A COMPARATIVE EXAMINATION OF SOME SAMPLES OF DIGITOXIN

By E. H. B. Sellwood*

From the Research Division, Allen and Hanburys Ltd., Ware, Herts.

Received June 17, 1957

Twelve samples of digitoxin have been examined. Little difference was found between them by physical constants or by colorimetric assays using sodium picrate or xanthydrol. Three samples were more potent biologically than the others but the difference is not a simple function of the gitoxin content which ranged from a few per cent to more than 20 per cent.

The British Pharmaceutical Codex, which describes Digitoxin as "the crystalline glycoside digitoxin associated with traces of more soluble glycosides and usually a small proportion of a sparingly soluble glycoside gitoxin", recognises the difficulty in preparing the pure glycoside. On the other hand the French Codex 1949, United States Pharmacopeia XV and International Pharmacopoeia, all describe single substances.

Further differences are shown in the standardisation for, while digitoxin B.P.C. is required to have a potency, biologically, of at least 900 units per gram and digitoxin U.S.P. to contain at least 90 per cent of $C_{41}H_{64}O_{13}$ determined by the Baljet reaction applied to a chromatographic eluate, digitoxin of the French Codex and of the International Pharmacopoeia is standardised by physical constants alone. These and other physico-chemical constants recorded in the literature show wide variations and Demoen and Janssen[1] suggested that the physico-chemical properties of pure digitoxin are still largely unknown; however with the rapid growth of chromatographic methods and improved chemical assays for digitoxin and gitoxin their comments do not describe the present position. Nevertheless a number of problems remain to be solved including the feasibility of eliminating the biological assay, and estimation of the gitoxin impurity.

In this communication the results of some physical measurements, and chemical and biological assays, of a number of samples of digitoxin are reported and their significance discussed.

Experimental Methods

Colorimetric assays with sodium picrate[2]. About 5 mg. of "digitoxin", accurately weighed, was dissolved in 100 ml. *iso*propanol and allowed to stand overnight to ensure complete solution. 5 ml. portions were then mixed with 5 ml. of sodium picrate reagent (1 per cent trinitrophenol 95 ml., 5 per cent sodium hydroxide, 5 ml., freshly mixed) and the maximum optical density measured using the EEL photoelectric colorimeter (Ilford filter 623, maximum transmission at 495 mμ), the instrument being balanced to zero with a blank consisting of 5 ml. *iso*propanol and 5 ml. reagent. Results were expressed as the extinction coefficient, E (1 per

* Present address: Bob Martin Ltd., Southport, Lancs.

cent, 1 cm.), or as percentage purity, taking the Canadian standard as 100 per cent.

Colorimetric assays with xanthydrol reagent. About 10 mg. of digitoxin, accurately weighed, was dissolved in 100 ml. of glacial acetic acid (A.R.) and 10 ml. of the solution diluted to 100 ml. with more acetic acid. 5 ml. portions of the diluted solution were placed in colorimeter tubes and 5 ml. of 0·25 per cent solution of xanthydrol in glacial acetic acid and 0·1 ml. of hydrochloric acid were added to each. A reagent blank containing no digitoxin was prepared in the same way. The contents of the tubes were stirred, the tubes corked and allowed to stand protected from light. Readings of optical density were taken at intervals from 2 hours onwards using Ilford filter 624 (maximum transmission at about 520 mμ) until the maximum density was reached. The results were calculated from the extinction coefficient E (1 per cent, 1 cm.) = 1520 obtained using digitoxose[2], on the basis of 3 molecules of digitoxose ($C_6H_{12}O_4$) ≡ 1 molecule of digitoxin ($C_{41}H_{64}O_{13}$).

Biological assays were carried out against standard digitalis, using guinea pigs, by the method of B.P. 1953 (page 830).

Melting points were measured by the B.P. capillary tube method.

Specific rotations were determined at 20° using the sodium D line on 1 per cent solutions in chloroform in a 2 decimetre tube.

Ultra-violet absorption. Determination were made using the Beckman Spectrophotometer Model DU fitted with photo-multiplier attachment. Absolute ethanol (A.R.) was refluxed with zinc dust and potassium hydroxide and redistilled rejecting the first and last 10 per cent. Sixteen to 18 mg. of the sample, accurately weighed, was dissolved in 50 ml. of the purified ethanol, 5 ml. of the solution was diluted to 50 ml. with more ethanol and the optical density measured at 217 mμ.

Gitoxin content. Paper chromatograms were run using the system carbon tetrachloride: ethanol: water and the glycosides eluted in xanthydrol reagent by the method described previously[2].

Results

Five commercial and six laboratory-prepared samples of digitoxin were examined. U.S.P. reference standard digitoxin was not available but a sample of the Canadian standard was tested. The results are summarised in Table I.

Discussion

The results of the colorimetric assays and determinations of gitoxin content and ultra-violet absorption are sufficiently close to justify considering all the samples as a single group. Only samples 6, 7 and 12 meet the requirements of the British Pharmaceutical Codex, although these differ but little from the others in their chemical and physical properties. Thus only half would pass the official test for melting point (at least 240°) although it is well known that lower values are frequently obtained. Similarly because of the small angular rotation of a 1 per cent solution of

TABLE I

ASSAYS AND PHYSICAL CONSTANTS FOR 12 SAMPLES OF DIGITOXIN

		Melting point (B.P.C. 240°)	$[\alpha]_D$ (B.P.C. about + 18°)	Xanthydrol method digitoxin (Calc. from assay of digitoxose) per cent	Picrate method calculated as		Biological assay		Gitoxin content per cent	Ultra-violet absorption Log ϵ
					E (1 per cent, 1 cm.)	Purity per cent (Sample 6=100)	Units/g.	Fiducial limits		
1	Commercial 1 ..	236°	+20°	99†	172	89‡	774	563–1064	14	4·13
2	" 2 ..	232°	+21°	97	172	89	655	440–976	23	4·15
3	" 3 ..	228°	+17°	97	185	96	788	—	7	4·19
4	" 4 ..	249°	—	100	177	92	760	—	12	—
5	" 5 ..	225°	+21·4°	98	190	98	1050	—	10	4·18
6	Canadian Standard	245°	—	98	193	100	1368	876–2136	11	4·17
7	Laboratory 1 ..	254°	+18°	95	193	100	1368*	876–2136	8	4·18
8	" 2 ..	245°	+20°	100	170	88	723	602–869	17	4·18
9	" 3 ..	255°	+15°	103	181	94	881	743–1045	20	4·18
10	" 4 ..	229°	+19·7°	92	192	99	894	641–1246	17	4·16
11	" 5 ..	230°	+18·9°	94	189	98	761	602–963	13	4·15
12	" 6 ..	251°	+17°	95	174	90	1401	865–2271	9	4·19

* Original assay 10 years ago, 1298 units/g.

† Standard deviation of 5 determinations ± 2·23.

‡ Standard deviation of 5 determinations ± 2·45.

digitoxin (approximately 0·4°), the specific rotation serves only as a confirmatory test of identity and is used in the B.P.C. as such.

Petit and colleagues[3] suggested that, although the low solubility of gitoxin does not permit the actual determination, its specific rotation in chloroform would be higher than that of digitoxin. However in the present work there is no indication that the values obtained are even a guide to the gitoxin content. Determinations of specific rotation may nevertheless be of value in detecting isomerisation as a sample of digitoxin which had become partially isomerised through accidental contact with alkali had a specific rotation of − 49·5° instead of the normal + 18°.

Colorimetric assays gave results of the expected order although the xanthydrol method showed three samples to be slightly low in digitoxose content. Values of E (1 per cent, 1 cm.) in the picrate method are 10 to 20 per cent lower than some recorded in the literature but this is attributed to the use of a filter type instrument which gives lower results than the prism type[4] and to the use of *iso*propanol in which optical densities are a few per cent lower than those obtained when using ethanol. The lowest extinction coefficients were about 10 per cent less than the highest but all the samples are probably normal in respect of the unsaturated lactone ring. This is confirmed by the ultra-violet absorption which in all samples showed a maximum at 217 mμ with log ϵ of the expected order. The partially isomerised digitoxin gave in the picrate method E (1 per cent, 1 cm.) = 61 and ultra-violet absorption, log $\epsilon = 3{\cdot}72$, thus showing a serious loss of the lactone function.

Whereas the colorimetric assay results varied from the mean by up to 6·5 per cent, the lowest biological assay was 31 per cent below the mean, and the highest 45 per cent above. Furthermore, there is no indication that the results of the biological assays reflect the equivalent of digitoxin as measured either by the picrate method or by the sugar content determined by the xanthydrol method, but neither the chemical nor the biological assays measure digitoxin alone.

Three samples by biological assay possess a distinctly higher potency than the others and, although these have a lower gitoxin content, the reduction in gitoxin does not account fully for the increase in potency. Apart from the lower gitoxin content these samples do not differ markedly physically or chemically from the others.

Assays of sample 7, initially and after storage for ten years, show that digitoxin in the solid state can be expected to maintain its potency indefinitely.

Chromatographic separation followed by colorimetric assay indicated that most of the samples contained a considerable proportion of gitoxin but, while with some a high gitoxin content was associated with a poor biological assay, this was not so with all. Samples 7, 8, 9, 10, and 12 were examined chromatographically by Tschesche who in a personal communication reported all to contain gitoxin while samples 8, 9, and 10 contained gitaloxin also.

From our chromatograms also gitoxin was found to be the main impurity. On some of our paper strips, however, between the spots of

digitoxin and gitoxin, there was a trace of a substance giving a blue fluorescence when dipped into phosphoric acid and its R_F value suggested it to be gitoxigenin. Tschesche's results indicate that this was not gitoxigenin itself, but the gitoxigenin glycoside gitaloxin (16-formyl gitoxin). This would, in contrast to gitoxigenin, react with xanthydrol and so might cause errors in quantitative determinations of the digitoxin and gitoxin zones but as it appears to be present only in traces such errors should be small.

While the potency of digitoxin containing an unknown proportion of gitoxin may readily be determined by biological methods, it is very desirable from the analytical viewpoint that a pure substance should be employed. The problem is essentially one of reducing the gitoxin content to an acceptable level. As gitoxin produces in the colorimetric assays optical densities comparable to those given by digitoxin and has similar ultra-violet absorption and optical rotation, a specific quantitative test for gitoxin is required. Fluorimetric[5–7] and spectroscopic methods[4] have been proposed which will detect the presence of less than 1 per cent of gitoxin in digitoxin. It is desirable, therefore, that a more sensitive test than the Keller-Kiliani should be introduced in the Official Standards in order to limit the gitoxin content, as digitoxin of commerce must contain at least 10 per cent before any appreciable red tinge is produced in this test.

The physico-chemical constants for the samples examined are, in general, consistent and the colorimetric assays of most are concordant both for a method based on the sugar side chain and a second on the lactone ring. Provided therefore that a suitable limit test for gitoxin could be applied, or the digitoxin freed from gitoxin chromatographically before a colorimetric assay is performed (as in U.S.P. XV), there seems to be no reason why digitoxin should not be standardised by physico-chemical methods alone, so rendering unnecessary the costly and time consuming biological methods.

Acknowledgements. The author thanks Dr. H. O. J. Collier for the biological assays and for his comments on the results and also Professor R. Tschesche of Hamburg for chromatographic examinations and Mr. R. V. Swann, Chemist in charge of the Physical Laboratory, for ultra-violet measurements.

REFERENCES

1. Demoen and Janssen, *J. Amer. pharm. Ass., Sci. Ed.*, 1953, **42**, 635.
2. Sellwood, *J. Pharm. Pharmacol.*, 1956, **8**, 1061.
3. Petit, Pesez, Bellet and Amiard, *Bull. Soc. chim. Fr.*, 1950, **17**, 288.
4. Küssner, Reiff and Voigtländer, *Arch. Pharm.*, 1955, 288, 284.
5. Pesez, *Ann. pharm. franç.*, 1950, **8**, 746.
6. Jensen, *Acta. pharm. tox. Kbh.*, 1952, **8**, 101.
7. Fruytier and Van Pinxteren, *Pharm. Weekbl.*, 1954, **89**, 99.

DISCUSSION

The paper was presented by the AUTHOR.

In a written communication DR. J. M. ROWSON said the results in the paper did not suggest that the application of a limit test for gitoxin would permit the evaluation of digitoxin by chemical and physical methods alone. The penultimate paragraph should be modified to read "freed from gitoxin and other glycosides or aglycones chromatographically". Why were samples 6, 7 and 12 of higher potency if gitoxin content is not related to potency, were these three samples free from gitaloxin, and what was believed to be the biological potency of pure digitoxin? For several digitoxin samples he had found "spots" on paper chromatograms in the region of gitaloxin and hoped to publish these results soon since it seems possible that this impurity (of high potency) may account for the occasional digitoxin of high potency.

DR. G. E. FOSTER (Dartford). Had the author any figures for digitoxin when the Keller-Kiliani and 3:5-dinitrobenzoic acid methods were used. In the B.P.C. a fundamental rule of biological assay was being broken in that digitoxin was compared with digitalis which was not a comparison of like with like and that might be the cause of some of the errors in the assay. If digitoxin were made too pure it was less soluble and as soon as injections were made up, it crystallised out.

MR. J. C. HANBURY (Ware). The activity of digitoxin appeared to depend on the botanical origin, and the very pure samples were usually either of French or Danubian origin, but no one knew what the glycosides were which contributed to the very high biological activities. Pharmacologists stated that they had examined samples which physically and chemically were nearly pure digitoxin, with biological activity around 1800 or 2000 units.

MR. W. SMITH (Ware). What was the potency the Canadians claimed for their standard, and how was this standardised?

MR. S. G. E. STEVENS (London) suggested that infra-red spectrophotometry might help in this investigation.

In reply MR. SELLWOOD said that gitoxin was the main impurity in digitoxin, others were present only in small proportions. He was unable to explain why the three samples 6, 7 and 12 were so different on the biological assay as it was not related to the gitoxin content. If Dr. Rowson had found a trace of another active substance it might help to solve the problem. He had used the 3:5-dinitrobenzoic acid and Keller-Kiliani methods, but had relied on the picrate method as being the most sensitive method based on aglycones. Xanthydrol was very satisfactory and sensitive and there was adequate coverage of aglycone and sugar content by the two colorimetric methods. English leaf was capable of yielding a very good sample of digitoxin and he had found a high proportion in the leaf grown by Dr. Rowson. There seemed to be traces of impurities which affected biological potency to a considerable extent, and they must be

extremely potent substances. It was difficult to purify digitoxin beyond a certain limit without great loss of material, and that was why it was necessary to accept a product which would contain only 90 per cent digitoxin as suggested by U.S.P. He had no further information about the biological potency of the Canadian standard. It was taken as a standard, but not as 100 per cent digitoxin, in Table I, because in common with sample 7 it gave the highest picrate assay, and also had the greatest biological potency. The only difference between digitoxin and gitoxin was a single hydroxyl grouping, which altered chemical properties and biological activity, though there was confusion about the biological potency of gitoxin.