluate on charcoal at pH 7, elution with acidified methanol, followed by evaporation at reduced pressure and precipitation of streptomycin by dilution of the concentrated methanol eluate with 5 volumes acetone or amyl acetate. An indication is given of the order of recovery, and the potency of the product obtained. The stability of streptomycin under the conditions of pH and temperature to which it may be subjected during the extraction is outlined. The process has been carried out in pilot-scale production equipment handling 1500 l. batches of culture filtrate.

The aim of this work was to devise a method of extracting streptomycin from culture filtrates prepared by the method of Ainsworth, Brown, Marsden, Smith & Spilsbury (1947) which could be applied to the large-scale and economic manufacture of the antibiotic. In particular, a method was required that could be carried out in an extraction plant previously used for penicillin production.

Extraction methods given by other workers can be summarized as follows:

- (1) A. Adsorption on charcoal at pH 7·0 and elution with 0·5 % HCl in methanol (Waksman & Schatz, 1945; LePage & Campbell, 1946; Carter, Clark, Dickman, Loo, Skell & Strong, 1945).
B. Concentration of neutral eluate (LePage & Campbell, 1946).
C. Precipitation of streptomycin hydrochloride, by addition of a large excess of ether or acetone to the eluates or concentrates (Waksman & Schatz, 1945; LePage & Campbell, 1946; Carter *et al.*, 1945).

(2) Charcoal adsorption at low pH to remove pigments followed by extraction of the streptomycin as in 1 (LePage & Campbell, 1946; Carter *et al.*, 1945).

(3) Adsorption on charcoal at pH 7·0 followed by elution of water-free charcoal cake with anhydrous methanolic hydrogen chloride, concentration of neutral eluate and precipitation of the streptomycin hydrochloride with acetone (Waksman & Schatz, 1945).

(4) A two-stage process involving solution of the first precipitate in water, adsorption on charcoal, elution with acid-methanol, concentration and precipitation (LePage & Campbell, 1946).

The adaption of the foregoing processes to large-scale manufacturing conditions presented the following difficulties: (a) the construction of acid-resistant plant; (b) the use of large volumes of methanol, with consequent high cost of production; (c) the production of large volumes of methanol-acetone mixtures,

presenting difficulties of fractionation; (d) the difficulty of recovering small weights of a hygroscopic product from large volumes of solvent.

Bearing in mind these difficulties, a process was developed along the following lines:

(1) Adsorption of the streptomycin from the culture filtrate on 1% (w/v) activated charcoal at pH 6–8.

(2) Elution of the streptomycin from the charcoal with 1.2% (v/v) aqueous phosphoric acid at pH 1.0–2.0.

(3) Adsorption of the streptomycin on 3–4% (w/v) activated charcoal from the neutralized phosphate eluate at pH 7.

(4) Elution of the streptomycin under anhydrous conditions with acidified methanol.

(5) Evaporation of the neutralized methanol eluate to one-eighth of its original volume.

(6) Precipitation of the streptomycin by dilution of the concentrate with acetone, or other precipitant of higher boiling-point, e.g. amyl acetate.

This process has some advantages. For example: (i) In the first stage of the extraction, streptomycin is eluted from the charcoal with dilute aqueous phosphoric acid, enabling stainless steel equipment to be used when volumes are large. (ii) The organic solvent elution stage is delayed until a considerable volume diminution has been effected, decreasing solvent requirements to at least one-tenth of those employed in other processes. (iii) Precipitation of streptomycin is not accomplished until volumes are comparatively small. (iv) The volume of methanol-acetone mixture produced during the process is at a minimum. (v) The use of aqueous phosphoric acid as an eluting agent gives pigment-free eluates and renders unnecessary the introduction of a special decolorizing stage.

METHODS

The method of streptomycin assay was a dilution test using *Escherichia coli* (*Bacterium coli*) as the test organism, and the unit employed was 1 μ g. streptomycin base (Brown & Young, 1947). It was necessary to remove methanol by evaporation from concentrated methanol solutions to avoid interference by the bactericidal properties of the alcohol. In practice, samples were evaporated under reduced pressure to 8% or less of their original volume and diluted back to the initial volume with distilled water. Unsatisfactory results were obtained with certain phosphate eluate samples, and data obtained for extraction efficiencies and potencies up to the phosphate eluate stage are approximations.

The potencies of the final dried products obtained from the pilot plant were also determined by a spectrographic assay method (unpublished) developed by Dr Tudor S. G. Jones of the Wellcome Chemical Research Laboratories (see Table 5).

Total solids were determined by drying in an oven at 70° for 18 hr. Such results agreed satisfactorily with determinations made by drying by sublimation of the water from the frozen state.

LABORATORY-SCALE EXTRACTION

Most of the work on adsorption and elution from charcoal was carried out on laboratory-size Metafilters (Metafiltration Ltd., Hounslow, Middlesex) designed to take a charge of 150 g. charcoal and 115 g. filter-aid. This gave a charcoal bed sufficient for a 15 l. culture filtrate cycle. The beds were washed with water prior to each elution and at the end of each complete and successive cycle.

The extraction process from culture filtrate to the first eluate may be referred to as *stage 1*; and from the first eluate to the final product as *stage 2*. All volumes of washing liquids and eluants used in stage 1 are given as volumes/100 vol. of culture filtrate. For stage 2, all volumes are given as volumes/100 vol. phosphate eluate. Thus a '40 volume' wash means, in stage 1, washing with a volume of liquid 40 % of the volume of culture filtrate taken, and in stage 2, 40 % of the volume of phosphate eluate. The ratio streptomycin to total solids ($\mu\text{g./mg.}$) is referred to as 'potency'.

After removal of the mycelial felts the culture fluid was clarified by filtration through cloth filters precoated with Hyflo Supercel (Johns Manville Ltd.). The culture filtrate assayed between 200 and 400 $\mu\text{g.}$ streptomycin/ml. on harvesting (potency 6–18). The pH of the culture filtrates ranged between pH 6 and 8.

Stage 1; First charcoal adsorption

'Farnell 14' was found to be the most suitable active charcoal for the adsorption of streptomycin from these culture filtrates and allowed elutions to take place with suitable eluants. A level of 1 % (w/v) charcoal to culture filtrate volume appeared optimal for pH 6–8. Pigment adsorption increased, whilst total solid and streptomycin adsorption decreased below pH 6, and at pH 2 almost pigment-free filtrates were obtained without appreciable adsorption of streptomycin. The effect of culture filtrate pH on streptomycin adsorption efficiency is indicated in Table 1.

Table 1. *Effect of pH on streptomycin adsorption efficiency*

Original titre of culture filtrate: 148 $\mu\text{g./ml.}$ Adsorption on 'Farnell 14' charcoal: 1 % (w/v).

pH of adsorption	Streptomycin in culture filtrate after adsorption ($\mu\text{g./ml.}$)	Adsorption efficiency (%)
4	67	53
5	30	80
6	< 11	> 93
7	< 11	> 93
8	< 11	> 93

No differences in efficiency of adsorption of streptomycin were observed as between passing culture filtrates through static charcoal beds, and adsorption on charcoal suspensions. Similarly, static elution appeared as satisfactory as elution by suspension. Static charcoal beds used for successive adsorptions and elutions retained their efficiency, and no appreciable quantity of solid

material accumulated in them. One bed could be used for at least seven complete operations. Table 2 indicates the total solid (mg./ml.) recovery between culture filtrate and eluate over seven successive cycles.

Table 2. *The use of one charcoal bed for a series of adsorptions and elutions*

	Cycle							Mean
	1	2	3	4	5	6	7	
Culture filtrate (500 ml.); total solids (mg./ml.)	18.4	18.4	18.4	18.4	18.4	18.5	18.4	—
Spent culture filtrate (500 ml.); total solids (mg./ml.)	—	15.4	15.9	18.4	16.0	15.6	15.4	—
Phosphate eluate (200 ml.); total solids (mg./ml.)	—	5.7	8.8	5.8	6.0	5.8	5.7	—
Washwater (200 ml.); total solids (mg./ml.)	—	2.4	2.6	3.8	2.7	3.0	2.1	—
Total solids of culture filtrate:								
Adsorbed on charcoal (%)	—	16	13	—	13	15	16	15
Recovered in phosphate eluate (%)	—	12	—	13	13	13	12	13

The effect of water washing the charcoal beds after the adsorption of streptomycin was studied. Approximately 30–40 % of the adsorbed solids could be removed without appreciable loss of streptomycin. The progressive change in total-solids content of the effluent when water-washing the charcoal beds is indicated in Fig. 1. A 40 volume water-wash appears to be optimum.

Fig. 1. Relation between water-wash volume and concentration of inactive material in effluent.

Stage 1; Elution of first charcoal adsorbate

The progressive change in streptomycin content and pH of the eluate on elution with 1.2 % phosphoric acid is shown in Fig. 2. The first 3–5 volumes of eluate were almost inactive. Elution was complete with 15 volumes. Provided a 40 volume water-wash was introduced between the adsorption and elution, the phosphate eluates were almost colourless. Elution of the water-washed charcoal bed with acid-methanol gave pigmented eluates.

An indication of the order of recovery and volume diminution obtained from culture filtrate to phosphate eluate is given in Table 3. The potency of the phosphate eluate was estimated as 30–60, but the accuracy of the recovery figures is affected by difficulties experienced in assaying streptomycin in the presence of phosphate.

Fig. 2. The progressive change in streptomycin content (continuous line) and pH (broken line) during elution with 1.2 % phosphoric acid. *A–B*, for 10,000 l. culture filtrate to 830 l. eluate, 8.5 % streptomycin loss; *C–D*, for 10,000 l. culture filtrate to 1200 l. eluate, 3.6 % streptomycin loss.

Table 3. *Volume diminution for culture filtrate to phosphate eluate and % recovery*

Culture filtrate volume (l)	15	15	15	15	15	
Phosphate eluate volume (l)	2.5	2.0	2.0	2.0	2.3	
Eluate volume as % filtrate volume	16	13	13	13	15	
Total streptomycin (g.):						
In culture filtrate	1.5	2.27	2.42	2.42	1.43	
In eluate	1.10	1.58	1.46	1.57	1.18	
% recovery	73	68	61	65	82	Mean 69.8
Culture filtrate: Titre ($\mu\text{g./ml.}$)	100	151	161	161	95	
Potency ($\mu\text{g./mg.}$)	—	6.3	7.5	7.0	3.1	

Neutralization of the phosphate eluate with caustic soda gave heavy precipitates of calcium phosphate, calcium ion having been extracted from the charcoal bed during elution at low pH. These precipitates were troublesome to handle, particularly on a manufacturing scale, and entailed an additional filtration. The amount of precipitate could be decreased, but never completely eliminated, by washing the charcoal beds with a large volume of dilute aqueous phosphoric acid prior to the adsorption of streptomycin.

Stage 2; Second charcoal adsorption and elution

The readsorption of streptomycin from the neutralized phosphate eluate was effected, as at the first extraction stage, with Farnell 14 charcoal, and the subsequent elution was by means of acidified methanol. Both operations were carried out with the charcoal suspended in the liquor; 3–4 % (w/v) charcoal was optimal.

Adsorption. After suspension for 30 min. the charcoal was filtered off and the bed washed first with 40 volumes of water and finally with 40 volumes of neutral methanol. The purpose of the neutral methanol wash was to remove some inactive material and to lower the water content of the charcoal bed to a minimum. Failure to remove water from the charcoal resulted in sticky and hydrated precipitates of streptomycin hydrochloride at the precipitation stage.

Elution. This was effected by suspension of the washed charcoal in methanol containing 2 % (v/v) conc. hydrochloric acid (pH 1.5–2.0) with agitation for 30 min. 15 volumes of eluant were used, and while 2 % (v/v) of conc. HCl was usually adequate to bring the suspension to pH 1.5, it was advisable to check the pH after 15 min. stirring, and to add more acid if required. The spent charcoal was removed by filtration and the charcoal bed washed three times on the filter with 5 volumes of acid-methanol. The bulked eluate and washings were adjusted to pH 6 with 80 % (w/v) caustic soda. Calcium phosphate was again precipitated, but the amount of this precipitate also could be decreased by pretreatment of the charcoal with aqueous acid. Recoveries and potencies for the second adsorption and elution are given in Table 4.

Table 4. *Volume diminution phosphate eluate to methanol eluate and % recovery*

Phosphate eluate volume (l.)	2.0	2.3	2.4	
Methanol eluate (l.)	0.38	0.44	0.40	
Methanol eluate vol. as % phosphate eluate vol.	19	19	17	
Total streptomycin (g.): In phosphate eluate	1.57	1.18	1.05	
In methanol eluate	1.06	0.81	0.66	
				Mean
% recovery	68	69	62	66.3
Methanol eluate potency (μ g./ml.)	248	245	—	

Stage 2; Concentration of second eluate and separation of streptomycin hydrochloride

The neutralized and filtered eluate was concentrated to one-eighth of its original volume by evaporation under reduced pressure at 30–40° and refiltered. Some inactive material, mainly sodium chloride, was thrown out of solution during concentration and the streptomycin potencies increased in consequence. There was no appreciable inactivation of streptomycin during concentration; the pH tended to fall.

White flocculent precipitates of streptomycin hydrochloride were obtained

by the addition to the filtered concentrates of five times their volume of commercial acetone. The precipitates were filtered off and dried under reduced pressure over sulphuric acid. Losses of streptomycin in the mother liquor were not more than 1% of that in the culture filtrate, provided the water content of the methanol-acetone mixture was at a minimum. No detectable increase in potency occurred during precipitation. The overall extraction efficiencies from culture filtrate to precipitate averaged 40%, and the potency of the precipitate was 200–500.

The solubility of the product in methanol was 10.9 g./100 ml., in ethanol 0.5 g./100 ml., and in methylated spirits 1.1 g./100 ml. The solubility of streptomycin hydrochloride in these solvents will be influenced to some extent by the water content, but it is apparent that, of the three solvents investigated, methanol alone is suitable as an eluting agent. With methanol eluates, care had to be taken to avoid concentration to such a degree that the solubility of streptomycin hydrochloride was exceeded.

The use of acetone as precipitant on a manufacturing scale would be uneconomic owing to the difficulties of fractionating mixtures of methanol and acetone. A search was made for alternative precipitants, of suitable availability, cost, boiling-point, solubility in methanol, water content at saturation, and ease of dehydration. Amyl acetate and monochlorobenzene were the most satisfactory and gave as good streptomycin recoveries as did acetone.

Stability

The stabilities of the crude culture filtrate, the methanol eluate and of aqueous solutions of streptomycin hydrochloride were investigated at various pH values and temperatures. The maximum stability of culture filtrates and aqueous and methanol solutions of streptomycin hydrochloride was in the range pH 3.0–6.0. Aqueous solutions of streptomycin hydrochloride were kept at pH 1.0 and 20° for 173 hr. without loss of activity; aqueous and methanol solutions were kept for 33 days at 20° and pH 7, without any detectable inactivation. Material dried from the frozen state has been kept at 20° for 67 days, without detectable loss of streptomycin content. Aqueous and methanol solutions of streptomycin hydrochloride were heated at 90° for 7 hr. in the range pH 3.0–6.0 without detectable loss of streptomycin content.

PILOT-PLANT EXTRACTION

Filtration and first adsorption. The culture fluid on the plant scale was harvested in batches of 1500 l. and was clarified by filtration through leaf cloth filters precoated with filter-aid; filter-aid was also continuously injected into the flow-line on the feed side of the filter. The temperature of the liquor was decreased to +3° in a brine-cooled heat exchanger. A battery of ten Metafilters, each filter having a bed of 1.5 kg. of Farnell 14 charcoal and 2 kg. Hyflo-Supercel, was used for the adsorption of streptomycin from each 1500 l. batch. The Metafilter beds were pretreated with 1800 l. 1.2% (v/v) aqueous phosphoric acid, followed by 1800 l. mains water, before being fed with the

harvested metabolism fluid. The adsorption of streptomycin was effected at a flow-rate of 4000 l./hr., i.e. 400 l./hr./Metafilter.

First elution. The beds containing the adsorbed streptomycin were washed with 625 l. mains water, and eluted with 250 l. 1.2 % (v/v) aqueous phosphoric acid, the first 50 l. of eluate being run to waste. The eluates were neutralized with 80 % (w/v) caustic soda and insoluble calcium phosphate removed by sedimentation.

Table 5. *Mean stage potencies, and % recoveries for twenty pilot-scale batches*

	Mean potency ($\mu\text{g./mg.}$)	Total strepto- mycin (g.)	Stage	Stage recovery (%)	Overall recovery (%)
Culture filtrate*	18.2	5928		—	—
Phosphate eluate	—	3263	Culture filtrate to phosphate eluate	55	55
Methanol eluate	231	2595	Phosphate eluate to methanol eluate	80	44
Methanol concentrate	362	2565	Methanol eluate to methanol concentrate	99	44
Precipitate	422	2099	Methanol concentrate to precipitate	82	36
Freeze-dried product	389 350.5†	1635	Precipitate to freeze-dried product	77	28

* Mean titre: 262 $\mu\text{g./ml.}$

† Spectrographic assay.

Second adsorption. Each 200 l. batch of neutralized and decanted phosphate eluate was agitated with 4 % (w/v) of previously acid-washed Farnell 14 charcoal, for 30 min. The charcoal was collected on Doulton vacuum filters and washed with 100 l. distilled water, followed by 100 l. neutral methanol. This neutral methanol wash was generally used in three fractions, the first 60 % (v/v) methanol-water, the second 98 % (v/v) methanol-water, and the final wash with 100 % methanol.

Acid-methanol elution. The water-free charcoal cake was suspended and agitated in 30 l. methanol, containing 2 % (v/v) conc. HCl. Elution was effected in 30 min. The spent charcoal was recovered on a Doulton filter and the charcoal bed washed with three 10 l. washes of acid-methanol.

Concentration. The bulked eluate and washings were adjusted to pH 5–6 with 80 % (w/v) caustic soda, and concentrated to one-eighth volume in a climbing film evaporator. The methanol concentrate, of 7–8 l., was filtered on a Buchner filter and the filtrate poured into 40 l. acetone. The precipitated streptomycin hydrochloride was filtered off, dried under reduced pressure, dissolved in pyrogen-free distilled water, Seitz-filtered, and freeze dried in phials, having approximately 160,000 $\mu\text{g./phial.}$ Stage and overall recoveries and potencies are given in Table 5. The overall and stage recoveries are calculated from the mean of twenty batches. The crude filtrate titres and final potencies were appreciably higher than the corresponding laboratory figures. The overall

recovery from culture filtrate to freeze-dried product was lower than that obtained in the laboratory, but stage recoveries on the plant approximately paralleled those obtained in laboratory extractions. There was a significant increase in potencies during the concentration of methanol eluates.

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