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PERSISTENCE OF AMPHOTERICIN B IN THE VAGINA*

ESTIMATION BY A NOVEL SAMPLING METHOD

BY

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This report describes a method of estimating the duration of fungicidal activity of amphotericin B in the vagina after its insertion in a cream base.

Various localized vaginal infections are treated by the insertion of a pessary or cream into the vagina. This method of treatment is not entirely satisfactory since failure to respond may be due to the method of application rather than to the limitations of the preparation or the resistance of the infection to the active principle.

The effectiveness of this procedure depends on the distribution of the active principle over the whole surface of the vaginal mucosa. A variety of factors may combine to prevent or delay this dispersal. When inserting a pessary the patient may fail to introduce it high enough into the vagina to ensure uniform spread throughout its surface. The composition of a pessary together with local anatomical, physiological, or pathological conditions may prevent its rapid and complete disintegration, thereby delaying the liberation of the active principle. Although much work has been done on the composition of pessaries to ensure their rapid disintegration in the vagina, the practical difficulties of application remain and make the efficacy of this particular form of treatment extremely difficult to assess in any individual patient.

In some respects the use of a cream inserted with an applicator is preferable to a pessary. The cream spreads more uniformly over the vaginal mucosa but the amount of leakage varies considerably between patients. A pregnant ambulant multiparous woman near term may lose a very substantial proportion of the total dose in this way.

Whichever vehicle is used, the convenience of the patient demands that the active principle should be present in effective concentrations at all vaginal sites for at least 24 hours, or a multiple thereof, so that the patient can insert the cream or pessary on going to bed. This also avoids much of the early leakage which occurs in the ambulant patient. But whether or not effective vaginal concentrations are maintained for 24 hours in any individual patient is unknown to the clinician. Failure to maintain such concentrations may account for a proportion of treatment failures. This single factor may explain the poorer response to treatment reported in pregnant as opposed to non-pregnant patients treated for vaginal candidiasis (Jennison, 1958; Ewing, 1967).

Until a satisfactory method is devised for estimating the concentration of a preparation in the vagina at a given time after its introduction, the effect of this factor on the results of treatment will remain unknown. No satisfactory method of estimating such concentrations has so far been described, and the results of assays can be extremely variable, especially after the use of a pessary. The sample for assay may be derived from a mass of pessary debris lying partly disintegrated in the posterior fornix. Alternatively the sample may be obtained from an area to which minimum dispersal has taken place. In either case the results obtained would give an inaccurate picture of the concentrations present in all areas of the vagina.

In this trial a cream* was preferred to a pessary since its more uniform distribution over the vaginal surface was thought likely to give more dependable assays.

The method of sampling vaginal concentrations here described† avoids the danger of obtaining an unrepresentative sample from a single vaginal site and is presented as an improved method for estimating the survival time of any preparation administered in this way.

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*The cream was specially made up by E. R. Squibb & Co.

†Suggested by Dr G. Ewart Cree, Consultant Venereologist, United Bristol Hospitals.

All patients were treated at clinic sessions, either in the morning or early evening, and thus were ambulant immediately afterwards for the whole or part of the day. If this had any effect on the vaginal concentrations subsequently recorded it could only be to reduce them as compared with those which would have been obtained if the patient had remained at rest in bed for approximately 8 hours after the insertion of the cream.

Method

With the patient in the lithotomy position, a known amount of amphotericin B cream was inserted high into the vagina by means of an applicator. After various sampling intervals a tampon was inserted and then left *in situ* for 24 hours. During this period any amphotericin B remaining in the vagina was partitioned between the tampon and the vaginal mucosa to an extent dependent on (*a*) its accessibility to the tampon, and (*b*) physicochemical factors. If the extent of this partition were found to be reasonably constant, then assay of the amphotericin B in the tampon, after removal from the vagina, could be used as a basis for estimating the amount of active principle actually present in the vagina during the sampling period.

Evidence is presented below to show that the extent of partition is reasonably constant, and the relation between the amount of amphotericin B inserted into the vagina and that recovered from the tampon is described.

After the sampling tampon had been removed from the vagina its absorbed amphotericin B was extracted in a solvent and assayed against a standard preparation by a microbiological diffusion-zone assay method of the parallel-line type commonly used for antibiotics.

Subjects

All the patients were ambulant adult females who volunteered to co-operate, but many failed to attend at the appointed times and had to be excluded from the trial, so that the analysis of results concerns only patients who completed the trial satisfactorily.

The first fourteen patients had a tampon inserted into the vagina immediately after the introduction of the amphotericin B cream; 24 hours later the tampon was removed and the amount of amphotericin B absorbed by the tampon was assayed. The results in these fourteen patients were used as a control. Two women in this group were pregnant, both primiparae. The age range was 17 to 28 years (average 20·7).

In the next ten cases the tampon was inserted 24 hours after the introduction of the cream and was left *in situ* for 24 hours to absorb any remaining amphotericin B. It was then removed for assay. Six of the ten patients in this group were pregnant, all primiparae. The age range was 16 to 20 years (average 18·3).

In a further group of ten patients the tampon was inserted 48 hours after the introduction of the cream, and was removed for assay after remaining *in situ* for 24 hours.

In a final group of ten patients the tampon was inserted 72 hours after the cream and removed for assay after 24 hours. Since no measurable amounts of amphotericin B could be detected after 48 or 72 hours, details of the patients in the last two groups are omitted.

Technique

Application of Cream

A plastic vaginal applicator was filled with amphotericin B cream containing 50mg./4g. cream base and weighed. After the cream had been introduced the applicator was re-weighed and the exact amount of cream inserted was recorded.

Sampling Amphotericin B Cream in the Vagina

For this tampons were used. Several brands were investigated for absence of inhibitory substances, absorption of cream, and ease of extraction of cream. Of the brands tested "Cameo" (Robinson and Sons Ltd.) proved the most satisfactory on the basis of these criteria.

The tampon was left in the vagina for 24 hours, after which time it was placed in a glass container and stored at +4°C. until examined.

Microbiological Assay of Amphotericin B

Assay of amphotericin B in extracts of the sampling tampons was accomplished by a diffusion-zone microbiological assay. Two special features affected the design of the assay method: (*a*) a *yeast* was used as the sensitive test organism, and (*b*) amphotericin B is appreciably soluble only at (or below) pH 2·0 or at (or above) pH 11·0, or in solvents such as form-dimethylamide.

These considerations led to an assay design in which amphotericin B, dissolved in buffer of pH 11·0, diffused from cups into an agar medium that had been adjusted to pH 6·6 in order to allow optimal growth of the indicator yeast. Clearly, under standard conditions of incubation, and with given concentration of buffer salt, the zone of diffusion of the buffer from the cups is reasonably constant.

Within the area where an effective buffer concentration has diffused, the size of inhibition zones is related solely to the dose of amphotericin B in the cup by the usual log-dose/response function. When higher concentrations of amphotericin B are employed, the size of inhibition zones is limited not only by the concentration of active principle but also by the rapid decrease in its solubility as the pH falls off beyond the effective diffusion of buffer salt. Although these factors determine the position of the zone edge, subsequent growth of the indicator yeast is made possible, even within the buffer-diffusion zone, because continued diffusion of the buffer salt throughout a large mass of agar gel dilutes out its effectiveness. In other words, the diffusion of buffer solution alone does not produce zones of inhibition.

A plot of zone diameter *versus* $\log_2$ (concentrations of amphotericin B) is shown in Fig. 1 (opposite). The region marked "A" is that where the response depended only on amphotericin B concentration; the region marked "B"

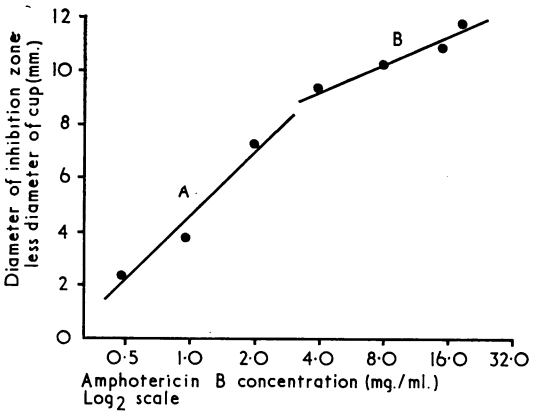

Fig. 1.—Assay of amphotericin B: log-dose/response plot showing the two linear regions *A* and *B* discussed in the text.

illustrated the additional pH-solubility effect. Tampon extracts were diluted so that their responses fell into region "A", thus allowing a true "parallel-line" method of comparison. Very occasionally an extract gave responses which fell into region "B" thus necessitating a "parallel-line" comparison with this valid but less sensitive region of the response function.

Assay Medium.—This was Oxoid blood agar base No. 2 Code No. CM271 adjusted to pH 6·6, dispensed in 100 ml. amounts and sterilized in the autoclave at 10 lb./sq. in. for 15 minutes.

Dessicating Dishes.—Heat-resisting glass dishes measuring $12 \times 12 \times 1$ in. were used to contain the medium (Simpson, 1958). Lids were made locally from sheet aluminium. The dishes with lids *in situ* were wrapped in kraft paper and sterilized in the hot-air oven at 160°C. for one hour.

Indicator Organisms.—Saccharomyces cereviseae was used as the sensitive indicator throughout these investigations. It was maintained on assay medium slopes stored at 4°C. with weekly subcultivation. For use in the assay, the growth from a slope was washed off into physiological saline and diluted to give a reading of 50 per cent. light transmission at 650 mμ in a Unicam spectrophotometer using 1 cm. cells.

Diluents

(*a*) Form-dimethylamide (British Drug Houses Ltd.)

(*b*) pH 11·0 buffer solution: K_2HPO_4 35*g*; N. NaOH, 20 ml; distilled water to 1 litre.

Amphotericin B Standard Solution.—50 mg. equivalents of amphotericin B (Squibb) were transferred to a 100 ml. volumetric flask and dissolved in form-dimethylamide by shaking for 15 minutes. This solution was then diluted 1 in 10 with form-dimethylamide to give 50 μg./ml.; all subsequent dilutions were made in pH 11·0 buffer solution.

The standard solution was made fresh each time it was used.

Preparation of the Assay Plate.—Two 100 ml. amounts of medium were used for each plate. The medium, after melting, was held at 50°C. in a water-bath.

The assay plate was set upon a specially levelled surface. 100 ml. of agar was poured into the plate to form a basal layer and allowed to solidify. The remaining bottle was inoculated with 2·5 ml. of *Saccharomyces cereviseae* suspension. After being mixed by gentle agitation, the contents of the bottle were poured over the basal layer to form the inoculum layer. The plate was placed at 4°C. for 30 minutes to harden the medium before cutting reservoirs and to arrest the growth of the indicator strain.

Preparation of Samples for Assay.—100 ml. form-dimethylamide was added to each tampon. The amphotericin B was extracted by shaking for 15 minutes on a mechanical shaker. Dilutions were made in buffer solution to give an estimated 2 and 1 μg./ml. The amphotericin B control was diluted to concentrations of 8, 4, 2, and 1 μg./ml. in order to span the regions "A" and "B" of Fig. 1.

Assay Procedure.—The plate was removed from the refrigerator and 64 cups of 9 mm. diameter were cut in the medium using a sterile No. 5 cork borer. Positioning was facilitated by placing a rectilinear (8×8) grid beneath the plate with 27 mm. between its intersections. The plugs of medium were removed with a sterile lancet-headed needle.

The eight treatments were allocated at *random* to the letters A to H and these letters were marked on the template in the form of a randomized 8×8 latin square. With the template in position the cups were filled using an automatic pipette. The plate was incubated at 37°C. for 18 hours.

Measurement of Inhibition Zones.—The assay plate was examined by obliquely transmitted light against a matt black background. The zone diameters were measured to the nearest 0·1 mm. with vernier calipers fitted with needle points. All zones were measured across a diameter diagonally opposed to the major axes of the plate, thus allowing a maximum distance between adjacent cups.

Computation of Potencies.—The mean common slope ($\bar{b}$) of the regression of the zone diameter on the dyadic logarithm (*i.e.* $\log_2$) of the treatment level was first calculated from the total level difference.

The dyadic logarithm of the potency of a sample relative to that of the standard (M_2) was then obtained by dividing the difference between sample means by $\bar{b}$; multiplication of M_2 by 0·3010 converted this value into a common logarithm (M_{10}); and the relative potency (R) of the sample was then obtained as the antilogarithm of M_{10}.

The final estimate of the amount of amphotericin B absorbed by a tampon (in mg.) was obtained by multiplication of (R) by the following factors:

(*a*) the concentration of the higher standard solution of the appropriate region "A" or "B" (see Fig. 1).
(*b*) any dilution factor.
(*c*) the volume of fluid (100 ml.) in which the tampon was extracted.

Results

It was found that all the sampling tampons were evenly stained, thus supporting the view that the cream had been distributed with reasonable uniformity throughout the vagina.

Table I shows the results obtained from fourteen patients in whom sampling tampons were inserted immediately after application of amphotericin B (zero time group) and from ten patients in whom sampling was begun 24 hours after application (24-hr sample group). The vital statistics of these patients are not given in detail since the whole experimental group was fairly homogeneous.

Table I reveals that the amount of active amphotericin B recovered from the "zero time" group was assayed as roughly 45 per cent. of that inserted, whereas only about 2 per cent. was recovered from the "24-hr sample" group.

TABLE I

AMOUNTS OF AMPHOTERICIN B RECOVERED FROM TAMPONS INSERTED IMMEDIATELY AFTER AND 24 HRS AFTER APPLICATION.

Sampling Tampon inserted immediately after Application (14)			Sampling Tampon inserted 24 hrs after Application (10)		
Patient No.	Amount Applied (mg.)	Amount Recovered (mg.)	Patient No.	Amount Applied (mg.)	Amount Recovered (mg.)
50	8·375	2·00	16	49·750	2·10
58	17·875	9·50	31	45·750	0·70
52	27·875	10·50	32	51·625	1·50
56	32·250	11·50	33	44·875	0·80
1	49·500	23·00	34	49·000	0·80
2	49·000	27·00	35	46·875	0·50
3	49·750	18·30	38	47·500	0·50
8	47·500	18·80	42	51·250	1·50
9	47·500	26·00	40	45·625	0·50
11	42·750	21·30	21	50·625	0·90
12	30·625	12·87			
15	47·500	19·10			
44	50·000	20·00			
45	53·500	24·00			

In order to attempt a more precise interpretation of the data, an Analysis of Regression was performed on the results of the "zero time" group. This showed that the amounts of active principle recovered (y) could be represented, within statistical limits of variation, by a simple linear function of the amount inserted (x).

Fig 2 shows a plot of this function, which can be represented as $y = mx$, where m represents the regression constant. Numerically, the "least squares" estimate of m was 0·429. Thus, over the range from 0 to 53·5 mg. active principle inserted, the amount recovered by the sampling method was 42·9 per cent. when sampling was begun immediately after insertion.

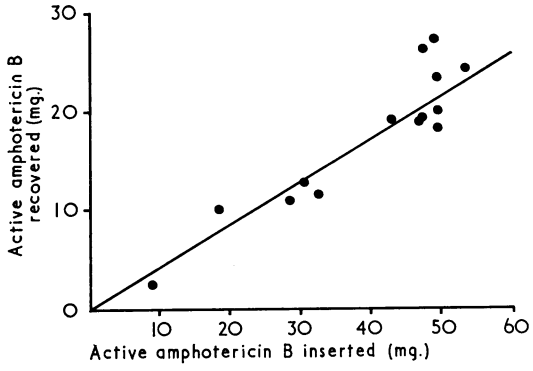

Fig. 2.—Relation between amount of amphotericin B inserted and amount recovered when sampling tampon was inserted immediately.

We next applied these findings to the data of the "24-hr sample" group, arguing that, if the amount of active amphotericin B recovered from a tampon was y mg., then it was likely that the amount actually in the vagina during the sampling period (x mg.) was:

$$x = y/m \quad \text{or} \quad x = y/0{\cdot}429$$

Table II (opposite) shows that, when this reasoning was applied to the relevant data of Table I, the estimated amount of active amphotericin B remaining in the vagina 24 hours after sampling was only about 5 per cent. of that originally inserted (*i.e.* about 2 mg.).

The data for the groups of patients in whom sampling was begun 48 hours or 72 hours after insertion are not presented since, although each of these patients received about 50 mg. active amphotericin B, in no case was a measurable amount detected by the assay of tampon extract. The lower limit of sensitivity of the assay method corresponds

TABLE II

ESTIMATED PERSISTENCE OF AMPHOTERICIN B IN THE VAGINA IN THE PERIOD 24–48 HRS AFTER INSERTION.

Patient No.	Amount Inserted x mg.	Amount Recovered y mg.	Amount estimated as persisting in Vagina $x^1 = y/0{\cdot}429$	$100\ x^1/x$ per cent.
16	49·750	2·10	4·89	9·83
31	45·750	0·70	1·63	3·57
32	51·625	1·50	3·50	6·77
33	44·875	0·80	1·86	4·15
34	49·000	0·80	1·86	3·80
35	46·875	0·50	1·17	2·49
38	47·500	0·50	1·17	2·45
42	51·250	1·50	3·50	6·82
40	45·625	0·50	1·17	2·55
21	50·625	0·90	2·10	4·14
		Averages	2·29	4·66

to an amount absorbed by a sampling tampon equal to about 0·5 mg. or to an amount of about 1·17 mg. actually present in the vagina.

Discussion

The activity and distribution of a drug introduced into the vagina cannot be assessed with accuracy chiefly because of the difficulty in obtaining representative samples for comparison. When assays involve more than one patient and two or more preparations valid comparisons are even more unlikely.

The primary purpose of this investigation was to determine the reliability of a new sampling technique. It was thought probable that a more accurate evaluation of the amount of active principle present in the vagina at a given time after its introduction would be obtained by assaying the amount absorbed by a tampon over a period of 24 hours than by assaying samples obtained by loop, pipette, or swabs.

A statistical analysis of the results of the investigation confirmed this expectation by demonstrating a correlation between the amount of active principle inserted into the vagina and the amount absorbed by the tampons. The results suggest that this method could introduce a measure of standardization to the sampling technique which is absent in other sampling methods and thus increase the validity of any comparative investigations into the persistence of different preparations used in this way. Further, a more reliable sampling method makes it possible to determine with greater accuracy the amount of active principle required in a base to ensure effective vaginal concentrations over a period of time.

The efficacy of amphotericin B as a fungicidal agent is well documented (Jennison, 1958; Stough and Blank, 1958; Hildick-Smith, Blank, and Sarkany, 1964), and it was not the purpose of this trial to assess the clinical effectiveness of this agent when incorporated in a cream base. The number of patients was too small to warrant firm conclusions on the clinical responses, nor is amphotericin B marketed as a vaginal cream. The results suggest, however, that such a cream might provide a very useful addition to the preparations at present available for the local treatment of vaginal candidiasis.

Table II shows the amount of amphotericin B inserted in the cases of ten ambulant patients (col. 2), and the estimated amounts still present in the vagina of each of these patients 24 hours later (col. 4). These estimated amounts are recorded with much greater confidence than would have been possible if the samples for assay had been taken by the usual methods. In addition it is likely that the amounts recorded underestimate the actual amounts present in these cases at 24 hours, since the amounts present at that time must have been at least as great as those which could be calculated from that absorbed by the tampon during the subsequent 24 hours.

The average amount of amphotericin B contained in an applicator full of the cream used in this trial was approximately 48 mg. After 24 hours the estimated amount still present in the vagina averaged 2·29 mg. Since the minimal inhibitory concentration of amphotericin B to *Candida albicans* is only 0·0005 mg./ml., it is very likely that in this small series of ambulant patients clinically effective amounts of amphotericin B persisted in the vagina for 24 hours in all ten cases. The validity of this observation can be determined only by clinical trials, since there is no way of proving that the presence of a given amount of active principle will result in a minimal inhibitory concentration at all vaginal sites for sufficient time to ensure successful treatment.

Summary

A method is described for estimating the persistence of any preparation inserted into the vagina. A statistical analysis of results is recorded.

A cream containing 48 mg. amphotericin B was shown to persist in the vagina for at least 24 hours, after which an average of 2·29 mg. of the active principle was recovered. It is considered that this dosage is likely to be clinically effective when repeated at intervals of 24 hours.

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La persistance de l'amphotericine B dans le vagin et son estimation par une nouvelle methode d'echantillonage

RÉSUMÉ

Une methode est décrite pour l'estimation de la persistance de n'importe quelle préparation placée dans le vagin. Une analyse des statistiques preparées des résultats est consignée.

Une crême contenant 48 mg. d'amphotéricine B a été demontrée comme étant encore présente dans le vagin pendant au moins 24 heures, après ce temps une moyenne de 2,29 mg. du principe actif a été recouvrée. Il est considéré que ce dosage serait vraisemblablement éfficace cliniquement s'il est répété à intervalles de 24 heures.