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USE OF CONCENTRATED EXTRACTS FOR MICROBIOLOGICAL ASSAYS OF PENICILLIN RESIDUES IN PORCINE TISSUES.

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INTRODUCTION.

The extensive medical and non-medical use of antibiotics in meat animals has made it necessary for food hygiene agencies to develop regulations to insure that residues do not reach the consumers through meat and meat products. Furthermore, meat inspection agencies have a special need to know whether in some cases, more or less atypical or indistinct post-mortem findings are the indirect result of a small residue of antibiotics. Such residues may interfere with the pathological picture normally developing during the progress of disease and eventually mask the symptoms of disease, thus making the diagnosis difficult. Therefore, it has become increasingly important to have easily applied and sufficiently sensitive methods to be able to detect the minute quantities often involved.

A variety of microbiological assay methods have been used to determine antibiotic residues in animal tissue.[3] The simplest procedures are those in which antibiotics are allowed to diffuse from the tissue material into the surrounding inoculated agar medium. Extraction methods will frequently be more accurate but may result in a high dilution of the antibiotic residue and loss of sensitivity.[9] Pensack et al.[10] employed a 20 % pyridine buffer solution, pH 6.o, for assay of 1-ephenamine penicillin in porcine tissues. Cover and Ludvig[4] used sterile saline for the extraction of procaine penicillin G from chicken tissues. Grove and Randall[5] suggested the use of a phosphate buffer (pH 6.o) for the extraction of penidllin from animal tissues and animal feeds. Multiple extractions with the same buffer or with aqueous acetone (1:1) is further recommended in the "Official Methods of Analysis"[8] for the quantitative determination of penicillin in animal feeds.

Very sensitive methods utilising concentrated tissue extracts have been reported recently. Hansen et al.[7] and Hansen[6] had excellent results using acetonitrile to extract penicillin and tetracycline from chicken tissue. Pedersen[9], comparing the effect of different extraction liquids, found an extraction and evaporation in vacuo technique with sodium oxalate, a tetrasemine (EDTA) buffer solution and acetone to be very efficient for the determination of both penicillin and tetracyclines from chicken tissue.

The above methods, however, were mainly used for extraction of chicken tissues. In our preliminary experimentation, we were unable to obtain the same satisfactory results when assaying porcine tissues. Results on porcine muscle tissue extracts were variable and inconsistent. The purpose of this study was to evaluate some of the factors involved in the variability of the assey for penicillin in porcine tissues when concentrated extracts are used.

MATERIALS AND METHODS

All tissues referred to as normal were taken from carcasses of pigs which had not been subjected to any antibiotic treatment or given any antibiotic-containing feeds for 21 days immediately prior to slaughter. Ten gram samples, fat and connective tissue removed, were extracted singly with 30 ml. of one of the following extractants:

1. Acetone[x]
2. Acetonitrile[x]
3. Phosphate buffer,[5] pH 6, made by dissolving 8.0 Gm. KH_2PO_4 and 2.0 Gm. K_2HPO_4 in 1000 ml. distilled water.
4. Buffered acetone I (acetone/buffer 5:1)
5. Buffered acetone II (acetone/buffer 2:1)
6. Buffered acetonitrile I (acetonitrile/buffer 5:1)
7. Buffered acetonitrile II (acetonitrile/buffer 2:1)
8. Acetone-water mixture (acetone/distilled water 1:1)
9. Extractants proposed by Pedersen[9]: Saturated solution of sodium oxalate made by dissolving 40 Gm. in 1100 ml of distilled water. EDTA-solution made by dissolving 0.4 Gm. of ethylenediaminotetraacetic acid in a phosphate buffer, pH 7.0. This buffer was made of 113 Gm. $Na_2HPO_4 \cdot 2H_2O$ and 18 Gm. KH_2PO_4 in 1000 ml distilled water. In our extraction procedure, 3 ml. oxalate solution, 3 ml. of EDTA-solution and 24 ml. acetone were added to each sample.

[x] Baker Analyzed Reagent.

Test organism. A stock suspension of Sarcina lutea was prepared by inoculating the surface of a blood agar base medium[x] in a large petri dish (diam. 150 mm.) from a 24 hr. slant. After incubation at 28 C. for 20 hrs. cells were washed from the surface of the medium with 30 ml. of physiological saline and the suspension adjusted so that a dilution of 1:30 would give a 50 % transmission on a Hitachi Perkin-Elmer UV-Vis spectrophotometer at 580 mμ with a square cell (1 cm). The adjusted suspension was stored at 2 C. as the stock suspension for a maximum of 14 days. The most satisfactory zones of inhibition were obtained by using 0.5 ml of this suspension per 100 ml. agar.

Assay medium and preparation of plates. A cylinder plate diffusion assay technique was used with a single layer of agar.[xx] Petri dishes, 150 mm. x 15 mm., with flat and even bottoms were placed on a leveled glass plate after which 25 ml. of inoculated medium were added to each dish to form a thin layer of uniform thickness. The inoculated dishes were stored in

[x] Bacto Blood Agar Base, Difco. [xx] Bacto Penassay Agar, Difco.

tight plastic bags at 2 C. for a maximum of 5 days. Before use, stainless steel cylinders (length 10 mm., outer diam. 8 mm., inner diam. 6 mm.) were placed on each plate and a filter paper placed under the glass lids to absorb excess moisture.

Assay procedure. The different extractants were added to 10 Gm. samples of tissue and homogenized in a Virtis "45" homogenizer for 5 minutes. After standing for 10 minutes, the homogenate was centrifuged and the supernatant was decanted into a 250 ml. beaker and placed in a hood for evaporation. To increase the rate of evaporation, a stream of air was circulated through each beaker. By this procedure, evaporation to dryness was complete within 3-6 hours. The residue was resuspended in 2 ml. of phosphate buffer and the suspension pipetted into the cylinders on the inoculated agar plates. Controls to exclude non-specific inhibition zones from those caused by penicillin were run simultaneously by adding 1 drop of a 1:10 dilution of penicillinase[x] in phosphate buffer to control cylinders. The plates were incubated at 26 C. for 16-20 hours before measuring the diameter of the zones.

Determination of lactic acid. Lactic acid was determined by the enzymatic method of Hohorst[2].

RESULTS AND DISCUSSION

Non-specific zones in concentrated extracts. Antimicrobial factors causing inhibition zones of growth have been observed during the assaying of tissues from various animals which have not been subjected to any form of antibiotic administration.[6,9] The occurence of those zones was even more frequent when concentrated extracts were used. For the remainder of this manuscript, the terms non-specific zones will be used for all zones which could not be attributed to antibiotics given directly to the animals from which the samples were taken. Table 1 presents the variation of non-specific zone diameters when different fluids were used for extracting muscle (M.longissimus dorsi), liver and kidney tissue from 10 porcine animals which had received no antibiotics for at least 3 weeks prior to slaughter. Acetone or acetonitrile extracts of the muscle in most cases yielded non-specific zones which varied greatly in size from one animal to another. The size of the zones also appeared to be dependent upon the post-mortem age of the sample, the largest zones occuring in the samples taken 24 hours post mortem. When 5 ml. of phosphate buffer were added to these extractants before homogenization, non-specific zones were found with samples from only 2 animals. However, when 10 ml. buffer was added before homogenization, no non-specific zones were observed. The use of oxalate

[x] Bacto Penase, Difco.

EDTA and acetone as extractants also gave zones in some cases when muscle tissues were extracted.

The most variable results were obtained with extracts from the liver. In contrast to muscle extracts, no zones were seen when pure acetone or acetonitrile were used. However, when distilled water or buffer were added to these extractants, non-specific zones occurred rather frequently. The non-specific zones were also frequently observed with unconcentrated phosphate buffer or saline extracts.

Non-specific zones were not observed with extracts of kidney tissue, except for a few very narrow zones with acetone-water as extractant. The frequent occurence of non-specific zones in aqueous extract of liver tissue would indicate that the growth-inhibiting effect in these extracts must be due to one or more water-soluble substances. As a whole, the non-specific zones observed were less distinct and more difficult to measure precisely than were zones produced by penicillincontaining extracts. The non-specific zones also had a tendency to diminish or disappear after prolonged incubation.

The possibility of the non-specific zones being due to a growthinhibiting effect of the extractants themselves can be excluded since no zones occurred around cylinders filled with the resuspended evaporation residues of the pure extractants. The only exception was the oxalate-EDTA-acetone evaporation residue which gave distinct inhibition when dissolved in buffer and plated.

The pH values of the experimental tissue and finally of the plated extract represent another factor of greatest importance to the occurence of non-specific zones.[6,9] Table 2 shows the results of an experiment in which the lactic acid content and the pH values of the muscle of 2 pigs were compared with the occurence of non-specific zones in muscle extracted with non-buffered acetonitrile. Muscle tissue frozen in liquid nitrogen immediately after killing of the animal had a low content of lactic acid and produced no zones of inhibition. After 4 hours post-mortem at 2 C., narrow inhibition zones were found from the extraction samples in which rapid glycogenolysis had taken place and the pH values dropped quickly, as is often seen in the case of pale, soft, exudative, porcine muscle[1]. After 24 hours, all samples produced non-specific zones when unbuffered acetonitrile was used as extractant. These findings are somewhat different from the results of Hansen,[6] who was only occasionally confronted with non-specific zones when acetonitrile extracts of muscle were used. Hansen,[6] however, used filter paper discs instead of cylinders and the buffering capacity of the substrate itself was probably sufficient to eliminate the effect of pH because of the smaller amount of test material absorbed on discs. As shown in Table 1, it was necessary, when using the cylinder plate technique, to add 10 ml phosphate

buffer to each muscle sample before homogenization in order to safeguard against the formation of non-specific zones. This addition of buffer only slightly prolonged the time necessary for evaporation.

Effect of concentration of extracts on zone diameter. The extractants, which did not give none-specific zones when muscle tissue samples were extracted within 4 hours after the death of the animal (Table I), were used in an experiment to demonstrate the effect of concentrating the extracts. Muscle samples were taken from M.longissimus dorsi of a pig which prior to slaughter had been given procaine penicillin in the neck muscles. Both the supernate and a suspension of the dry residue in 2 ml. buffer were plated. From the physiological saline and phosphate buffer extracts only the supernatant was plated because of the long time required for evaporation and because of microbial growth that at time might occur in these extracts during evaporation and incubation. As shown in Table 3, the penicillin content of the samples was so low that only very narrow zones or no zones at all could be demonstrated in the unconcentrated extracts. After the extracts were concentrated and resuspended in 2 ml buffer, wide zones varying from 19.5 - 23.5 mm were produced by all extracts, thus showing that a significant increase in the sensitivity of the test was obtained by applying the concentration procedure.

Sensitivity of assay procedure. Table 4 presents the results of an experiment designed to determine the sensitivity of the assay procedure when procaine penicillin G was added to the tissue samples prior to homogenization and extraction with different extractants. Only extractants previously shown not to produce non-specific zones (Table I) were used in these trials. Controls to which penicillinase was added were all negative. The zone sizes given in mm. are the means of 5 determinations made with each extractant in 10 different pigs. In extracting muscle tissue, the best results were obtained with buffered acetonitrile which produced distinct zones around the cylinders with 0.0025 units and only a slight, not measureable inhibition at the 0.00125 u. level. In extracting livers and kidneys, acetonitrile and oxalate-EDTA-acetone gave similar results yielding narrow but still measurable zones down to the 0.0025u. cylinders. Because of its higher evaporation rate, acetonitrile was considered the preferable extractant for liver and kidney tissues. For muscle tissue, however, acetonitrile buffered with 10 ml. buffer per sample was preferred in spite of its somewhat lower sensitivity (Table 3) in order to avoid the interference of non-specific zones.

The sensitivity of the method could be improved further by using less than 2 ml. of buffer for resuspension of the dry residue. When 0.5 ml. was used for suspending muscle extract residues, non-specific zones occurred

occasionally. Consequently, the use of 2 ml. buffer was preferred as a routine because its buffering capacity was sufficient and because it permitted plating of more cylinders from each sample.

In these studies, only minor attention was given to factors of more special interest to quantitative determinations such as the use of a double layer technique to improve distinctness and readability of zones and the influence of suspended material in the cylinders upon diffusion rates and zone sizes. More emphasis was put upon the selection of extractants and an evaporation and resuspension method that could provide a fast and simple but still sensitive and reliable procedure for qualitative assays of low-level penicillin residues in tissues.

TABLE 1. Growth-inhibiting Effect of Tissue Extracts at Different Times After Slaughter.[x]

Extractants	Muscle Time after slaughter			Liver Time after slaughter			Kidney Time after slaughter		
	4 hrs	24 hrs	8 days	4 hrs	24 hrs	8 days	4 hrs	24 hrs	8 days
Acetone	8-13	11-17.5	12-14.5	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Acetone-dist. water	Neg.	Neg.	8-10	13-18	13-16	16-18.5	8-12	8-9.5	8-11
Buffered acetone I	Neg.	8-9.5	8-9.5	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Buffered acetone II	Neg.	Neg.	Neg.	8-10	8-9	Neg.	Neg.	Neg.	Neg.
Acetonitrile	8-14	10-15.5	10-15	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Buffered acetonitrile I	Neg.	8-9	8-9.5	8-10.5	8-10.5	Neg.	Neg.	Neg.	Neg.
Buffered acetonitrile II	Neg.	Neg.	Neg.	12.5-15	11-15	10-16.5	Neg.	Neg.	Neg.
Oxalate-EDTA-acetone	Neg.	8-12.5	8-11.5	8-9.5	Neg.	Neg.	Neg.	Neg.	Neg.
Phosphate buffer [xx]	Neg.	Neg.	Neg.	8-13	8-14	8-14	Neg.	Neg.	Neg.
Physiological saline [xx]	Neg.	Neg.	Neg.	8-12	8-10.5	8-12	Neg.	Neg.	Neg.

[x] Figures express in mm the variation in the diameter of the non-specific zones. Outer cylinder diameter 8 mm.

[xx] The phosphate buffer and saline extracts were plated directly from the supernatant. All the other extracts were concentrated and the resuspended residue plated.

TABLE 2. The Relationship Between Lactic Acid Formation, p H Changes in Muscles and the Occurrence of Non-specific Zones of Growth Inhibition at Various Times Following Slaughter.[x]

	0 hrs.			4 hrs.			24 hrs.		
	pH	Acid.lact. mg./Gm. of muscle	Zone diam. mm	pH	Acid.lact. mg./Gm. of muscle	Zone diam. mm	pH	Acid.lact. mg./Gm. of muscle	Zone diam. mm
Pig. no. 1									
M. long. dorsi	6.3	3.1	Neg.	5.8	5.1	10	5.5	7.7	14
M. rect. femoris	6.6	1.3	Neg.	6.1	4.2	Neg.	5.7	7.0	11
PIG. no. 2									
M. long. dorsi	6.3	3.6	Neg.	5.5	8.7	12	5.3	9.5	15
M. rect. femoris	6.5	2.7	Neg.	5.8	6.7	11	5.6	8.0	14

[x] Acetonitrile as extractant. Outer diameter of cylinder 8 mm.

TABLE 3. Effect of Concentration of Extracts Made With Different Extractants From the Same Penicillin Containing Muscle Tissue.[x]

Extractants	Unconc. supernat., mm	Conc. resuspended residue mm
Acetone-dist. water	Neg.	19.5
Buffered acetone I	9.5	22.0
Buffered acetone II	Sl.[xx]	21.0
Buffered acetonitrile I	9.5	23.5
Buffered acetonitrile II	9.0	23.0
Oxalate-EDTA-acetone	Sl.[xx]	23.5
Phosphate buffer	Neg.	....
Physiological saline	Neg.	....

[x] Outer diameter of cylinder 8 mm. [xx] Slight inhibition, but no distinct zone.

TABLE 4. Sensitivity of Concentration Method Using Different Extractants.[x]

Tissue and extractant	I.U. penicillin added per Cm. of tissue				
	0.05	0.01	0.005	0.0025	0.00125
MUSCLE					
Buffered acetone II	27.8	18.8	14.5	11.0	Neg.
Buffered acetonitrile II	28.9	19.8	16.1	12.2	Sl.[xx]
LIVER					
Acetone	26.0	15.7	11.9	Sl.[xx]	Neg.
Acetonitrile	29.8	18.9	14.4	10.0	Neg.
Oxalate-EDTA-acetone	29.3	18.4	14.2	10.1	Neg.
KIDNEY					
Acetone	25.5	14.5	9.5	Neg.	Neg.
Acetonitrile	29.9	19.8	15.5	10.6	Neg.
Oxalate-EDTA-acetone	30.0	19.7	15.1	10.2	Neg.

[x] Figures express in mm. the mean values for 5 determinations made with each extractant in each of 10 different pigs. Outer cylinder diameter 8 mm.

[xx] Slight growth-inhibition, but no measurable zones.

SUMMARY

Several solvents were compared for the extraction of penicillin from porcine muscle, kidney and liver tissues using a cylinder-plate monolayer technique with Sarcina lutea as the test organism. The extracts were concentrated to dryness by the use of a flow of air making it possible to extract, evaporate and plate the resuspended residues on the same day. The occurrence of non-specific zones of growth inhibition were frequently observed. These could be avoided by using acetonitrile as extractant in kidney and liver tissue and phosphate buffered acetonitrile in muscle tissue and by using phosphate buffer for resuspension of the evaporation residue. The methods used allowed the detection of 0.0025 I.U. penicillin per Gm. of tissue.

Z U S A M M E N F A S S U N G.

Mehrere Lösungsmittel wurden für die Extraktion von Penicillin aus der Muskulatur, Niere und Leber verglichen. Eine Diffusions-Methode mit Extrakt-gefüllten Stahlcylindern auf einschichtiger Agarplatte und Sarcina lutea als Teststamm wurde benutzt.

Die Extrakte wurden mit Hilfe eines Luftstromes zur Trockenheit konsentriert, wobei es möglich war, die Extraktion, Verdampfung und Applizierung der desuspendierten Residuen auf die Platte an demselben Tag durchzuführen.

Das Vorkommen von nicht-spesifischen Hemmungszonen wurde haüfig registriert. Diese Zonen kamen nicht vor wenn Acetonitril als Extraktant für Nieren - und Lebergewebe und phosphatgepufferter Acetonitril für Muskulatur benutzt wurde und wenn derselbe Puffer für das Desuspendieren der trockenen Residuen gebraucht wurde. Die angewandten Methoden gestatten Mengen von 0.0025 I.E. Penicillin pro gram Gewebe zu messen.

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