STUDIES ON THE QUALITY OF LIVER EXTRACT PREPARATIONS

by

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ABSTRACT

Results of analyses of different brands of Liver Extract have been presented. It has been recommended that in regard to the 'B_{12}' content the higher limit should be fixed at 150 percent of claim. 'Total solids' and 'Protein Nitrogen' have not been found to bear any relationship either between themselves or with the contents of 'B_{12}' vitamin and 'histamine like substances' respectively. It has therefore been recommended that these standards should be further investigated. A content of 'histamine-like substances' higher than 50 mcg/ml has been shown to be related with toxicity of the preparation. It has been recommended that standards in relation to the 'toxicity test' and 'limit of histamine like substances' should be finally arrived at after further collaborative work. A dose of 1·0 ml/Kg rabbit weight has been recommended for the Pyrogen Test; a pH range of 5 to 7 has been confirmed.

Introduction

Crude liver injection occurs as a monograph in the Indian Pharmacopoeia (1955 Ed.), wherein it is specified that the preparation should contain 5 mcg of vitamin B_{12} per ml. The United States Pharmacopoeia (15th revision) has included two different preparations, *viz.*, liver injection (10 to 20 mcg/ml) and 'Liver injection crude' (1 to 2 mcg/ml). The pharmacopoeial tests include estimation of potency in terms of vitamin B_{12} activity and general tests for injections. These standards are considered inadequate, because (*a*) the quality of these preparations available in the market has been found to vary considerably in physical, chemical and biological properties, and (*b*) they are reported sometimes to produce allergic reactions. Chowdhary et al[1] have prescribed limits for undue toxicity, pyrogenicity, histamine like substances and pH in respect of crude liver extract (injection). These limits (with the exception of histamine like substances) have been accepted by the Drugs Technical Advisory Board, Ministry of Health, who have recommended additional standards[2] in respect of 'total solids' and 'limit for protein' in the preparation.

While studying these standards, it was felt that (*i*) the requirement in respect of 'limit of histamine-like substances' should have been incorporated in the recommendations of DTAB, and (*ii*) for the sake of uniformity the standards should have been arrived at by more comprehensive and collaborative work in several laboratories on a large number of preparations. Consequently we examined a number of different brands of liver injections and have observed that there is lack of standardisation in the manufacture of this preparation.

Experimental

Hydrogen ion concentration (pH)—This was determined potentiometrically by means of the glass electrode and a suitable pH meter. Colourimetric method could not be employed due to the colour of the preparation.

Total solids—An accurately measured volume of the preparation was evaporated in a tarred glass basin on a water bath, dried at 105°C for an hour and then at 60°C in vacuum for two hours. The solid material was accurately weighed and expressed as percentage (w/v) of the preparation.

Total nitrogen and proteins—The total nitrogen content was estimated by the well known Micro-Kjeldhal's method. The proteins were precipitated by adding an equal volume of 20 percent trichloracetic acid; the precipitate was separated by centrifugation and the non-protein nitrogen estimated in the filtrate. The difference between the total nitrogen content and the non-protein nitrogen gave the protein nitrogen content. Calculations were made on percentage basis.

Freedom from undue toxicity—The test was performed on a batch of 10 healthy albino mice (male) weighing between 23 to 25 g; the selection of mice in a particular group was made by ensuring that the fasting weights of the lightest and the heaviest mouse do not differ by more than 3 g. A dose of 0·4 ml/20 g. body weight injected intraperitonally. The mice were observed for a period of seven days; the sample was deemed to have passed the test if there was no mortality during this period.

Limit of Pyrogens—This test was performed on normal healthy non-pregnant rabbits weighing not less than 1·5 Kg. and whose rectal temperature did not exceed 39·8°C. Intravenous injection of the preparation in dosage of 1·0 ml/Kg. body weight of the animal was given. Temperatures were recorded at regular intervals of not more than 30 minutes, beginning at least 90 minutes before injection and continuing for 3 hours after injection. The mean initial temperature of each rabbit was the mean of the temperature readings recorded for that rabbit at the 60th and 90th minutes; the first two temperatures at zero and 30th minutes indicate whether the animal has kept up the normal range of temperature or has shown significant variation. The maximum temperature of each rabbit was the highest temperature recorded for that rabbit in the three hours after injection (the maximum temperature is invariably attained by the animal within this period). The difference between the mean initial temperature and the maximum temperature recorded by a rabbit was taken to be its response. The temperature was recorded with the help of a rectal thermometer (standardised against NPL standards) and while doing so the animals were gently held on the lap. Other procedural details regarding selection of animals, housing them during and between the test, food and water restrictions and the interpretation of the data were in accordance with the stipulation specified in the British Pharmacopoeia (1958).

Content of Histamine-like substances—This was determined by estimating the depressor effect on a suitable dog preparation (anaestheised with Phenobarbitone, 140 mg/Kg body weight), comparisons being made with known concentrations of histamine acid phosphate.

Estimation of Vitamin B_{12} activity—Vitamin 'B_{12}' activity of the samples was estimated by the microbiological method of assay. The cup-plate technique was used employing Lactobacillus Leichmannii No. ATCC 7830. Zones of exhibition on agar plates were measured for estimating the potency. The B. P. (1958) procedure of (2+2) dose assay was used employing a dose ratio of 1 : 2. The validity of the test procedure for each assay was tested by the procedure outlined in B.P. under "Assays depending upon measured effects". The fiducial limits of error (P=0·95) in the estimations, when four replicates were put up for each dose of the standard and test material were found to be between 85 and 120 percent of the estimated potency. Known concentrations of cyanocobalmine BP quality, were used as standard; the dilutions employed in the assays being 1·0 and 2·0 mcg/ml. The composition of the assay medium, the procedure for maintaining and preparing the organism were the same as described in standard literature[3] for the assay of vitamin B_{12} with the difference that Folic Acid and Riboflavine were omitted from the composition. Other details of the test procedure were essentially the same as followed by Cuthbertson et al[4] in the microbiological assay of vitamin B_{12} with Lactobacillus lactis Dorner by the Plate method. When the estimated potency was abnormally higher than the manufacturers' claim as in samples 2, 5 and 8 pertaining to Table 2, the initial results were taken to serve for guidance only and further estimations were made by suitably diluting the preparations so that the doses of the test and standard preparations during the assay were approximately the same.

Results and Discussions

Results of analyses of different brands marketted by the various manufacturers in the country are given in Tables 1 and 2. Table 1 contains laboratory results in respect of all the tests given above, while Table 2 gives 'B_{12} content', 'toxicity results' and content of 'histamine like substances'. The reason for giving results in respect of these three tests only in Table 2 is that in this series of experiments it was intended to study the relationship between these only in order to have some idea of the extent of standardisation at the manufacturing stage.

pH.—According to the requirements set forth by the Health Ministry, the pH of liver extract preparations should be between 5 and 7. From Table 1, it will be seen that all the fifteen different brands investigated by us have a pH value falling within this range. Thus our findings are in conformity with the recommendations of DTAB in this respect.

'Total solids' content—The standard laid down by the Ministry of Health in regard to this requirement is that it shall not be less than 15 per cent w/v in case of preparations containing 2 mcg of vitamin B_{12}/ml and not less than 7·5 percent w/v for preparations containing 1 mcg of B_{12}/ml respectively. From Table 1, it will be seen that the 'total solids' content of the brands analysed by us, varies from 5·84 to 20·65 percent, the claims of firms in respect of 'B_{12}' in their products being 2, 3·5, 5·0, 10·0 and 15·0 mcg per ml. In the eight samples claimed to contain 2 mcg/ml of vitamin B_{12}, only three brands (1, 6 & 7) satisfy the requirement of Ministry of Health with regard to 'Total solids' content; out of these brand No. 7 falls short of claimed 'B_{12}' potency; out of the five brands (2, 3, 4, 5 and 8) which do not conform to the

requirement in respect of 'total solids content', only two, *viz.*, brands 3 and 5 fall short of the claimed vitamin 'B_{12}' potency, while the remaining brands conform to the claim in respect of 'B_{12}' content. Samples with higher claims for vitamin 'B_{12}' (3 to 15 mcg/ml) do not show a relationship between the 'total solids' and 'B_{12}' contents. The estimation of 'total solids content' in each sample was repeated to confirm these variations. This may be due to the different manufacturing and processing techniques employed by the manufacturers in the country.

Total nitrogen and protein nitrogen—From table 1, it will be seen that there is no evidence of relationship between either 'total nitrogen' and 'histamine like substances' or 'non-protein nitrogen' and 'histamine like substance' in the samples examined by us. It will therefore, be worthwhile having further collaborative investigations in respect of this standard also.

Vitamin 'B_{12}' content—From table 1, it will be seen that majority of the samples (9 out of 15) were found to contain 'B_{12}' potency vayring between 100 and 120 per cent of claim. Table 2, which contains results on 8 brands, different from those pertaining to Table 1 reveals wide variations in respect of the vitamin 'B_{12}' content, sample No. 8 showing a content as high as 850 per cent of the claimed value. It will also be seen from Table 2 that samples with abnormally high 'B_{12}' contents in comparison to the claim, do not pass the test for undue toxicity and have been found mostly to show high contents of 'histamine like substances'. These abnormal findings are due to the fact that in these brands there is lack of standardisation at the manufacturing stage. However, so far as the tolerance for vitamin 'B_{12}' content in the liver injection is concerned, it would be appropriate to follow USP. Consequently no lower limit should be specified, while the maximum permissible limit on the higher side should be kept at 150 per cent of claim.

Histamine like substances and freedom from undue toxicity—From Tables 1 and 2, it will be seen that a majority of the brands have been found to contain 'histamine-like substances' below 50 mcg/ml in terms of histamine acid phosphate, B.P. Chowdhary et al[1] have specified a limit for 'histamine like substances', which works out to be 110·6 mcg. of histamine acid phosphate per mil. We observed (as will be seen from Table 2) that if the histamine-like substances were present to the extent of more than 50 mcg of histamine acid phosphate per mil., the samples invariably failed to pass the test for 'absence of undue toxicity'. On comparing our dose with that employed by the above workers in respect of the toxicity test, it is seen that we employed a dose of 0·4 ml/20g. body weight of of albino mice, while Chowdhary et al have recommended a dose of 0·25 ml/20g. body weight; we observed the amimals for a period of seven days, while they have recommended observation for 72 hours. We did not consider it necessary to bring down the toxicity dose, as a large majority of samples which were satistactory in regard to the 'B_{12}' content and 'histamine-like substances' invariably proved to be satisfactory in respect of freedom from 'undue toxicity'. It is therefore, considered advisable to have more comprehensive and collaborative data in regard to the toxicity test and the limit of 'histamine-like substances', and if necessary, clinical investigations may also be undertaken to finally arrive at the latter standard.

Pyrogen Test—So far we have not come across a sample which failed in respect of the Pyrogen test, although the dose employed by us (1·0 ml/kg. body weight) is higher than that employed by Chowdhary et al (·75 cc/kg. body weight). It will, therefore, be worthwhile collecting data from different laboratories to arrive at a uniform dose for this test.

References

1. Chowdhary, B.N., M.D. Chakravarty and S. C. Bhattacharya,—*J. Indian, Med. Assoc*, 26, 92, 1956.

2. *Proceedings of the Drug Analysts Conference*, 1957.

3. Methods of Vitamin Assay, *Inter Science Publishers*, Inc., New York, 1951.

4. Cuthbertson, W.F.J., Pegler, H.F., and Lloyd, J.T., *Analyst, 76* 133, 1951.

TABLE 1

Results of estimation on different brands of liver injections

Brand No	Vitamin B_{12} content mcg/cc		Pyrogen test	Test for absence of undue toxicity	Histamine like substances (mcg/cc)	pH	Total solids (percent)	Total Nitrogen (percent)	Protein Nitrogen (per cent)
	Claimed	Estimated							
1	2·0	2·3	Passes	Passes	2·5	5·2	19·71	0·8399	0·0675
2	2·0	2·0	,,	,,	7·2	5·0	10·38	0·955	Negligible
3	2·0	1·5	,,	,,	7·2	5·0	10·91	0·9868	,,
4	2·0	2·0	,,	,,	5·8	5·0	10·84	0·9868	,,
5	2·0	1·5	,,	,,	5·8	5·0	0·05	0·8820	0·0710
6	2·0	2·2	,,	,,	47·0	6·0	20·65	1·97	0·083
7	2·0	1·58	,,	,,	Negligible	6·5	15·46	1·906	0·185
8	2·0	2·06	,,	,,	..	5·0	8·8	0·6955	·0011
9	3·5	1·45	,,	,,	12·5	6·0	10·0	1·007	0·0158
10	3·5	1·75	,,	,,	50·0	7·0	9·52	0·6554	0·0158
11	5·0	3·08	,,	,,	5·0	5·0	5·84	0·5786	Negligible
12	10·0	10·49	,,	,,	Negligible	5·2	19·74	0·3622	,,
13	10·0	10·16	,,	,,	6·6	5·2	18·72	0·3449	,,
14	15·0	18·2	,,	,,	4·7	5·2	9·0	0·6680	,,
15	15·0	18·1	,,	,,	4·7	5·4	11·22	0·7791	,,

NOTE—The above samples were found to be satisfactory with regard to tests pertaining to sterility, alkalinity of glass and volume of injection.

TABLE 2.

Relationship between B_{12} content, Histaminic substances and toxicity of certain liver injections

Brand No	Vitamin B_{12} content (mcg[1] cc)		Test for absence of undue toxicity	Histamine like substances (mcg/cc)
	Claimed	Estimated		
1	15·0	11·5	Passes	8·0
2	3·0	13·3	Does not pass	16·5
3	10·0	6·2	Passes	Negligible
4	5·0	5·9	,,	20·0
5	5·0	35·5	Does not pass	200·0
6	2·0	7·9	,,	100·0
7	1·0	3·95	,,	500·0
8	10·0	85	,,	100·0

NOTE—These samples are different from those detailed in Table 1.