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THE USE OF SEPHADEX LH-20 COLUMN CHROMATOGRAPHY TO SEPARATE UNCONJUGATED STEROIDS

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SUMMARY

Sephadex LH–20 chromatography has been used successfully to separate many steroids, particularly the unconjugated biologically active ones. Since it gives very low or negligible blank values, it has been especially useful to separate steroids prior to competitive binding assays employing antibodies, transins and receptors. Separation is temperature dependent, with good results below 25°C. After appropriate washing, Sephadex can be re-used many times. Microcolumns using pipettes have been found advantageous as they can be packed very quickly, the eluate flow can be controlled by input so that stopcocks are unnecessary, and the eludate volume can be made very small. Where lengthy separations are required, or where many fractions are being collected, automated systems have been employed although their application presents several problems: because of the changes in flow rate which occur with gravity flow it is preferable to use a constant infusion pump and reverse flow; in so far as possible only glass, stainless steel and Teflon may be allowed to come into contact with the solvents; due to low surface tension it has not been feasible to split the eluate stream and an alternate fraction technique has been used in locating tracers. Constant specific activity in multiple solvent systems has been used as a means of confirming the identity of various steroids in fluids and tissues.

INTRODUCTION

The introduction of lipophilic Sephadex (LH-20) has had a considerable impact on steroid methodology for several reasons: 1. it gives excellent separations for a great variety of steroids, 2. it provides an excellent means of purifying substances prior to mass spectrometry, and 3. it does not contribute any significant blank value to assays employing binding proteins (radioimmunoassays, radiotransinassays, and radioreceptorassays).

Lipophilic Sephadex was developed in Europe in 1965 and was investigated very largely by the Scandinavians, particularly by Sjövall, Vihko and their colleagues who provided a great deal of basic information regarding its use [1]. Several years later it was found, in our own laboratory [2, 3] and in that of Mikhail *et al.*[4], that, unlike other chromatographic materials, it gives negligible blank values in binding assays. It continues to be widely used for both routine and research procedures involving steroids.

Sephadex LH-20 is prepared by hydroxypropylation of Sephadex G-25 to give a Dextran gel with both hydrophilic and lipophilic properties. It is stable in all solvents which are not strongly acidic and do not contain strong oxidizing agents. Separation depends on molecular size (gel filtration), adsorption, partition, ion exclusion and retardation, and probably other mechanisms still unknown.

Columns

For manual separations, ordinary 10 or 25 ml glass burettes with Teflon stopcocks are often used with gravity flow (Fig. 1). Commercial columns are also available, *e.g.* from the Pharmacia Company itself. Commonly, one or two ml aliquots are collected and split, half being assayed and the other half evaporated to dryness and counted to determine recovery. Where only short columns are required, *e.g.* for separating progesterone from other steroids in serum, very small columns can be used. Pipettes (disposable ones if the columns cannot be re-used) and syringe barrels are often suitable. We have found 1 ml (tuberculin) syringe barrels with or without a No. 20 needle attached to be very convenient for some separations, *e.g.* progesterone from 17-hydroxyprogesterone, since with the solvent system used (heptane 50: chloroform 50; ethanol 0·25), the columns automatically stop when the solvent level reaches the top of the gel. Spontaneous stopping depends on the combination of column bore and solvent and is a considerable convenience when coloumns are operated manually since no stopcocks

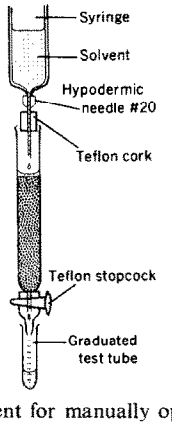

Fig. 1. Arrangement for manually operated columns.

are required. Very long, narrow columns (spahgetti columns) can be made from Teflon tubing. For automated collection, columns require special fittings (see automated separations).

Column preparation and maintenance

Packing. Enough solvent is prepared for the whole experiment. The amount of Sephadex required is weighed and put into a beaker containing solvent, for an hour or more. The column is rinsed with solvent which is discarded. A minimal amount of glass wool (just enough to cover the hole in the stopcock) is wrapped loosely around the end of a glass rod and inserted into the column. The glass wool is dislodged and tapped into place. The column is filled with solvent, and Sephadex added, a little at a time. From time to time the stopcock is opened to let some solvent through. If bubbles form they can be dispersed by moving a glass rod or copper wire through the solvent. After all the Sephadex has been added, a further 20 ml of fresh solvent is passed, which may be saved and checked for blanks. The columns are then ready for the addition of the sample, which is added in a small amount, *eg.* 0·2 ml, of the column solvent, followed by a washing of the sample container with another aliquot of solvent.

Running the columns. The columns can be left for weeks or months provided they are not permitted to dry out. If the Sephadex floats and dries at the top leaving a solvent space at the bottom, the stopcock is opened, the Sephadex allowed to pass to the bottom and more solvent is added to the top. Columns may be stopped one day and re-started the next to complete a long collection. To prevent Sephadex from floating, the top of the Sephadex can be packed down with a large wad of glass wool. This can be removed when a sample is added and later replaced. Quite often air bubbles form but unless these are quite large, they do not affect the separation appreciably.

Re-using the columns. Provided all the substances which might interfere in subsequent assays are removed in the course of elution, columns can be re-used without re-packing or further elution. The solvent system which we use to fractionate corticoids in serum prior to assays using transcortin is such an example. Cortisol, the most polar assayable substance present in serum is eluted last and, once it emerges, the next sample can be applied immediately. In our laboratory columns for this purpose are used eight successive times before repacking.

If all the interfering substances present are not eluted in a convenient volume, then the column must be unpacked, the Sephadex washed, and the column repacked. Unless solvents have exactly the same bed volume, one cannot change from one solvent to another in the same column.

Unpacking and washing the Sephadex. The Sephadex can usually be floated out of the columns with chloroform, aided by stirring with a copper wire or glass rod; it is collected in a beaker, covered and saved until a large amount has been accumulated. The glass wool remaining in the column is washed out and discarded. The columns can then be washed, rinsed with the solvent to be used, and repacked.

The accumulated Sephadex is decanted, a little at a time, into a large Buchner funnel with a filter paper on the bottom. The chloroform is removed by suction. The Sephadex is washed with about 100 ml of Chloroform, then three times with 100 ml of redistilled ethanol. The gel is stirred about gently in the ethanol with a glass rod, then suctioned for about 30 min. It is spread out on aluminum foil and dried at 30°C in an oven for about 2 h or in room air overnight. The material is stored in the original plastic container with air-tight seal. It is usually discarded after eight washings, or when the separations are less satisfactory.

Automated separations

Since manual fraction collection is very tedious, we have attempted to automate our systems. However we have run into many problems, all relating to the corrosive nature of the solvents used, especially methylene chloride.

In our experience, collection by drop of volume (for volumes of 2 ml of less) was not sufficiently precise and a constant infusion pump with timed collection was adopted. This proved to have several advantages. Since the columns were operated against gravity the column packing remained more uniform; flow rates could be easily and accurately adjusted, and the time required for the collections was known in advance.

Many types of equipment are available for use with aqueous solvents but for organic solvents only glass, Teflon and stainless steel have been found to be entirely satisfactory. One system used frequently in our own laboratory separates testosterone, dihydrotestosterone, and other androgens using heptane 50: methylene chloride 50: ethanol 1. Unlike solvents containing larger amounts of methylene chloride, this solvent can be used with acid-flex tubing. The materials used routinely for this separation include the following: glass columns (No. 3500, 0·9 cm. i.d. × 60 cm.) were obtained from Glenco Scientific Inc., 3121 White Oak Dr., Houston, Texas. Lower end plates and supports for 0·9 cm. i.d. columns, two for each column No. 3500-G-9) were used at both ends. Teflon tubing (No. 3114–030, 0·03 in. dia.), about 10 feet per column, was connected where necessary by multifit connectors (1/8th in., size 2, No. 3020–2 × 2). In the perfusion pump, Acid-flex tubing (i.d. 0·065 in., No. 116–0538–13), obtained from the Technicon Co., was used.

The constant infusion pump was obtained from Canlab (polystaltic, No. P–8510). The fraction collector was obtained from the Brinkman Co., Montreal (Brinkman Linear II fractionator, No. 23–30–200–4 with distributing head No. 23–30–390–6, extension track set No. 23–30–310–8, extra carrier No. 23–30–260–8, and eight tube racks No. 23–30–470–8). These are assembled as shown in the

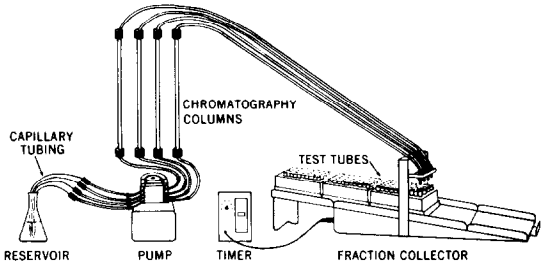

Fig. 2. Arrangement for automatically operated columns.

diagram (Fig. 2). Samples are added through capillary tubing (No. 3010–407, Chemical Rubber Co., Cleveland, Ohio 44128) which otherwise remains in the reservoir.

The polystaltic pump could not be used with 98% methylene chloride because it dissolved the Acid-flex tubing and all other forms of flexible tubing tried. Alternatively, a minipump with a sapphire plunger and sapphire ball valves and seats (Model No. 3365–160, obtained from the Milton Roy Co., St. Petersburg, Phila.) was investigated. Although it withstands all solvents so far tried, including 100% methylene chloride, it is much less convenient to use and only supplies a single column.

Due to the low surface tension of organic solvents it has not been possible to split the eluate stream as with aqueous solvents. Taking alternate fractions for counting and assay has resulted in accurate splitting provided that relatively small fractions (say 12), are taken for each substance being collected. The

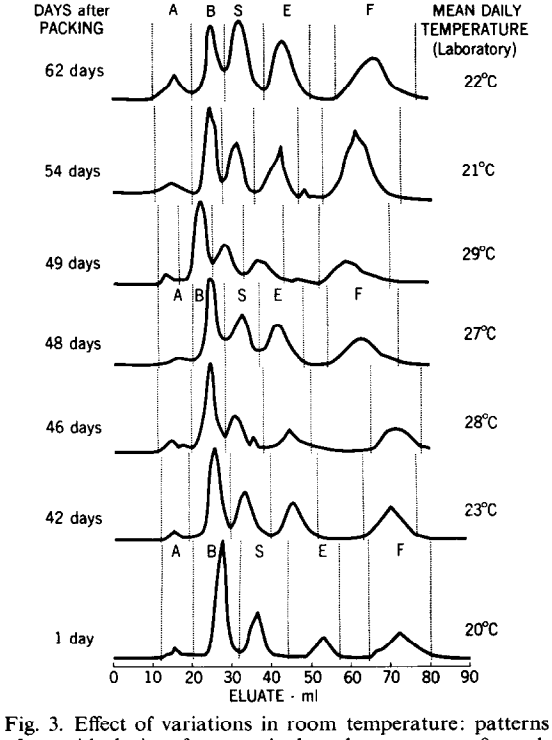

Fig. 3. Effect of variations in room temperature: patterns of steroid elution from a single column over a 9 week period. Elution by gravity flow is more rapid and peaks emerge earlier at higher temperatures. (A, B, S, E, F: see Fig. 4).

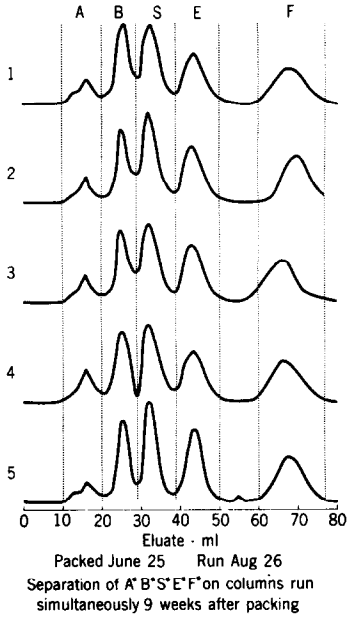

Fig. 4. Patterns of steroid elution in 5 columns operated simultaneously 9 weeks after packing. (F: cortisol, E: cortisone, S: 11-desoxy cortisol, B: corticosterone, A: contaminant). Solvent system methylene chloride 98: methanol 2. Temp. 22°C).

fractions can then be pooled if desired. When such a technique was employed in the separation of testosterone (T) and dihydrotestosterone (DHT), where the DHT was eluted from 40 to 54 ml, and the T from 56 to 70 ml, summing alternate 1 ml fractions resulted in a recovery of total counts added of 43·5 ± 2·8% S.D. for DHT and 44·7 ± 4·0% S.D. for T. Thus this technique is satisfactory for clinical purposes.

Temperature dependence

In general, good separations are obtained at cool room temperature and below, but above 20°C the separation becomes rapidly poorer. Thus in a warm room (80°F, 26·5°C) the separation is much less satisfactory than in a slightly cool room (68°F, 20°C) (Fig. 3). Therefore air conditioning is desirable in separating substances with close elution volumes. Provided environmental conditions are kept reasonably constant, highly reproducible patterns can be obtained, as shown in Fig. 4.

Blanks

Despite the high specificity of many stereospecific proteins (antibodies, transins, and receptors), the great diversity of steroids in biological fluids frequently makes it necessary to do some form of chromatography prior to assay. In our experience Sephadex LH-20 is greatly superior to any other chromatographic medium for this purpose because of its negligible blank values [2, 3]. In fact, whenever we have observed blanks with this medium it has always been possible to trace them to some source other than the Sephadex itself—usually column solvents which have deteriorated. This can be easily

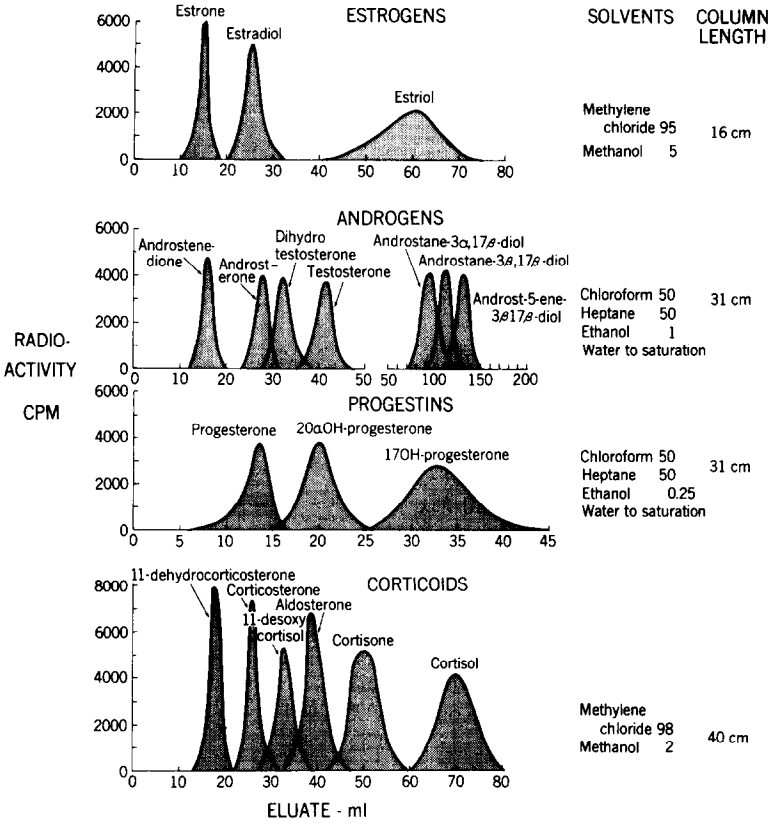

Fig. 5. Solvent systems for various types of steroids. (Reprinted from Ref. 2.)

verified by assaying the column solvent alone. In one instance it was found to be due to washing the Sephadex with deteriorated ethanol. Thus it is better to assay the solvent emerging from the column as a check. Commercial reagent grade solvents which have been distilled just prior to delivery have been found to be satisfactory for long periods (months) provided they are kept in brown bottles in the refrigerator. The blank values of all solvents used in preparing samples for binding assays should be checked regularly.

Available solvent systems

Many different solvent systems are currently being employed with Sephadex LH-20 to separate unconjugated steroids for many different purposes. In general, steroids are eluted in order of increasing polarity [1] (Fig. 5). Substitution of methylene chloride for chloroform in the androgen system in Fig. 5 has been found by us to give a better separation of testosterone from dihydrotestosterone. In addition to the solvent systems shown here, benzene:methanol 85:15 has been used very extensively to separate estrogens [4], and has also been used to separate estrogens from some androgens [5]. Methylene–chloride–methanol 95:5 v/v has also been used to separate some androgens from estrogens [6]. Relative mobilities of various steroids in LH-20 have been compared to those using thin layer chromatography [7]. Resolution by the two methods was comparable. Corticosteroids of rat plasma (desoxycorticosterone, 18-hydroxydesoxycorticosterone, aldosterone and corticosterone) have been separated using benzene–cyclohexane 2:1 v/v equilibrated with 80% methanol [8]. To separate various neutral and phenolic steroids, combinations of heptane, isooctane, hexane and cyclohexane with small amounts of other solvents have been used [9] as well as combinations of cyclohexane and ethanol [10].

Sephadex LH-20 analogues

Sephadex analogues related to LH-20 were used to obtain reverse phase systems where compounds separate in order of decreasing polarity [11]. Hydroxyalicyclic derivatives of LH-20 have been investigated recently [12].

Special applications

Sephadex LH-20 has been used extensively as one means of purification prior to gas–liquid chromatography and mass spectrometry as described in the preceding paper. In some instances, highly purified materials can be obtained after even a single passage through Sephadex. Thus we were able to obtain a mass spectrum for dihydrotestosterone in plasma which was indistinguishable from that of a pure standard dihydrotestosterone using the eludate obtained by passing a diethyl ether extract of plasma once through Sephadex LH-20 employing heptane–chloroform–ethanol–water 50:50:1:0·03 by vol. as the solvent system[13].

Another frequently used means of identifying or confirming identity of steroids is to demonstrate constant specific activity of material passed through multiple solvent systems using paper or thin layer chromatography. This can be accomplished equally well using LH-20. For example, we used 4 different Sephadex LH-20 systems to confirm the identity of cortisone in placental extracts [14].

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