A COLORIMETRIC METHOD FOR THE DETERMINATION OF SMALL AMOUNTS OF PROCAINE PRESENT AS PROCAINE BENZYLPENICILLIN B.P. IN VETERINARY FOOD PRODUCTS

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The inclusion of trace quantities of antibiotics in animal foods as growth stimulants is already an established practice in the United States and it seems likely that British legislation will shortly be amended to permit the general sale in this country of foodstuffs containing penicillin. The development of such products will obviously necessitate suitable analytical control to check the stability of the penicillin present and the uniformity with which it has been distributed in the food. For the former, a preliminary extraction followed by microbiological assay will probably be necessary but for the latter a simpler alternative can be offered, if, as at present seems likely, the penicillin is added in the form of procaine salt. This method is based on the determination of procaine and the object of the work described here was to devise a process capable of determining concentrations of procaine penicillin of the order of 3·0 per cent. to 2·5 p.p.m. in various foodstuffs, premixes and concentrates. It is generally accepted that animal food products can be classified into three types depending on the procaine penicillin content and method of use:— (i) Premixes are usually compounded by the penicillin manufacturer and supplied to the foodstuff manufacturer for further dilution with meal to produce concentrates or final foods. (ii) Concentrates are produced as above, by the foodstuff manufacturer and supplied to the user for further dilution with meal. (iii) Final foods are supplied by the foodstuff manufacturer for direct administration by the user without further dilution.

The usual concentrations of procaine penicillin encountered in premixes are of the order of 3·0 to 0·1 per cent., in concentrates 30 to 20 p.p.m. and in foodstuffs 10 to 2·5 p.p.m. These products differ considerably in their composition, but for the purpose of this investigation they can be classified into two main groups:—*Group* 1: Products formulated with a single organic or inorganic substance, e.g. kaolin, lactose or chalk; this group is confined to premixes. *Group* 2: Those formulated with a variety of cereal products containing, in some cases, small amounts of animal proteins, together with vitamins and mineral salts; certain premixes, all concentrates and final foodstuffs fall into this group.

Several methods for the determination of procaine have been described in the literature. The spectrophotometric[1] method for the determination of procaine penicillin has been applied in these laboratories to certain formulations of Group 1 premixes. Low recoveries were experienced with limestone and kaolin formulations due to adsorption, and the method

is not specific for procaine, giving occasional erratic results due to irrelevant absorption in the ultra-violet region derived from impurities present in the diluent and from penicillin decomposition products if present. When applied to formulations of Group 2 the method is rendered useless due to ultra-violet absorbing impurities extracted with the procaine. The nitrite titration method developed by Shaw[2] has been successfully applied to premixes in Group 1 formulated with chalk and containing procaine penicillin of the order of 2·0 per cent., the procaine penicillin being first extracted by means of chloroform. The method gave low results when applied to premixes formulated with kaolin, this being attributed to adsorption of procaine penicillin on kaolin. This method also requires a large amount of sample to obtain accurate nitrite titres and was therefore abandoned in favour of the colorimetric method subsequently found which can be applied to all products in Groups 1 or 2.

Experimental

The sample of procaine benzylpenicillin used throughout this investigation conformed to the requirements of the British Pharmacopœia.

(*a*) *Investigation of a Suitable Reaction for Colour Development.*

For the following series of experiments an aqueous solution of procaine penicillin containing 3 μg. per ml. was used. This was considered to be the approximate concentration likely to be encountered in the final solutions after extracting the procaine from the foods. Diazotisation and coupling to H acid[3] in alkaline solution was attempted; this resulted in a stable red colour but due to the unstable nature of the reagent in alkaline solution the method was abandoned in view of the more satisfactory coupling method subsequently found. It is well known that *p*-dimethylaminobenzaldehyde forms yellow colours when coupled in acid solution to primary and secondary amines. Due to its simplicity this method appeared at first promising but subsequent work proved that the reaction was not stoichiometric and the intensity of the colour was sensitive to slight variations in *p*H of the solution. *N*-β-Sulphatoethyl-*m*-toluidine has been used[4] for the determination of a variety of drugs in biological fluids. The reagent, however, was found to be not sufficiently sensitive for the low concentrations of procaine penicillin encountered in this work. Bratton and Marshall[5] recommend the use of *N*-(1-naphthyl)ethylenediamine as a coupling reagent for certain diazotised amino groups in acid solution and this reagent, when added to diazotised procaine penicillin solution freed from excess of nitrous acid by means of sulphamic acid, yielded a bluish-red colour, the optical density of which was found to be suitable for the colorimetric determination of quantities of procaine penicillin up to 5 μg./ml. in the final solutions. It was found that diazotisation at room temperature produced colours slightly paler than those obtained by diazotising solutions of the same procaine penicillin content between 5° and 10° C. It was therefore decided to diazotise at the lower temperature. Furthermore, it was found that the intensity of the colour reached a maximum after 20 minutes and that no appreciable fading

took place on standing for some hours in subdued light. It was also found that colours of the same intensity were obtained by diazotising and coupling solutions of procaine penicillin and of procaine hydrochloride of equivalent procaine content, from which it appears that penicillin does not take part in the reaction. It has also been established that penicillin decomposition products produced by procaine penicillin do not interfere with the procaine determination when carried out by the above method. A typical calibration graph for the Spekker photoelectric absorptiometer is shown in Figure 1. This was obtained by taking aliquots of a standard solution of procaine penicillin in chloroform, making each up to 200 ml. with chloroform and shaking each of these solutions with dilute sodium hydroxide solution as detailed in the general method.

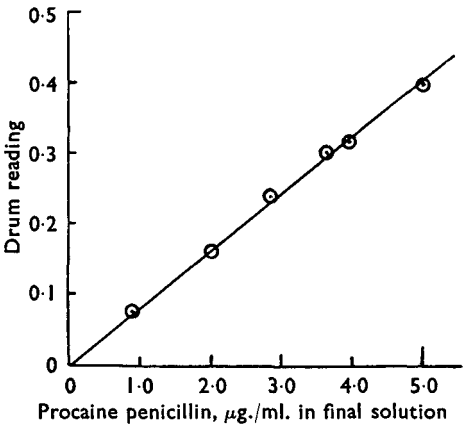

FIG. 1. Calibration curve of procaine benzylpenicillin.

(*b*) *Extraction of Procaine.*

Application of the above colorimetric method to Group 1 products necessitates extraction of procaine from inert diluent. This has been achieved by shaking with chloroform an aqueous suspension of the sample rendered alkaline by sodium hydroxide. An aliquot of the chloroform is shaken with dilute hydrochloric acid and an aliquot of the acid solution diazotised and coupled. The more obvious approach of extracting with a water-miscible solvent (e.g., methanol) followed by diazotisation and coupling in acid solution gave slightly low results when applied to those premixes formulated with chalk as diluent and very low results when applied to the kaolin formulation. The quantitative isolation of procaine from Group 2 products proved more difficult, the problem being analogous to the determination of alkaloids in powdered vegetable drugs. Due to the insolubility of procaine base and of procaine penicillin in water it is essential to extract with an organic solvent. This also extracts other ingredients from the cereals and yields dark solutions which have to be further purified to produce a virtually colourless solution containing the procaine in a form suitable for diazotising and coupling. Chloroform has been found to be the most suitable solvent for extraction of the procaine base and the formation of emulsions has been minimised by using a single extraction consisting of a relatively large volume of solvent. The most successful procedure so far discovered is quoted below. Typical results obtained by the general method on products in Groups 1 and 2 are given

TABLE I

RESULTS AND DETAILS OF METHODS FOR PRODUCTS IN GROUPS 1 AND 2

Formulation				Details of Method			
Class	Group	Procaine penicillin added	Procaine penicillin found	Weight of sample taken g.	Volume of chloroform and aliquot taken.	Volume of 0·1N hydrochloric acid ml.	Cell size cm.
Premix (Limestone)	I	2·43 per cent. 2·20 " 2·20 "	2·43 per cent. 2·25 " 2·25 "	0·1	200 ml., 10 ml. aliquot	25	1·0
Premix (Kaolin)	I	2·33 "	2·41 "	0·1	200 ml., 10 ml. aliquot	25	1·0
Premix (Limestone)	I	0·27 " 0·36 " 0·38 " 0·31 "	0·28 " 0·38 " 0·38 " 0·33 "	0·2	50 ml., 10 ml. aliquot	25	1·0
Premix (Kaolin)	I	0·32 "	0·31 "	0·2	50 ml., 10 ml. aliquot	25	1·0
Premix	II	0·131 " 0·083 " 0·091 " 0·091 "	0·140 " 0·082 " 0·090 " 0·091 "	2·5	200 ml., 10 ml. aliquot	25	1·0
Concentrate	II	33·0 p.p.m. 33·4 " 33·4 " 33·4 "	34·2 p.p.m. 33·6 " 33·8 " 34·0 "	4·0	200 ml., 100 ml. aliquot	10	1·0
Concentrate	II	26·9 " 31·2 " 25·0 " 25·0 "	24·9 " 29·8 " 24·9 " 24·5 "	4·0	200 ml., 100 ml.	10	1·0
Foodstuff	II	13·9 " 11·2 " 10·0 "	12·7 " 10·1 " 9·7 "	10·0	200 ml., 100 ml. aliquot	10	1·0
Foodstuff	II	5·0 " 5·0 " 5·0 "	5·5 " 5·0 " 4·5 "	10·0	200 ml., 100 ml. aliquot	10	1·0
Foodstuff	II	2·8 " 2·5 " 2·8 " 2·4 "	2·5 " 2·7 " 2·0 " 1·9 "	10·0	200 ml., 100 ml. aliquot	10	2·0

in the Table together with the weight of sample and appropriate aliquots to be used in the general method.

(*c*) *General Method.*

Special Reagents. (i) Sodium nitrite—a freshly prepared 0·1 per cent. w/v solution.

(ii) Ammonium sulphamate—a 0·5 per cent. w/v solution which must be used within 14 days of preparation.

(iii) *N*-(1-naphthyl)-ethylenediamine dihydrochloride—a 0·1 per cent. w/v solution which must be stored in the dark, preferably in a refrigerator and discarded when it becomes yellow.

Method. Place in a separating funnel an accurately weighed amount of sample, depending on the expected procaine penicillin content, add 35 ml. of water, 15 ml. of 0·1N sodium hydroxide, and, by means of a burette or pipette, the stated amount of chloroform. Shake the contents of the separating funnel vigorously for about 3 minutes and allow to separate

(see Note 1). Run off the lower layer through a No. 1 Whatman filter paper and transfer, by means of a pipette, an aliquot part of the filtrate to a second separating funnel. Add to the contents of the second separating funnel the appropriate exact amount of 0·1N hydrochloric acid, shake vigorously for about 3 minutes and allow the layers to separate. Transfer the aqueous layer to a centrifuge tube and centrifuge for 10 minutes at 2000 r.p.m. Transfer 5 ml. of the upper layer to a 10 ml. graduated stoppered cylinder and cool to 5° to 10° C. Maintain the solution between 5° and 10° C. during the following operations until the coupling reagent has been added. Add 1·0 ml. of sodium nitrite solution and mix thoroughly. After 3 minutes add 1·0 ml. of ammonium sulphamate solution, mix thoroughly and allow to stand 2 minutes. Add 1·0 ml. of *N*-(1-naphthyl)-ethylenediamine dihydrochloride solution and dilute to 10 ml. with water; mix thoroughly and allow to stand for 20 minutes. Repeat the above procedure to obtain a blank solution using the same weight of diluent as the weight of sample taken (Note 2). Measure the optical density of the test solution using a cell of specified thickness (see Table) by comparison with the blank solution using a Spekker photoelectric absorptiometer and Ilford 605 filters. By the same method, and with suitable dilutions of a standard solution of procaine penicillin in chloroform covering the required range, prepare a calibration graph. Read from the graph the amount of procaine penicillin contained in the sample.

Note 1. Group 2 products have a tendency to form stable emulsions which will not separate on standing. In such cases it has been found that the emulsion can be broken by centrifuging at 2000 r.p.m. until separation is affected (this does not normally exceed half an hour).

Note 2. In the case of Group 1 products it is not necessary to carry out a blank on the diluent—the blank solution being prepared by the addition of 1 ml. of sodium nitrite solution to 5 ml. of 0·1N hydrochloric acid cooled to 5° to 10° C. and contained in a 10 ml. stoppered cylinder, and adding the ammonium sulphamate solution and other reagents as detailed in the method. For Group 2 products it has been found necessary to prepare a blank solution using the diluent material without added procaine penicillin as some colouring matter is occasionally extracted with the procaine.

Summary

A colorimetric method for the determination of procaine penicillin based on extracting, diazotising and coupling procaine base has been described for animal food products containing between 3·0 per cent. and 2·5 p.p.m. of procaine penicillin.

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