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THE PREPARATION OF HIGH RESOLUTION MICROGRANULAR CARBOXYMETHYL CELLULOSE COLUMNS AND THEIR APPLICATION TO THE SEPARATION OF GUANETHIDINE AND ITS METABOLITES IN URINE

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SUMMARY

Columns (1.2 × 6 cm) with up to 100 theoretical plates/cm were prepared using CM cellulose which had been graded by back-washing. The columns were fitted with specially made polythene column ends to allow samples to enter and leave the column bed with minimum distortion and were rapidly packed at high flow rates of buffer. The columns could be operated at low pressures and used repeatedly over several weeks.

Four columns connected in series were used to separate radioactive products excreted in the urine of rats which had been treated with guanethidine. Four radioactive products which accounted for 99% of the applied radioactivity were identified as 1-(6-carboxyhexyl)-2-iminoimidazolidine, 2-(6-carboxyhexylamino)-ethylguanidine, guanethidine N-oxide and guanethidine.

INTRODUCTION

A recent study[1] reported the identification of two metabolites of the antihypertensive drug guanethidine as guanethidine N-oxide and 2-(6-carboxyhexylamino)-ethylguanidine. The latter product is readily transformed in alkaline solution to 1-(6-carboxyhexylamino)-ethylguanidine.

In order to determine the quantities of these three products which were formed *in vivo* and to find out whether other major metabolic products were formed, a procedure for quantitatively separating mixtures of these compounds from each other and from unchanged drug in biological samples prior to radioactive assay was required. Since the metabolites of guanethidine could not be extracted into organic solvents and the amount of radioactivity in samples was low, only procedures to which reasonably large volumes of biological fluids could be applied directly would be suitable.

Large volumes of these fluids cannot be applied to thin layer chromatograms or to paper for electrophoresis since the inorganic salts present alter the phase compo-

sitions of the solvents used in thin layer chromatography and the electrical conductivity of the buffers used in electrophoresis.

Partial fractionation of the metabolites of guanethidine can be achieved using a synthetic sulphonic acid cation exchange resin[2]. This procedure however is not very suitable for the quantitative assay of metabolites since in order to elute guanethidine and guanethidine N-oxide from the column of ion exchange resin 6 *N* hydrochloric acid must be used and under these conditions the products are eluted as a very broad trailing peak in incomplete yield and are not resolved. When a synthetic carboxylic acid resin was tested it was found that guanethidine could only be eluted at pH values less than 4 and under these conditions elution was again slow and not quantitative.

Recently the application of a 'microgranular' DEAE cellulose for the high resolution chromatography of nucleic acids[3] has been described. Tests with the corresponding CM cellulose showed that guanethidine and its known metabolites could be eluted from this material at between pH 9.5 and 10.5 as symmetrical peaks. In order to prepare high resolution columns in which the cellulose would not bed down on repeated use a number of aspects of column preparation were studied.

This paper reports a method, based on these investigations, for the preparation of short CM cellulose columns with approximately 100 theoretical plates/cm for a test dye which require low pressures to achieve a given flow rate and do not bed down on repeated use.

EXPERIMENTAL

Materials

Polythene rod, 0.5 in. diameter was obtained from Xlon products Ltd.; neoprene 'O' rings, 7/16 in. O.D. 5/16 in. I.D. from George Angus Ltd.; precision bore glass tubing, 12 mm bore, from Jencons Ltd.; polythene cannula, pp 25, 0.4 mm I.D., 0.80 O.D. and pp 100, 0.86 mm I.D., 1.52 mm O.D. from Portex Plastics Ltd.; CM 32, microgranular carboxymethylcellulose from Whatman Ltd.; ARW-7 wetting agent from Technicon; and guanethidine sulphate from these laboratories. 2-(6-Carboxyhexylamino)-ethylguanidine, guanethidine N-oxide and [^{3}H]guanethidine were prepared as previously described[1].

Design of column ends

Column ends were made from soft polythene rod using a lathe to produce the shape shown in Fig. 1. 'O' rings were fitted into grooves which were cut to such a depth that the rings sealed against the inside wall of a 12 mm bore precision bore glass tubing but allowed the column end to be slid in and out of the column with reasonable ease. The 'O' ring nearest the end of the polythene was used to hold a piece of fine nylon cloth which was tightly stretched over the end of the polythene section. The edges of the cloth were carefully trimmed so that no leakage of buffer could occur past the second 'O' ring and out off the column. These column ends were fitted into sections of 12 mm bore precision bore glass tubing cut to lengths of 9 cm. To facilitate the introduction of the column ends the edges of the glass sections were flared slightly. Columns were connected using lengths of pp 25 polythene cannula the ends of which were inserted into a short length of pp 100 which in turn could be

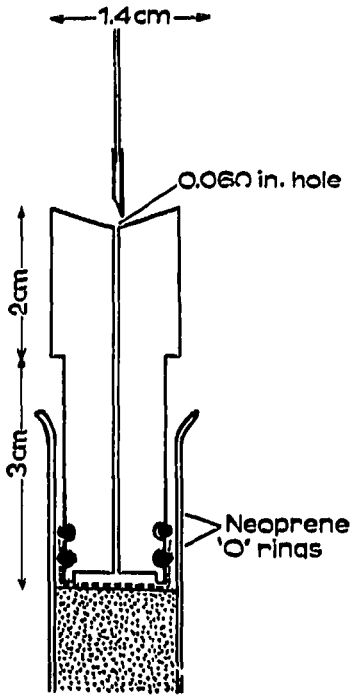

Fig. 1. Column end made from soft polythene.

inserted tightly into the hole in the polythene column end. When the column connection was being changed the hollow in the top of the polythene end was filled with buffer to prevent air from entering.

Fractionation of CM cellulose

This was carried out using a back-washing procedure based on a method for classifying sulphonic acid resin particles developed in these laboratories by Mr. E. J. STEVENS following the work of HAMILTON[4]. In a typical preparation 300 g of CM 32 was swollen and recycled according to the manufacturer's instructions and finally equilibrated by repeated washing with bicarbonate buffer, pH 9.7, 0.5 *N* with respect to sodium ions, prepared by mixing 2 vol. of 0.5 *M* sodium bicarbonate with 5 vol. of 0.25 *M* sodium carbonate. During these operations "fines" were decanted. The powder was gently slurried to give a final volume of just under 2 l and transferred to a 2 l pear-shaped separating funnel clamped tap downwards with its axis vertical. The diameter of the separating funnel was 14.5 cm at its broadest point. The outflow from the top of the separating funnel was led to a sintered glass filter funnel attached to a buchner flask. Filtrate was withdrawn from this flask through the side-arm and pumped upwards through the separating funnel by a peristaltic pump the flow rate of which could be varied from 6 to 20 l/h. By initially filling the buchner flask with buffer and sealing the tube leading from the flask to the side-arm with rubber sleeving, continual recycling of buffer through the separating funnel could be achieved. Cellulose fibres light enough to be eluted at the flow rate being used accumulated on the glass sinter.

Back washing was started at 6 l/h. In the first 2–3 h considerable amounts of fine particles appeared in the outflow from the funnel. When the quantity of these

particles in the eluate had become low, after about 4 h, the material which had collected on the sintered glass filter was removed by very gentle swirling with buffer added to a depth of 2–3 cm and decantation. The flow rate of circulating buffer was then increased to 10 l/h and elution was again continued from 3–4 h until only small amounts of material were being eluted from the separating funnel. Eluted material was then collected from the sintered funnel as above and the washing procedure repeated at a number of increasing flow rates to give a series of fractions of powder as shown in Table I. Small portions of suspension of certain fractions were transferred

TABLE I

EFFICIENCIES OF CARBOXYMETHYL CELLULOSE COLUMNS

1.2 × 6 cm columns packed with fractionated CM cellulose as described in Methods. Plates/cm were calculated for a 15 sec pulse of thymol blue in bicarbonate buffer, pH 10, applied at 0.16 ml/min and eluted with buffer at this flow rate.

Fraction no.	*Flow rate used for fractionation (l/h)*	*Plates/cm*
1	6	100
2	10	80–100
3	14	80–100
4	18	75
Unfractionated	—	45

to a microscope slide using a Pasteur pipette, covered with a cover slip and photographed at × 80 magnification. The particles took the form of fibres of differing lengths and histograms could be constructed showing the numbers of particles falling within certain limits of length in a given photographic field (Fig. 3).

Procedure for packing columns

The procedure for producing efficient columns was critical and is therefore described in detail. A column end fitted with about 15 cm of pp 25 polythene cannula was inserted into the lower end of a section of glass tubing. Buffer was poured into the column and air bubbles under the nylon cloth stretched over the polythene column end were removed by placing the tip of a Pasteur pipette with a partially compressed teat just over the bubble and sucking the bubble through the cloth by releasing the pressure on the teat suddenly. Buffer was then allowed to drain from the column and the end of the cannula was placed level with the top of the glass column.

A slurry of powder of suitable consistency was prepared by decanting excess buffer from a thin layer (1–2 cm) of settled powder in a large beaker or conical flask (200–500 ml) and slowly adding buffer to the vessel with gentle swirling until the slurry had a thick but smooth consistency with no tendency to form clumps on swirling or pouring. Such a slurry requires the addition of a volume of buffer which is approximately 20% of the settled volume of powder after settling for 2 h. After thorough mixing by gently swirling this slurry was poured at once into the column section prepared as above and a polythene end, which had been wetted with buffer

and freed of air bubbles but was not fitted with cannula, was immediately carefully inserted into the top of the column and pushed down steadily until both 'O' rings were properly sealed against the inner wall of the glass tube. It was important that during insertion buffer should be free to flow through the hole in the upper column end. A polythene cannula connected to a pump tube on a Technicon multichannel peristaltic pump delivering buffer at 1.6 ml/min was then inserted tightly into the upper polythene column end for 20–30 sec and removed as soon as the powder had stopped settling rapidly. A cannula delivering buffer at 0.16 ml/min was inserted into the column end and after allowing the packed column to adjust itself at this flow rate for 15–30 min the cannula was temporarily disconnected while the column end was pushed into the column until the nylon cloth just touched the upper surface of the powder. The column could then be sealed for storage by inserting the free end of the cannula attached to the bottom of the column into the top of the column or it could be connected to a pump or other columns for use. Columns produced by this method have a bed height of 6–7 cm.

Testing of columns

A 15 sec pulse of a mixture of dextran blue 2000 (0.5%, w/v) and thymol blue (0.0125%, w/v) in bicarbonate buffer (pH 9.7) was pumped onto the column at 0.16 ml/min using a Technicon multichannel peristaltic pump. The column was then eluted with buffer at the same flow rate using the same pump tubing. The dead volume of the pump tubing is small and this method of sample application which does not involve breaking the connection of the pump and the column is thus simple and adequate. The behaviour of dye on entering the column was carefully observed to make sure that it formed a thin completely horizontal zone. To obtain a quantitative estimation of peak broadening the eluate from the column was passed through a 12 mm path length flow cell in a Technicon colorimeter and its absorbance at 570 mμ was recorded. The number of theoretical plates/cm for thymol blue was calculated using the formula

$$n = \frac{d}{W\frac{1}{2}} \times \frac{5.45}{L}$$

where

n = number of theoretical plates/cm
d = recorded displacement of peak maximum from point of sample application
$W\frac{1}{2}$ = recorded peak width at half maximum absorbance
L = length of column (cm)

Assay and identification of guanethidine and its metabolites

The apparatus shown in Fig. 2 was used for the identification and estimation of guanethidine and its metabolites in urine. Four columns were connected in series and part of the eluate was examined by a continuous colorimetric procedure while the remainder was collected for radioactive assay.

The colour reaction was based on the Sakaguchi procedure described by VAN PILSUM *et al.*[5] for the assay of samples in test tubes. In their method an alkaline solution containing α-naphthol and thymine was added to the sample followed by

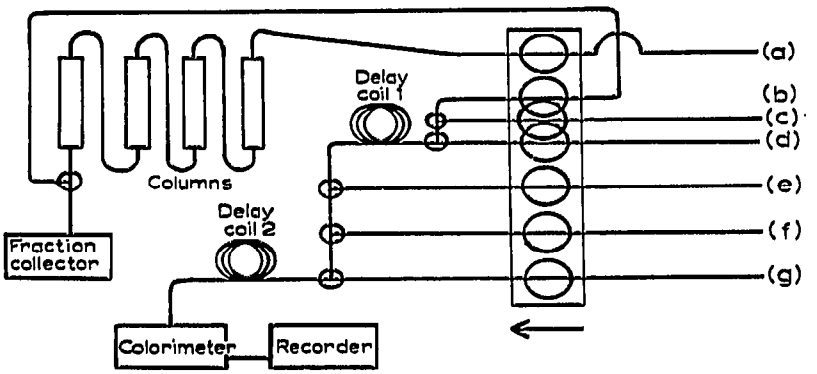

Fig. 2. Apparatus used for the separation and assay of guanethidine and its metabolites. The solutions and flow rates supplied by the Technicon peristaltic pump were: (a) Borate–NaCl buffer, pH 9.7, containing 0.056 *M* sodium tetraborate, 0.044 *M* NaOH, and 0.2 *M* NaCl/l at 0.16 ml/min. (b) Column eluate at 0.1 ml/min. (c) 2% (v/v) ARW-7 in 1.3 *N* HCl at 0.1 ml/min. (d) 1.4% (w/v) sodium metabisulphite at 0.1 ml/min. (e) 2 ml of 0.5% α-naphthol in ethanol (w/v) diluted to 56 ml with water at 0.16 ml/min. (f) 1.6% thymine (w/v) in 12% NaOH (w/v) at 0.1 ml/min. (g) Aqueous sodium hypochlorite containing 3% chlorine (w/v). Delay coils 1 and 2 were made of pp 25 and pp 100 and introduced delays of 3 and 1 min respectively.

sodium hypochlorite. After 1 min at 0° the reaction was terminated and the breakdown of chromophore arrested by the addition of thiosulphate. In the continuous procedure the addition of thiosulphate was omitted since a constant time elapsed between the addition of chlorine and the passage of products through the colorimeter. α-Naphthol was unstable in alkali and was pumped into the flow system independently of the alkaline thymine solution.

Guanethidine N-oxide gave a much weaker response in the assay procedure than guanethidine or 6-(2-carboxyhexylamino)-ethylguanidine but this could be rectified by treatment with sulphite in acid solution as the first step in the reaction sequence. The addition of sulphite did not alter the sensitivity of the assay for other components provided extra chlorine was added at the end of the reaction to oxidise the sulphite and leave sufficient excess chlorine for colour development. Details of the flow rates and reagents are given in Fig. 2.

Bicarbonate buffer was replaced by borax buffer for the elution of the column to prevent carbon dioxide gas from being generated when the buffer was acidified in the assay procedure. However, borax buffer could not be used to pack efficient columns since suspensions of CM cellulose in this buffer were not free flowing. Columns were therefore prepared as described above using bicarbonate buffer and thoroughly eluted with borax buffer before use. Once the columns had been packed changing to borax buffer did not reduce their efficiencies.

There was a time delay of approximately 7 min between samples leaving the column and entering the colorimeter cell. Short pulses of drug applied directly to the assay procedure gave peaks with a width at half height of approximately 1 min.

RESULTS AND DISCUSSION

The use of short columns

In preliminary experiments using CM cellulose columns with bed heights of approximately 30 cm, pressures of 30–40 p.s.i. were required to maintain a flow rate

of 0.33 ml/min. At this flow rate after 2 or 3 days the cellulose started to bed down and the pressure rose, slowly at first, then with increasing speed, making it impossible to continue using the column.

It was thought that this unstable situation might arise if certain flow rates and column lengths were exceeded so that the pressure drop across the column bed reached a value which caused compression of the bed to occur. When this happened the resistance to flow would rise and the pressure drop would increase resulting in further compression. If this explanation were correct it might be possible to prevent pressure build up and compression of the column bed by replacing a long column with several shorter columns connected in series so that the pressure drop across each column bed would be reduced.

When, instead of a 30 cm column, five 6 cm columns connected in series were tested the beds did not pack down after 2–3 weeks running at 0.33 ml/min and only 4–5 p.s.i. was required to maintain this flow rate. Since columns with these properties could be used repeatedly and could be driven with a peristaltic pump, which simplifies sample loading, the preparation of short columns suitable for high resolution chromatography was further investigated.

The design of column ends

The successful use of a number of short columns connected in series required that the column ends should not distort the flow of samples into and out of the cellulose beds and that the dead volume between the beds should be small. This was important because any peak broadening which occurred in the column ends would accumulate as the sample passed through a series of columns.

In order to minimise the dead volume, ends which could be adjusted to touch the upper and lower edges of the column bed were used. In these ends fluid travels in a narrow capillary hole (to reduce dead volume) and must be allowed to flow freely

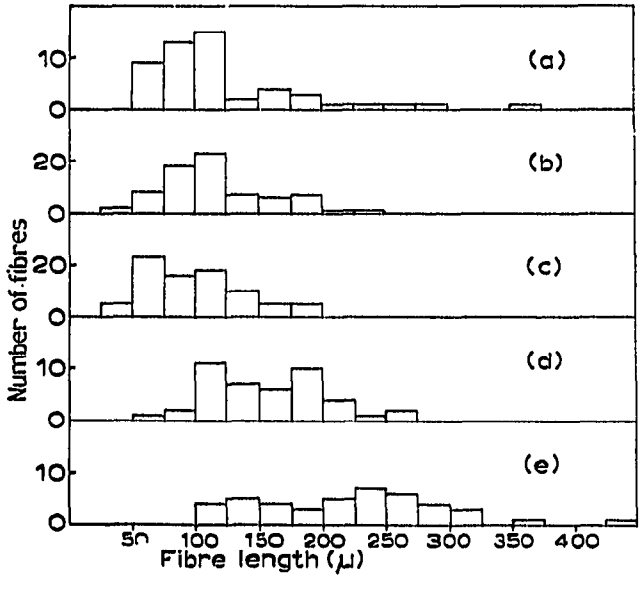

Fig. 3. Histograms showing the distribution of fibre length in fractions of CM cellulose prepared as described in Methods. (a) Unfractionated powder, (b) fraction 2, (c) fraction 2 after shaking by hand in a stoppered test tube for 30 sec, (d) fraction 3, (e) fraction 4.

in a radial direction on entering and leaving the column bed. Columns were fitted with different ends which might achieve this and the ends were tested by applying small samples of water-soluble dyes to the columns and observing the shape of coloured zone formed on the column.

With the design shown in Fig. 1 sharp horizontal zones of dye could be applied which showed either no distortion or very little distortion at the edge of the column. To test whether the band of dye was horizontal throughout the cross-section of the column, dye was applied and when it had travelled about 1 cm down the column, buffer flow was stopped, the column end removed and the cellulose bed extruded and divided longitudinally with a scalpel. The bed was firm and it could be seen that the zone of dye took the form of a flat horizontal disc.

When the nylon cloth in the column ends was replaced by inserted sintered teflon or polythene discs the bands of dye applied to columns were much more diffuse and the tendency for streaking of dye at the edges of the column was greater.

Preparation of columns using graded cellulose

Histograms showing the distribution of fibre length in samples of fractionated and unfractionated powder are shown in Fig. 3.

The different fractions were tested using a number of packing procedures and it was found that fractions 2 and 3 gave good columns when packed and tested as described in Methods. A typical trace is shown in Fig. 4.

Table I shows the number of theoretical plates/cm for thymol blue obtained with columns packed with different fractions of powder and eluted at 0.16 ml/min. Large numbers of plates/min indicate a good column which gave sharp peaks.

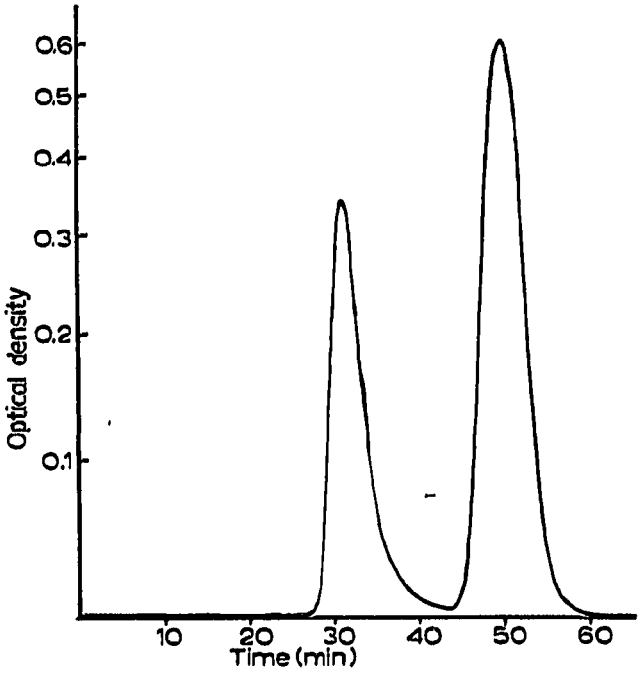

Fig. 4. Optical density at 570 mμ of the eluate from a 7.2 cm column packed with fraction 2 to which a mixture of dextran blue 2000 and thymol blue had been applied and eluted with bicarbonate buffer, pH 9.7, at 0.16 ml/min. The second peak to emerge was thymol blue and for this compound the number of theoretical plates/cm was 101.

Fractions 1, 2 and 3 gave columns with up to 100 plates/cm but fraction 1 was less suitable than the other fractions because on columns prepared with this fraction the dextran blue 2000 peak was distorted and higher operating pressures were required. Fraction 4 gave slightly less efficient columns and columns prepared from unfractionated powders were much less efficient than those prepared from the best fractions.

The packing procedure for columns was based on the method recommended by Whatman Ltd., for packing short columns with unfractionated microgranular ion-exchange cellulose. Several details of the procedure finally adopted were found to be critical for the regular preparation of efficient columns with fractionated powder.

In early experiments the consistency of the slurry used for packing columns was adjusted by adding a 20% excess of buffer to powder which had been allowed to settle in a measuring cylinder. However series of columns packed consecutively from powder kept in a measuring cylinder were progressively less efficient and it was thought that the agitation required to resuspend powder which had settled in a deep bed in a narrow vessel might damage the fibres. Examination of samples of fractionated powder (Figs. 3b and c) showed that on brief shaking the relative number of short fibres increased. To reduce breaking of fibres the procedure for preparing slurries described in Methods, which involves only gentle swirling, was used and found to give more consistent results.

The use of high flow rates of buffer and of a concentrated slurry were found to be essential for the preparation of good columns. Columns packed at lower flow rates or with more dilute slurries appeared to have good flow characteristics since samples of dye travelled down the column as completely horizontal zones but in these columns the zone became diffuse more rapidly. Thus it appears that the reduced efficiency of these columns was not due to a gross distortion of flow rate across the column bed but must be due to a lack of uniformity in flow on a much smaller scale. The high degree of uniformity of flow in a well packed column is indicated by the fact that the height equivalent to a theoretical plate (HETP) is 100 μ and thus approaches the dimensions of the cellulose fibres.

THOMPSON[3] suggested that rapid packing prevented classification of particles of different size during packing which might give rise to unevenness in the column bed but this explanation does not seem likely to apply to columns prepared from powder which had already been graded according to sedimentation velocity. An alternative explanation might be that rapid packing of a thoroughly mixed slurry results in freezing a random arrangement of fibres which might otherwise have become organised into groups of partially interlocked or aligned fibres and given a less homogeneous column bed.

In order to establish the best flow rate for using and testing columns the relationship between flow rate and column efficiency was investigated. The effect of flow rate on the HETP (the reciprocal of plates/cm) of a well-packed column for thymol blue is shown in Fig. 5. At flow rates greater than 0.16 ml/min the HETP rises and the column becomes less efficient so that for a given length of column the best separations of low molecular weight compounds are likely to be achieved at flow rates of this value or less.

If however the concentration of components emerging from the column does not have to be as high as possible and the shortest separation time is desired, a flow rate which gives a large number of plates/min for the compounds to be separated

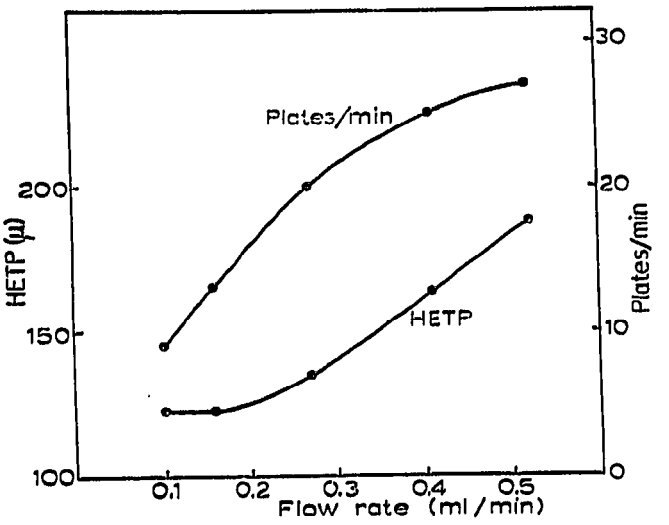

Fig. 5. The effect of flow rate on the HETP and the number of theoretical plates/min for thymol blue of a CM cellulose column packed with fraction 2 as described in Methods.

should be used. The length of column bed can then be chosen to give the necessary number of theoretical plates for the required separation. The results obtained for thymol blue (Fig. 5) showed that for this compound the number of plates/min increases with flow rate for flow rates of up to 0.52 ml/min and that at this flow rate thymol blue passed through more than twice as many theoretical plates in a given time than it did at 0.16 ml/min. Flow rates greater than 0.52 ml/min were not tested because at this flow rate the column bed packed down slightly and to achieve higher flow rates pressures were required for which the peristaltic pump and column connections were not suitable.

Metabolism of guanethidine in the rat

Fig. 6 shows the application of a series of columns to the identification and assay

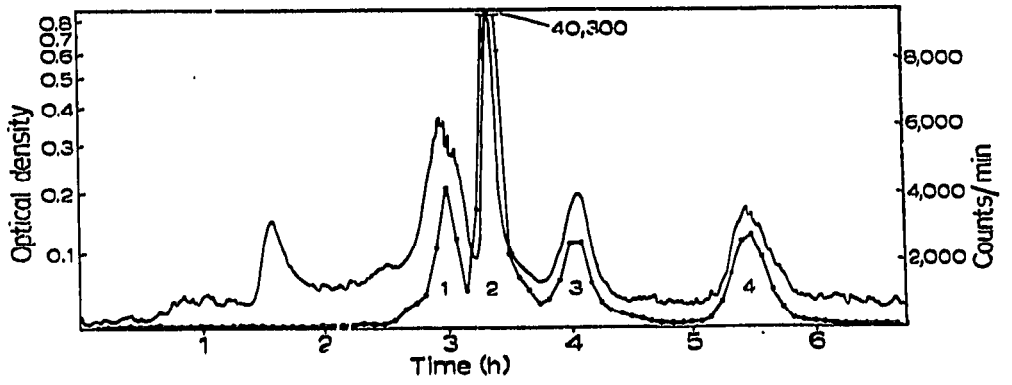

Fig. 6. Chromatography on CM cellulose columns of 1 ml of pooled rat urine collected 0–7 h after 3 mg/kg [^{3}H]guanethidine sulphate had been administered i.p. 2-(6-Carboxyhexylamino)-ethylguanidine (100 μg), and 300 μg each of guanethidine N-oxide sulphate and guanethidine sulphate were added to the urine as unlabelled markers. Four columns in series were used and the eluate assayed as shown in Fig. 2. The continuous trace shows the colorimetric recording and the circles connected by lines represent radioactivity. Peaks 2, 3, and 4 correspond in position to 2-(6-carboxyhexylamino)-ethylguanidine, guanethidine N-oxide and guanethidine, respectively. The colorimetric peak at 1 is due to naturally occurring Sakaguchi-positive components.

of metabolites of guanethidine excreted in rats urine. The flow rate used to elute the columns was 0.16 ml/min so that the concentration of products would remain high, the peaks could be sharp and their positions would provide a good criterion for identification.

It can be seen that four radioactive components were resolved from each other into symmetrical peaks. The radioactivity in these peaks accounted for 99% of that applied to the column. Radioactive peaks 2, 3, and 4 correspond in position and shape exactly to the colorimetrically determined peaks due to added guanethidine, 2-(6-carboxyhexylamino)-ethylguanidine and guanethidine N-oxide. The other radioactive peak (1) appeared in a position where normal urinary components which were Sakaguchi-positive were eluted and where 1-(6-carboxyhexylamino)-2-iminoimidazolidine, which can be formed from 2-(6-carboxyhexylamino)-ethylguanidine[1], would be expected to appear.

To confirm the identity of peak 1 a sample of rat urine was heated to transform the 2-(6-carboxyhexylamino)-ethylguanidine to 1-(6-carboxyhexyl)-2-iminoimidazolidine and then applied to a series of four columns. Fig. 7 shows the distribution of

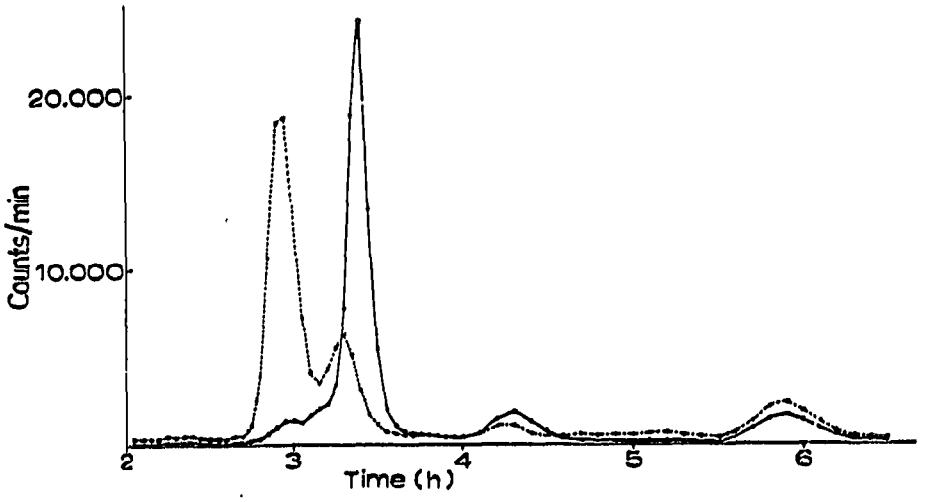

Fig. 7. Chromatography on CM cellulose columns of rat urine containing radioactive metabolites of guanethidine before (continuous lines) and after (broken lines) heating at 100° for 1 h at pH 7. Four 6 cm columns connected in series were eluted with borate–NaCl buffer, pH 9.7, at 0.16 ml/min. After heating peak 2 due to 2-(6-carboxyhexylamino)-ethylguanidine has decreased and peak 1 has increased indicating that peak 1 is due to 1-(6-carboxyhexyl)-2-iminoimidazolidine which is known to be produced from the former product on heating[1].

radioactivity in the eluate of columns to which unheated and heated urine had been applied. After heating, the major radioactive peak corresponding to 2-(6-carboxyhexylamino)-ethylguanidine was greatly reduced and was replaced by a large radioactive peak which corresponded in position to the unidentified component in unheated urine.

On the basis of this evidence it seems reasonable to conclude that 1-(6-carboxyhexyl)-2-iminoimidazolidine, 2-(6-carboxyhexylamino)-ethylguanidine, guanethidine N-oxide and unchanged guanethidine were the only radioactive products excreted in significant quantities in the urine of rats which had been treated with radioactively labelled drug.

CONCLUSION

DEAE and CM cellulose have been widely used for the fractionation of macromolecules. This study shows that CM cellulose can also be applied to smaller molecules for which synthetic ion exchange procedures are not adequate. DEAE columns prepared in a similar manner to the columns described in this paper have been used[6] for the separation of metabolites of chlorpropamide. In this case again the drug and metabolites could not be fractionated using synthetic ion exchange procedures. Further tests have shown that the barbituric acid derivatives amylobarbitone and phenobarbitone can be separated as two symmetrical peaks on DEAE cellulose columns and that the primary amine ephedrine will give a symmetrical peak on CM cellulose columns.

It seems likely therefore that CM and DEAE cellulose columns will be suitable for high resolution chromatography of a number of acidic and basic drugs and their metabolites.

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