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APPLICATIONS OF CHROMATOGRAPHY IN THE STANDARDIZATION AND CONTROL OF BIOLOGICAL PRODUCTS

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SUMMARY

The problems posed in the standardization of medicinal biological products and in the development of official specifications for such products, *e.g.*, in pharmacopoeias, are outlined. Information derived from bioassay can be extended and complemented by that from physico-chemical studies. The role of chromatography, including gas–liquid, thin-layer and column methods, is assessed and illustrated with examples from the literature and from the author's studies on antibiotics and polypeptide hormones.

INTRODUCTION

Biological products are substances and preparations used in medicine that cannot be defined completely by chemical and physical methods, and include vaccines, blood fractions, polypeptide hormones (both natural and synthetic) and their synthetic analogues, and antibiotics. Their potency or activity is determined by a biological assay. A distinctive feature of results obtained from bioassay is that they are subject to random error arising from the inherent variability of biological responses. It is essential, therefore, to assay the test material against a standard preparation so that the relative responses can be measured at the same time and under the same circumstances. This compensates for some of the variation inherent in the assay (use of different strains of animals or diets for them, lack of an absolute measurement, inability to define precisely the assay system) and, without adherence to it, results from different laboratories usually cannot be compared.

Many biological assay procedures are capable of highly sensitive and selective measurement of the characteristic activity of the biological product. However, they are usually complex, expensive, lengthy and of relatively poor reproducibility and there is a strong incentive to replace them with chemical or physical methods which are simpler, cheaper, faster and more precise. This has been achieved with many steroids, vitamins and antibiotics and such substances no longer feature in the list of biological substances made available by the World Health Organization[1]. The structural determination or synthesis of a biological product is in itself insufficient to guarantee that complete characterization is possible in the medical context solely by non-biological means. Nevertheless, developments over the past few years in chro-

matographic techniques have increased the prospects that biological assay methods will become less important and finally be replaced by other methods in some instances. This is due to the improvements in resolution, for example in high-performance liquid chromatography (HPLC), in sensitivity and selectivity of detection and in quantitation. This paper outlines the problems involved in the examination of biological products and considers the contribution that has, and will be, made by chromatography in this field.

INTERNATIONAL STANDARDS

The chemical and biological reference substances made available by the World Health Organization are intended for use as the primary standards against which national or local standards can be calibrated. Once sufficient need is demonstrated for the establishment of a biological standard, the main requirements are that the material selected should be representative of that in widespread use, that it should be stable and that its suitability should be confirmed through an international collaborative biological assay. Note that such a standard does not necessarily have to be of the highest attainable purity but its composition should be known if possible.

Antibiotics, as obtained by fermentation, are often mixtures of substances with closely related structures. Such mixtures may not be resolved during isolation and purification and the components may possess differing microbiological potencies. Before selecting material for the International Standard of an antibiotic, which is widely available in large amounts, it may be necessary to obtain and compare samples of it from throughout the world. This examination may be the first such carried out and can reveal the consistency or otherwise of material in therapeutic use. Thus, a preliminary step in setting up a new International Standard for erythromycin was the examination of samples obtained from 17 countries. As the microbiological activity of erythromycin C is much less than that of erythromycin B, which is itself 15–20% less active than erythromycin A, and bioassay cannot separate them, a rapid and sensitive separation procedure was required. Erythromycin is not amenable to quantitative analysis by gas–liquid chromatography (GLC) because of the number of reactive groups and its lability. In our hands, the best separation by thin-layer chromatography (TLC) of the erythromycin components was obtained using the system of Banaszek *et al.*[2]. Fig. 1 shows eight of the samples applied at high loads (100 μg) to reveal the minor components. One sample (127/75) was unusual, containing erythromycin C as the second component and very little erythromycin B, the remainder contained erythromycin B as the second component (up to 10%) and less than 5% of erythromycin C. The system also separates erythromycin from its estolate and from some other macrolide antibiotics (Fig. 2). As the amounts of the minor components fall within narrow limits, it was concluded that their presence was unlikely to affect significantly the parallelism of response between standard and test in the microbiological assay. In the light of our results, it has been suggested that tests to control composition should be added to the monograph for erythromycin in the European Pharmacopoeia (Ph.Eur.). Examination of erythromycin by HPLC is complicated by the absence of a suitable chromophore. The optimal wavelength for detection is about 207 nm but, because of the low extinction values, the procedure is not very sensitive to the low levels of minor components.

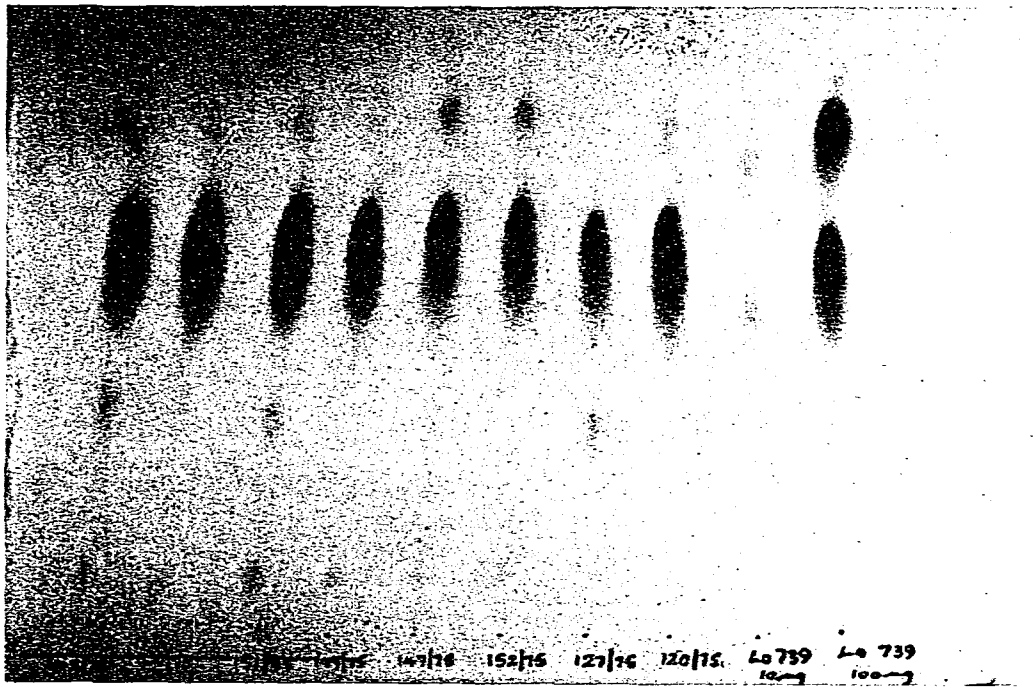


Fig. 1. TLC of samples of erythromycin on Kieselgel 60–Kieselgur F254 (Merck) plates impregnated with 15% formamide in acetone, dried at room temperature, spots applied then run in dichloromethane–*n*-hexane–ethanol (65:30:5, v/v/v). Detection: 50% sulphuric acid. Approx. R_F values: erythromycin A, 0.6; erythromycin B, 0.8; erythromycin, C, 0.45. The slowest component visible in some samples is microbiologically inactive.

Another antibiotic standard in the course of replacement is that for nystatin, one of the polyene group of macrocyclic lactones. Samples from eight countries were screened initially by TLC using *n*-butanol–pyridine–water (4:1:1) on silica gel and detection under UV light, which revealed some differences in the minor components. As nystatin is a tetraene with excellent UV properties (maxima at 291, 305 and 319 nm), the constraints on solvent composition for HPLC analysis apparent with erythromycin do not apply. We therefore examined the nystatin samples by reversed-phase HPLC as part of a wider study of the polyene antibiotics. Fig. 3 shows the analysis of material obtained from Australia, with the pattern of a main component with a number of minor components. We preferred a weakly basic two-component solvent (acetonitrile–0.01 *M* aqueous ammonium hydrogen carbonate, 27:73) with monitoring at 320 nm to the more complex system described previously[3]. The resolution improved at higher temperature. The pattern and relative proportions of the impurities varied from sample to sample but did not exceed 12%. The total amount of polyene, however, measured by UV absorption at 305 nm varied by one third between the samples. One impurity in nystatin is a heptaene which can be monitored selectively in the column effluent at 380 nm.

Different problems are posed by the polypeptide hormones and their analogues. The amounts available either from natural sources or by synthesis are usually small

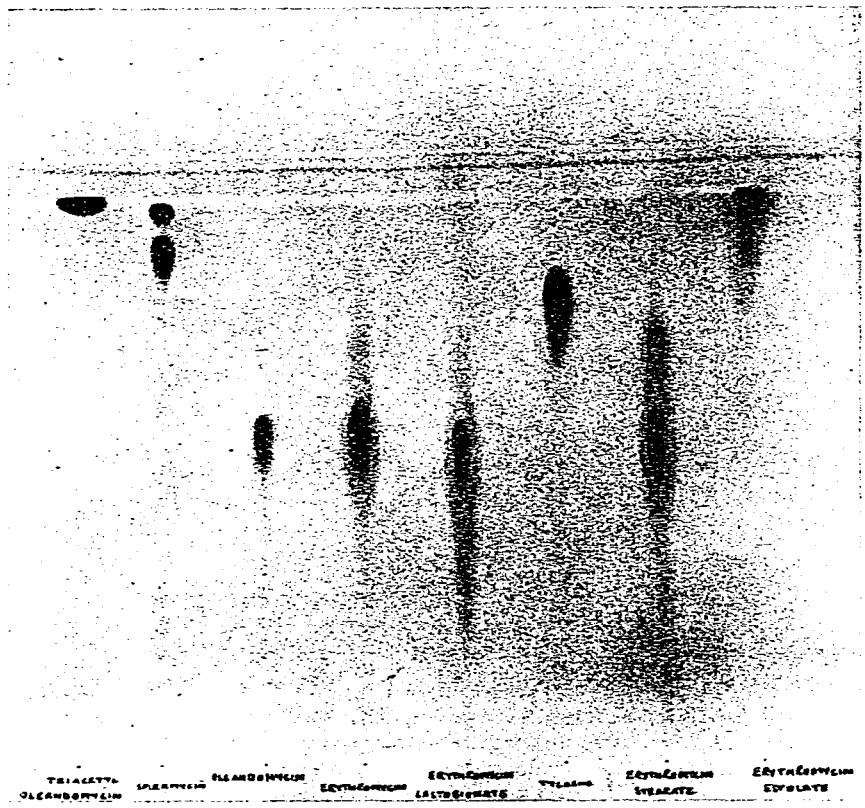


Fig. 2. TLC of erythromycin and related macrolides. Conditions as in Fig. 1.

and, because the substances are active at low levels, standards are prepared with an excess of inert carrier present. Often material is available from only a single source and comparative examination is unnecessary. The composition of synthetic substances must be studied because the by-products from the synthesis may themselves activate or inhibit the assay systems in which the standard will be used. The widespread use of radioimmunoassay methods has raised another difficulty as the presence in the standard of a synthetic fragment of the parent molecule or of a natural biological precursor containing the same peptide sequence may be detected by the antiserum employed. This not only leads to erroneous results but may also have serious consequences when treatment depends on the outcome of the assay. Thus the potential standard may have to be submitted to collaborative assays by both biological and radioimmunological methods and a standard suitable for one purpose may not be suitable for both. Any other evidence for purity is therefore very valuable.

As part of a programme to replace the standard for posterior pituitary gland by individual standards for the hormones present, we examined three samples of arginine vasopressin of high biological activity, obtained from two laboratories, by HPLC using a reversed-phase system and methanol–aqueous phosphate buffer as solvent. An extensive study of the separation properties of five similar nonapeptides has been published[4]. Fig. 4 compares the profiles obtained with 30 μg of each sample

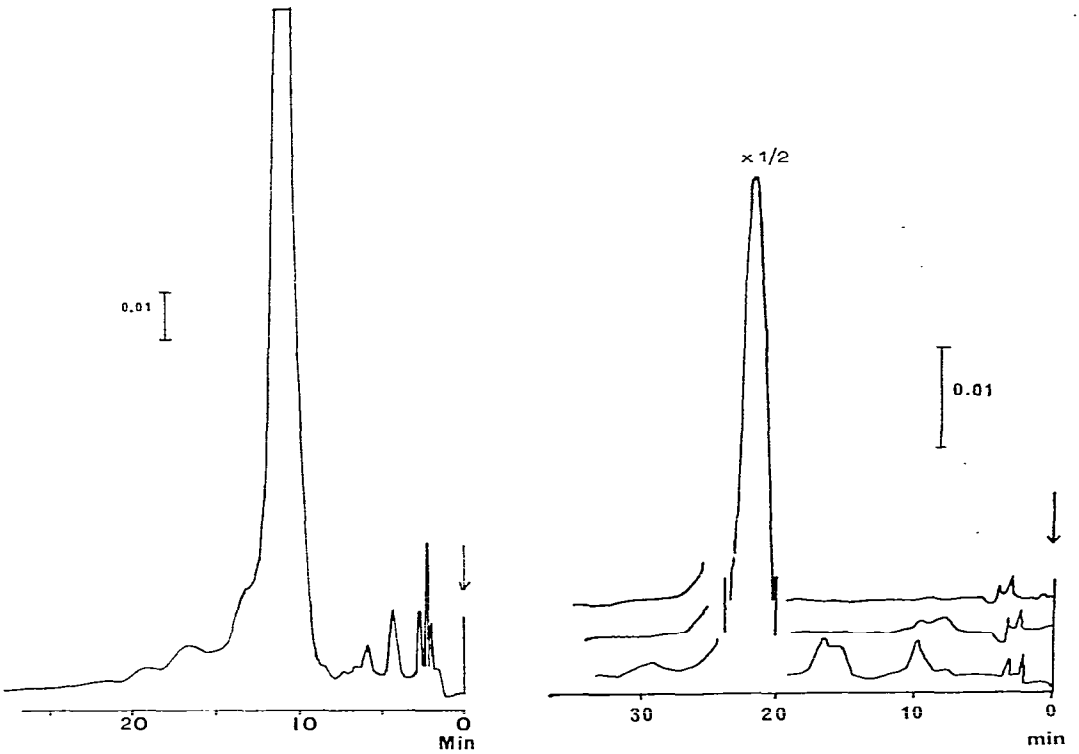


Fig. 3. Separation of nystatin by HPLC. Column: 25 cm × 4.6 mm I.D. μBondapak C_{18}, 10 μm. Pressure, 100 atm; solvent, acetonitrile–0.01 *M* ammonium hydrogen carbonate (27:73); flow-rate, 1 ml/min; column temperature, 60°; detection, UV at 320 nm; attenuation, 0.2 a.u.f.s.
Fig. 4. HPLC of arginine vasopressin. Column: 15 cm × 4.6 mm I.D. Spherisorb S10-ODS. Solvent, methanol-0.1 *M* sodium phosphate, pH 7.0 (40:60, v/v); flow-rate, 1 ml/min; detection, UV at 210 nm.

and reveals small differences in composition that are not apparent from the biological data.

The value of chromatographic methods as an aid to interpretation of data from other procedures is illustrated by our experience with human corticotrophin. A few milligrams of natural material were distributed, for use as a possible standard, into capillary "micro-ampoules"[5] each nominally containing 4 μg of corticotrophin and 100 μg of mannitol. Estimates in two other laboratories of the amount of peptide by radioimmunoassay varied by a factor of 2 and there appeared to be considerable variation of the contents between individual ampoules (Table I). We then determined the amount of mannitol present by silylation *in situ* in the ampoules followed by GLC, with the results also shown in Table I. It seems that the variability lies not in the contents but in the recovery of peptide from the ampoules.

BIOLOGICAL PRODUCTS FOR THERAPEUTIC USE

The legal standards for purity and strength of biological products for medicinal use in Great Britain are laid down in monographs in the British Pharmacopoeia.

TABLE I

RECOVERY OF CONTENTS OF MICROAMPOULES

Reproducibility of recovery as shown by the coefficient of variation $\left(\frac{\text{standard deviation}}{\text{mean estimate}} \times 100\right)$ of estimated contents.

Laboratory	*Procedure**	*No. of ampoules*	*Coefficient of variation (%)*
A	Radioimmunoassay	8	10.3
B	Radioimmunoassay	16	35.5
B	Radioimmunoassay	16	38.4
B	Radioimmunoassay	7	28.7
C	Chemical	12	7.6

* The radioimmunoassay procedures are not identical and are directed to the peptide present. The chemical determination is of the mannitol carrier, determined by GLC after *in situ* silylation, using an internal standard.

These monographs may be for the active ingredient or a formulation and apply from the time of manufacture of the substance throughout its shelf-life. When a manufacturer draws up a specification for material which he has isolated or synthesized, the origin and quality of raw materials and the procedures employed are known, in-process checks and controls can be applied, and this information can be taken into account. The situation in constructing a pharmacopoeial monograph is different. No assumptions can be made about the origin or manufacturing methods used for a substance purporting to be of a given nature or quality. Because the monograph applies throughout the expected life of the product, degradative changes may affect both composition and potency. Further, the methods described must be applicable in a variety of laboratories, with different facilities and expertise. For example, requirements accepted in the European Pharmacopoeia are mandatory in the thirteen member countries involved in its preparation: no tests can be omitted from or added to a European Pharmacopoeia monograph by a national authority. Thus, in Britain the European Pharmacopoeia requirements supersede those for the same product in the British Pharmacopoeia. The methods employed should be as simple as possible, consistent with the information they are designed to provide, be capable of precise description and have good reproducibility between laboratories. Because a specification is taken in its entirety, a test which in isolation may not be conclusive for quality or potency may, in combination with the remainder of the monograph, contribute to a tight specification. Demonstration that a procedure, devised and performed in one laboratory, has high reproducibility and precision is insufficient: it must be validated generally before it can be adopted in a pharmacopoeial monograph. A number of published methods have failed to meet this objective when applied under varied conditions and to different samples.

There is a growing awareness that other techniques are capable of providing more detailed information about the quality and consistency of biological products than can be provided by biological assay alone. Ultimate replacement of biological assay depends upon the adoption of such techniques and the contributions of different modes of chromatography in this area are considered in turn below.

GAS-LIQUID CHROMATOGRAPHY

The molecular size, chemical complexity and relative instability of most biological products means that GLC has a limited role in their examination. Even when the preparation and chromatography of volatile derivatives is possible, the method has failed to gain general acceptance because of poor reproducibility. Notable exceptions are the antibiotics chloramphenicol and griseofulvin, where collaborative studies using rigorously defined procedures have demonstrated the superiority of the GLC assay to microbiological methods[6,7]. GLC is mainly used for the examination for and quantitation of solvents, water and acetic acid but could be adopted more widely for this purpose. For example, the present European Pharmacopoeia test to limit alcohol in streptomycin sulphate involves distillation, reaction of the distillate with dichromate and subsequent titration[8], although the same information can be obtained in a few minutes by dissolving the sample in water containing an internal standard and performing a GLC determination.

THIN-LAYER CHROMATOGRAPHY

By contrast, TLC procedures have been employed extensively. However, despite the clear advantages of relatively simple apparatus, versatility and low cost, various problems arise in transferring a method from one laboratory to a general procedure. Resolution and separation may be affected by changes in laboratory conditions (ambient temperature and humidity), by use of ready-coated instead of home-made plates, by use of supports from different suppliers. For example, a separation of kanamycin complex was found to depend upon the presence of a particular binder in the support and not wholly on the support itself. If quantitation is desired, the reliance placed on densitometry may be justified within one laboratory using a single instrument to examine samples from one source but may be much less so when the qualitative composition of the samples differs with origin and the procedure cannot be standardized. Provided that semi-quantitative measurement suffices, as in a limit test, possible approaches are to provide a reference specimen which is applied at the same concentration as the test sample and to limit minor components in the test to levels present in the reference; to apply the reference at, say, 80% of the loading of the sample and use the same criterion thus limiting levels in the test below those present in the reference; or to apply the reference or, often, the test sample at a low loading (*e.g.*, 5%) and relate the levels of minor components in the sample to the intensity of the major component at the lower loading. Examples of all these approaches are to be found in the British Pharmacopoeia. The option of applying each impurity separately at an appropriate level, as in the test for related impurities in tetracycline hydrochloride[9], is rarely possible.

The examination of gentamicin, an aminoglycoside antibiotic complex, illustrates several of these points. TLC has a higher resolving power than the paper chromatographic method used to identify gentamicin in the British Pharmacopoeia[10] and, in a suitable solvent system, a number of minor components are separable, representing about 10–15% of the total. Fig. 5a shows one plate to which relatively heavy loads have been applied and Fig. 5b shows a duplicate plate developed by direct bioautography, the heavy loads being necessary to reveal the minor components by

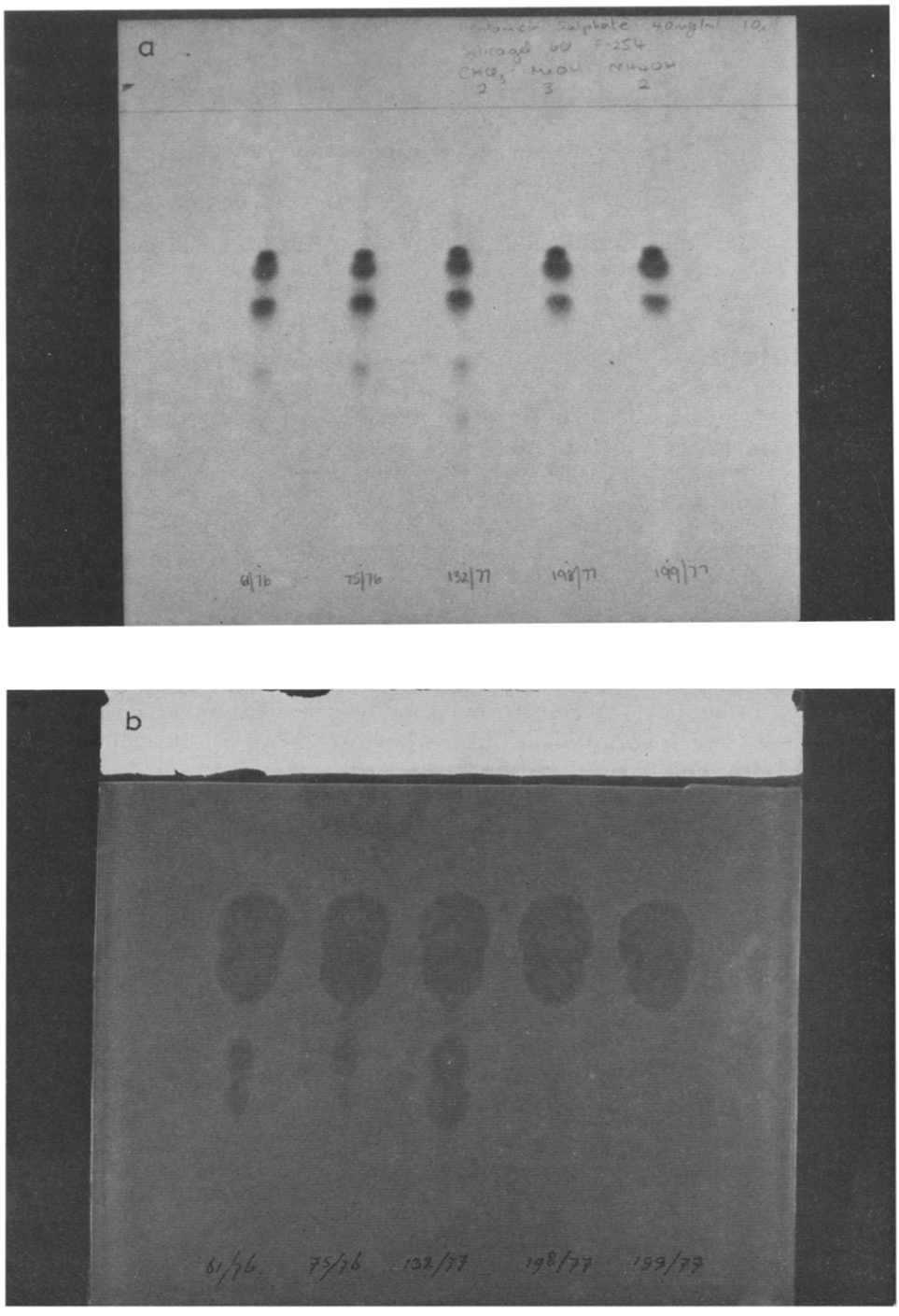
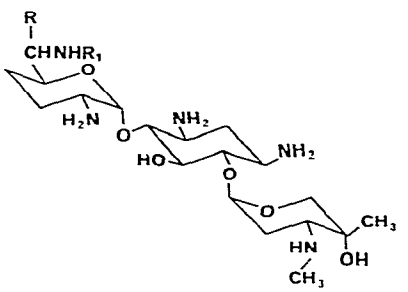


Fig. 5. TLC of samples of gentamicin on Kieselgel 60 F254 (Merck). Solvent, chloroform–methanol–ammonia (sp.gr. 0.88) (2:3:2, v/v/v). Load, 400 μg per sample. (a) Developed with ninhydrin; (b) developed by bioautography using nutrient agarose containing a spore inoculum of *Bacillus subtilis*.

this procedure. Some of the minor components possess microbiological activity and one with the highest activity in this respect may not be the same as that with the highest colour yield (*e.g.*, sample 132/77). An estimate of the level of the minor components is complicated by the fact that ninhydrin reacts with primary amino groups and the colour yield will differ according to the number of such groups in the molecule: gentamicin C_1 (Fig. 6) with three will give less colour on an equimolecular basis than gentamicin C_2 and C_{1a} with four each. However, some of the other components of the mixture are of unknown structure and the behaviour of those of known structure has not been established in this TLC system. The estimates can, therefore, only be relative. Another difficulty is that the separation depends critically on the composition of the three component solvent system. A change in the ratio of chloroform–methanol–ammonia (sp.gr. 0.88) from 60:68:60 to 60:72.5:60 by volume results in transition of a two-phase system to a single phase with marked change in behaviour (Fig. 7a and b). A similar transition can be observed with a fixed solvent ratio but a change in temperature. Thus, for adoption of such a procedure in an official monograph it might be necessary to specify the temperature (*e.g.* ,4°) at which the solvents are held and mixed and chromatography is performed. We have yet to explore the effect of different types of support on this separation.



Gentamicin C_1: R = R_1 = CH_3

C_2: R = CH_3; R_1 = H

C_{1a}: R = R_1 = H

Fig. 6. Structure of the major components of gentamicin.

Sisomicin, didehydrogentamicin C_{1a}, has been examined in parallel with gentamicin and, at loadings in the usual range, is separated from the three main gentamicin components. Minor components travel to positions similar to those of some present in gentamicin and might be presumed to be identical (Fig. 8a). That this may not be so is clear from the bioautograph of a duplicate plate (Fig. 8b), which shows that one component in sisomicin is active but that in the same position in gentamicin is not.

It should be noted that several of the difficulties associated with the evaluation and interpretation of gentamicin separations by TLC also apply to possible separations by HPLC. The gentamicins have no chromophore suitable for UV detection and refractometry would probably be too insensitive to permit the detection of all of the

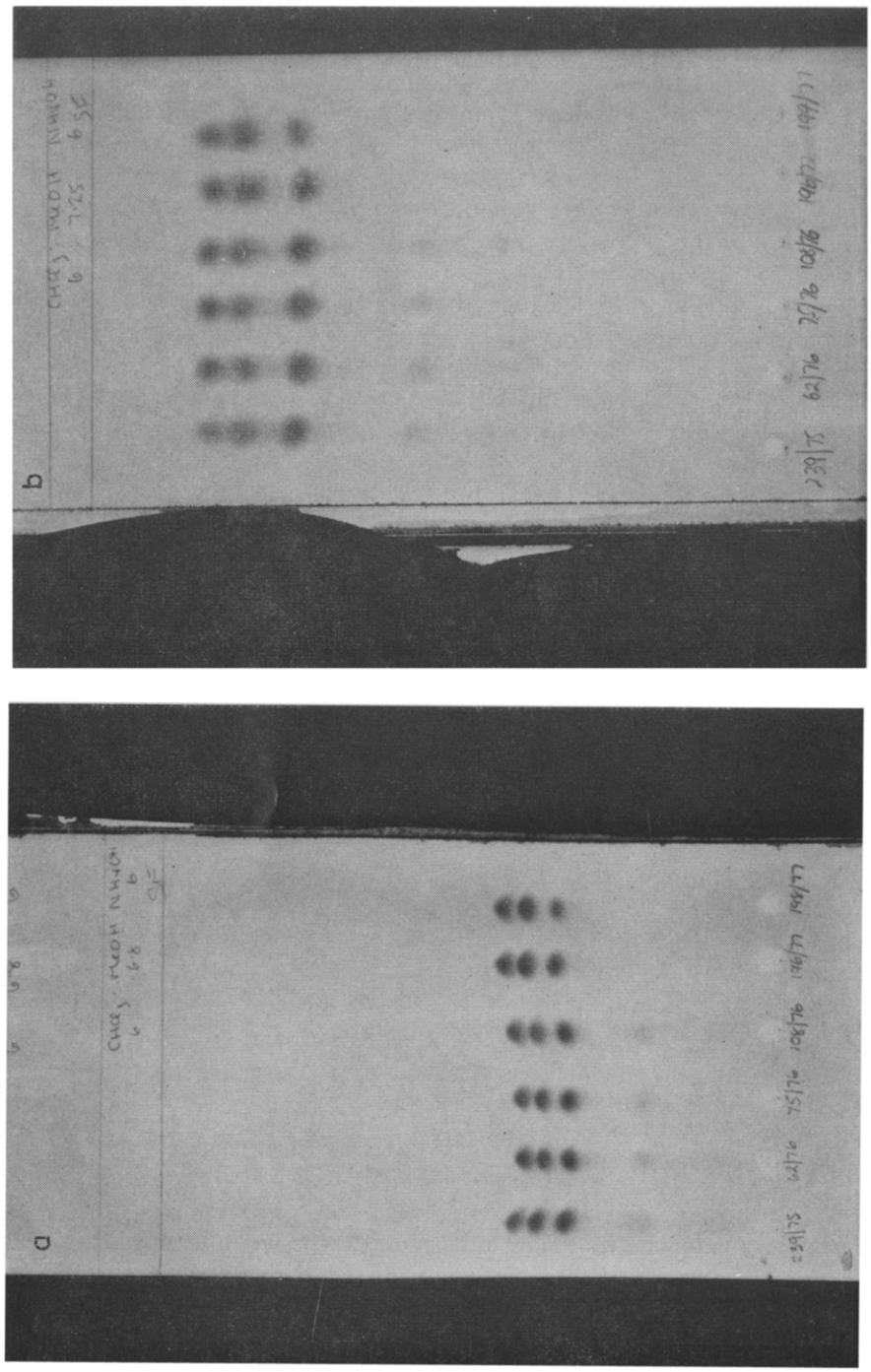


Fig. 7. TLC of samples of gentamicin on Kieselgel 60 F254 (Merck). Load, 100 μg per sample. Developed with ninhydrin. Solvent: (a) lower phase of chloroform–methanol–ammonia (sp.gr.0.88) (60:68:60, v/v/v); (b) chloroform–methanol–ammonia (sp.gr. 0.88) (60:72.5:60, v/v/v) (single phase).

minor components even if a suitable system without gradient elution could be devised. Derivatives with appropriate UV-absorbing or fluorescent properties might be obtained, either before or after separation, but the problems of relative yield and meaningful interpretation of the results remain.

Among biological products, the potential problem of detecting and quantitating impurities arising from different synthetic routes is most likely to occur with polypeptides. Detection may be facilitated by employing more than one TLC system but the dependence on a single test for impurities can be reduced by inclusion of others using different techniques. While an individual manufacturer may be able to chromatograph a known by-product simultaneously to control its level in the test sample, this approach cannot be adopted for a general method. The usual course taken is to use the test sample as its own control by applying one or more light loadings and to make a relative assessment of the impurities, the chain length of which will depend on the synthetic route employed (Fig. 9). The response to ninhydrin will depend on the total number of primary amino groups in each molecule and some impurities might remain undetected, whereas the response to the chlorine–starch–iodine reagent depends on the number of peptide bonds present and hence on the chain length.

COLUMN CHROMATOGRAPHY

Apart from the specialized use of column chromatography for amino acid analysis, this technique has not been adopted extensively in official monographs. An unusual ion-exclusion procedure is employed for the measurement of neomycin C in framycetin[11]. The general use of gel filtration separations in biochemical laboratories is reflected in the test for the molecular weight distribution of natural porcine calcitonin[12] and proposals for a similar test in a monograph for insulin under consideration for the European Pharmacopoeia.

An interesting example is presented by the vaccine used for prophylaxis of meningococcal meningitis. There is no suitable animal model to check the efficacy of the vaccine so, for the first time, international requirements are based on assessment by non-biological means[13,14]. Because the vaccine contains purified capsular polysaccharide with polydisperse molecular size, the requirement for the determination of molecular weight by gel filtration on Sepharose 4B is that a minimal amount of polysaccharide is eluted before a particular K_D value is reached.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Although the number of published examples of HPLC separations of biological products is increasing steadily, no such separation has yet been accepted for inclusion in an official compendium. The reason has been discussed above: inadequate evidence of reproducibility when the proposed method is examined in different laboratories using different equipment.

Thus, the tetracycline group of antibiotics have been intensively studied and a number of procedures using either ion-exchange or reversed-phase systems (see the references cited in ref. 15) have been published. While the correlation between HPLC and microbiological assay has been good in individual laboratories[16], attempts to establish by collaborative study that an HPLC method could replace the present

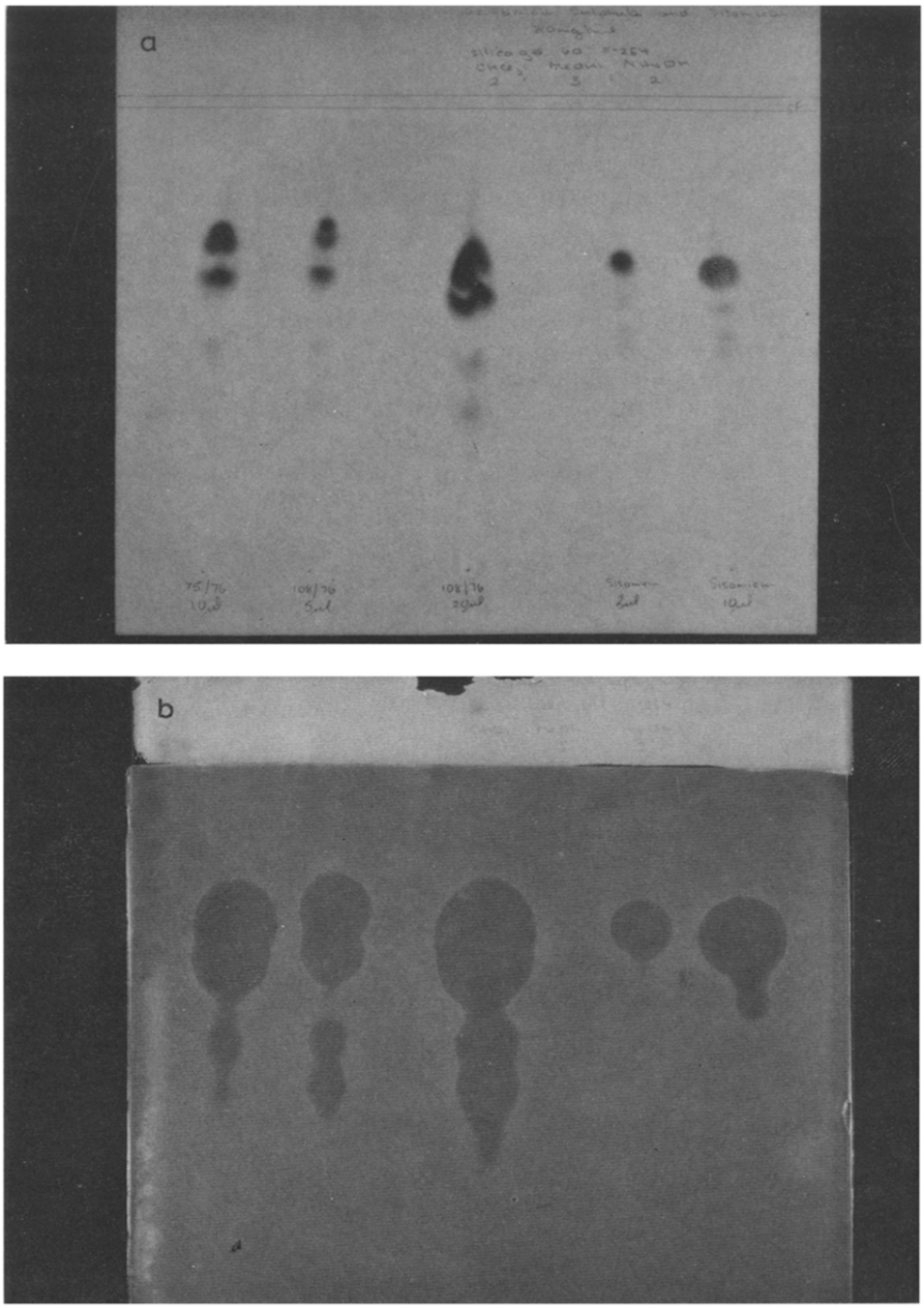


Fig 8. TLC of gentamicin and sisomicin. Conditions and development as in Fig. 5.

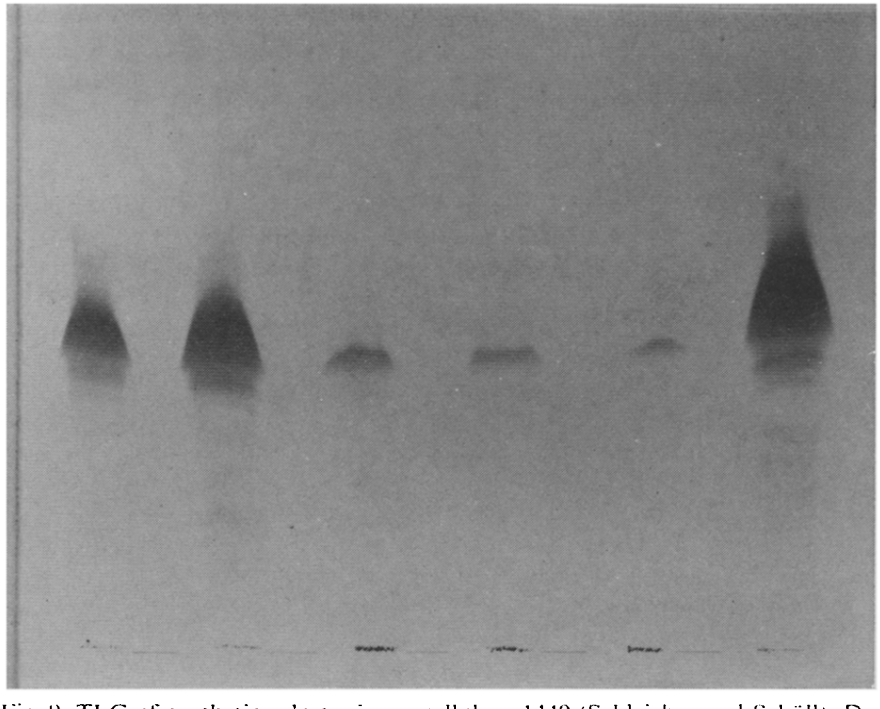


Fig. 9. TLC of synthetic salcatonin on cellulose 1440 (Schleicher and Schüll). Developed with ninhydrin–cadmium acetate. From left: sample A, 100 μg; sample B, 100 μg, 10 μg, 5 μg, 2 μg; sample C, 100 μg. Solvent: *n*-butanol–acetic acid–water–pyridine (42:4:30:24, v/v/v/v).

TABLE II

UNIFORMITY OF CONTENT OF OXYTOCIN TABLETS MEASURED BY HPLC

Each tablet extracted with 2 ml of water containing 0.005% (w/v) of chlorocresol (internal standard). Aliquots of 10 μl analysed on a 10 cm × 4.9 mm I.D. column packed with Spherisorb S5-ODS. Solvent, methanol–sodium phosphate buffer, pH 7.0 (40:60); flow-rate, 2 ml/min; detection, UV at 210 nm. Peak heights measured and reported as ratio of that for oxytocin to that for the internal standard.

Tablet No.	*Group*			
	*1**	*2*	*3**	*4*
1	0.96	1.06	1.00	0.97
2	0.96	1.02	1.01	0.95
3	1.03	1.04	1.00	1.05
4	0.99	1.05	0.96	0.98
5	0.96	1.04	0.92	1.02
6	0.95	1.04	1.01	1.04
7		0.99	0.97	1.01
8	0.94	1.07	1.00	1.01
9	0.99	1.05	0.97	1.01
10	1.03	1.06		0.99
Group mean	0.98	1.04	0.98	1.00
Range (%)	4 to −5	5 to −3	6 to −3	3 to −5
Coefficient of variation (%)	3.4	2.3	3.0	3.1

* Series of 9 only; one ratio not valid.

microbiological assay for oxytetracycline in the British Pharmacopoeia have not yet been successful. In the case of such an antibiotic, where the bioassay can be performed with high precision and results between laboratories readily compared because of the assay design, the demands on reproducibility of the physico-chemical method are severe. In general, biologists rely on statistical evaluation to validate their assays and are reluctant to accept an alternative method without a statistical demonstration that it is superior. It may be that reproducible tetracycline analysis, with the unresolved controversy about incorporation of chelating agent in the mobile phase[15] and critical dependence upon the support used[17], presents greater problems than have been recognized.

The potential of HPLC is illustrated by the extensive studies on the nonapeptide hormones oxytocin and lypressin[4,18] which established a significant correlation between results obtained by HPLC and those by bioassay, and demonstrated that dosage forms containing 2 μg/ml of active ingredient could be examined using injection volumes up to 750 μl. Although the bioassays for oxytocin are specific and relatively precise, the additional information about by-products obtainable by HPLC and the reproducibility of the method favour its use, particularly in stability studies. We have employed a similar reversed-phase HPLC procedure, but with methanol–phosphate buffer, pH 7.0 (40:60) as mobile phase and chlorocresol as internal standard, as a specific method to examine the uniformity of content of oxytocin tablets. Table II reports the results obtained with groups of ten tablets.

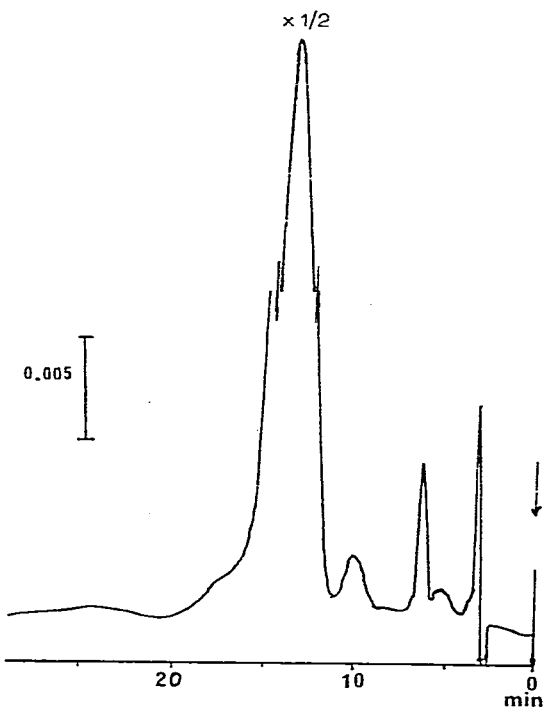


Fig. 10. HPLC of tetracosactrin. Column: 10 cm × 4.9 mm I.D. Spherisorb S5-ODS. Solvent, methanol–water–trifluoroacetic acid (53:46:1, v/v/v); flow-rate, 1.5 ml/min; detection, UV at 280 nm.

We are currently exploring the use of HPLC for the analysis of larger peptides. For example, tetracosactrin is a 24-residue fragment of corticotrophin, with a molecular weight approaching 3000 and obtained by synthesis. It is the subject of the first detailed monograph for such a peptide in the British Pharmacopoeia[19]. An opportunity arose to examine samples of tetracosactrin, undergoing stability tests in connection with establishing a biological standard, which were being submitted simultaneously to bioassay. An isocratic procedure based on the gradient system[20] was employed (Fig. 10), which, although of lower efficiency than observed with smaller molecules, was adequate for following the appearance of degradation products. The correlation between results of analyses by bioassay and HPLC is shown in Fig. 11 and offers the prospect of the eventual development of an HPLC assay to replace that in the British Pharmacopoeia.

Insulin is an even larger polypeptide (molecular weight about 6000) which can be examined by HPLC. Insulin undergoes spontaneous degradation in solution by deamidation of side-chain groups. Desamidoinsulin, being of different charge to the native hormone molecule, can be separated from it readily by polyacrylamide gel electrophoresis. This technique is employed extensively but is relatively time consuming, involving preparation of the gels, separation and development of the bands. Gels are quantitated by densitometry although relative assessment is possible by comparison with markers run in parallel. We have examined the separation of insulin and desamidoinsulin by HPLC although the choice of conditions is constrained by the limited solubility range of the hormone. As with tetracosactrin, the efficiency is not as high as with a smaller molecule, but Fig. 12 shows that adequate resolution is

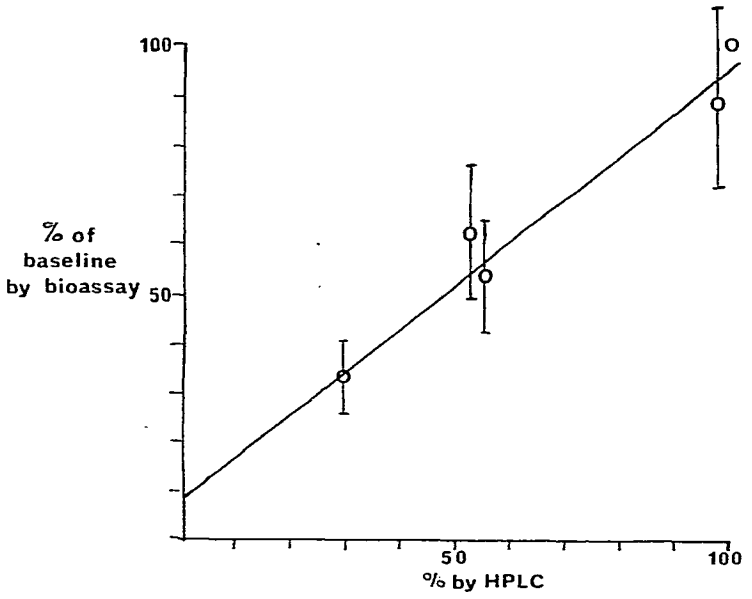


Fig. 11. Correlation between bioassay and HPLC results for tetracosactrin. Bioassay data shown as means with 95% confidence limits. Regression line found: $Y = 0.86X + 9.28$ (Y = bioassay, X = HPLC).

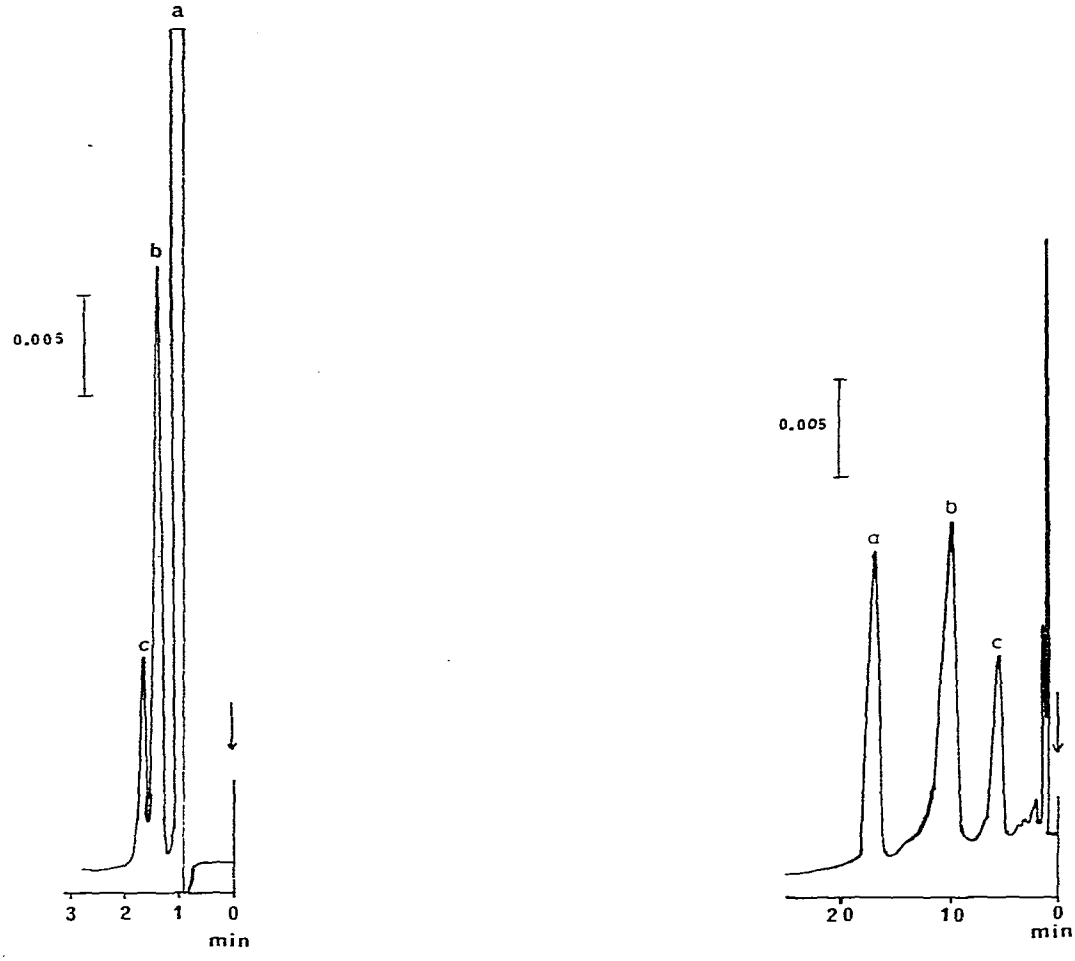


Fig. 12. HPLC of insulin. Column: 10 cm × 4.9 mm I.D. SAS-Hypersil. Solvent, methanol–0.1 *M* tris(hydroxymethyl)methylamine/0.01 *M* EDTA (pH 7.5), (74:26, v/v), containing 1% of cetrimide; flow-rate, 2 ml/min; detection, UV at 280 nm. Sample: formulation of pork insulin, 80 i.u./ml, stored at 45° for 2 weeks. a = Preservatives; b = insulin; c = desamidoinsulin.

Fig. 13. HPLC of thyroid hormones. Column: 25 cm × 4.6 mm I.D. Partisil-10 ODS-2. Solvent, methanol–0.1 *M* sodium acetate, pH 5.0 (60:40, v/v); flow-rate, 1.5 ml/min; detection, UV at 230 nm. a = Fluorenone (internal standard); b = thyroxine; c = triiodothyronine.

achieved. This procedure has been employed to follow the formation of desamidoinsulin in formulations of insulin stored at elevated temperatures.

It is recognized that the thyroid hormones triiodothyronine and thyroxine (T_3 and T_4) are entrained during the isolation of calcitonin from pork thyroid. It is desirable medically that these hormones should be present in no more than residual amounts, and we are involved in the development of a limit test to ensure this. Although the levels present are unlikely to exceed about 0.1% and interference from other components is a potential problem, sensitivity is not because the compounds possess high UV extinction coefficients. A sensitive HPLC procedure for the sepa-

ration of all of the thyroid hormones has been developed[21] using ion-pair partition. We have succeeded in developing a satisfactory separation in the reversed-phase mode (Fig. 13) and selecting an internal standard with suitable retention characteristics on one support. However, when attempts were made to transfer the procedure to other C_{18} columns, considerable changes in the retention behaviour were observed requiring marked alterations in solvent composition to restore the separation. This investigation will be described in detail elsewhere and it suffices here to point out the difficulty in framing a general test based on a separation so dependent upon the conditions employed.

The presence of vitamin D_3 in the list of biological standards[1] arises from its use in the biological assay for the vitamin in cod-liver oil, required by many pharmacopoeias. The problem is one of trace analysis: the limit set in the British Pharmacopeia is equivalent to a maximum of 2 ppm. The assay itself is complex, depending on the prevention of rickets in rats fed a vitamin-deficient diet, and the difficulties associated with it are reflected in the fiducial limits of 60–170% of the stated potency[22]. There are, of course, a number of published procedures for the analysis of vitamin D by HPLC[23] and of formulations of fat-soluble vitamins[24]. Many of these have adequate sensitivity to detect vitamin D at the levels in the oil but are subject to interference by other components present. We are examining ways in which this problem might be overcome, possibly permitting an assay of vitamin A and D in a single procedure.

CONCLUSION

The analysis of biological products by physico-chemical methods presents a variety of problems, some of which may be overcome through developments in chromatographic techniques. Even when methods are shown to possess adequate resolution and selectivity, and quantitation is possible, it is further necessary to demonstrate that they are reproducible under a variety of circumstances before they can be adopted for an official specification, as in a pharmacopoeia. This is usually only possible through a collaborative study. In addition to providing information about composition, chromatographic methods offer advantages of speed, versatility and precision over bioassay techniques and, in some instances, should ultimately replace them.

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