CHROM. 4144

THE SPECIFIC ASSAY OF BASIC DRUGS IN URINE BY CM-CELLULOSE CHROMATOGRAPHY USING CONTINUOUS DRUG–DYE COMPLEX EXTRACTION AS A DETECTION SYSTEM

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(Received April 21st, 1969)

SUMMARY

An automated assay procedure suitable for detecting basic compounds in the eluate from CM-cellulose columns is described. Using this procedure the behaviour on CM-cellulose columns of a number of drugs and of naturally occurring interfering compounds in human urine was investigated. It was found that the drugs could be eluted as sharp symmetrical peaks which in most cases could be resolved from interfering urinary components by suitable choice of the pH of the buffer used for elution. Chromatograms obtained from cyclizine, pethidine and amphetamine at 1 μg/ml and morphine at 2 μg/ml in urine showed that these drugs could readily be resolved from urinary components at this concentration. The resolution of guanethidine and its metabolites required the successive use of two buffers for elution. Quantitative aspects of the method were investigated for guanethidine and its metabolites, and a linear relationship was obtained between the peak area due to each of these compounds and the amount added to drug-free urine in the range of 1–20 μg/ml.

INTRODUCTION

In 1945 BRODIE and his colleagues described a series of simple and comprehensive methods[1–5] for estimating drugs in biological samples. One of these methods can be applied to many drugs containing basic groups and depends on the formation of a coloured complex between the drug and the sulphonic acid indicator dye Methyl Orange. The complex can be separated from excess dye by extraction into a suitable organic solvent, and the quantity of complexed drug can be estimated spectrophotometrically. The replacement of Methyl Orange with other dyes has been described[6].

Although the method is inherently sensitive, its lack of specificity limits its application to biological samples which normally contain appreciable and variable amounts of compounds which form solvent-extractable complexes with the dye. Only samples containing concentrations of drug which give responses several times greater than this blank material can be analysed with confidence using the simple

procedure. The assay of small amounts of drug is also made difficult by the rapid and erratic adsorption of small amounts of drug–dye complex onto glass.

Recent work on methods for the separation of guanethidine and its metabolites, guanethidine N-oxide and 2-(6-carboxyhexylamino)ethylguanidine, showed that these compounds can be chromatographed with good resolution on suitably prepared CM-cellulose columns[7]. These columns raised the possibility of separating drugs from interfering compounds in biological samples, so that the dye-extraction procedure could be applied specifically to the desired compound.

To achieve this most simply, a continuous extraction procedure was set up so that the column eluate could be monitored for compounds which formed solvent-extractable dye complexes.

An automated procedure for the direct assay of relatively large amounts of drug in pharmaceutical preparations using solvent extraction of a dye complex has been described previously[8]. The method described below was designed to achieve a high degree of sensitivity, to extract relatively polar metabolites, and to avoid the exposure of solutions to glass surfaces which might adsorb the drug–dye complex.

This paper describes the application of CM-cellulose chromatography to the assay of a number of drugs including guanethidine and its metabolites in urine, using continuous dye complex extraction as a detection system.

MATERIALS AND METHODS

Ephedrine and amphetamine were obtained from British Drug Houses Ltd. Bethanidine and cyclizine were kindly supplied by Burroughs Wellcome. Guanethidine was obtained from these laboratories. 2-(6-Carboxyhexylamino)ethylguanidine and guanethidine N-oxide were synthesised as described previously[9].

Bromocresol Green was obtained from British Drug Houses Ltd. Where possible other reagents were Analar grade. Polythene cannula sizes P.P. 220, P.P. 100 and P.P. 25 were obtained from Portex Plastics Ltd. and soft polythene rod and P.T.F.E. rod from Xlon Products Ltd. P.T.F.E. capillary tubing 1.6 mm O.D. was obtained from L.K.B. Produkter.

The following buffers were used:

1 *M* citrate, pH 3.7, for the dye extraction assay procedure: 126 g citric acid monohydrate and 117 g trisodium citrate made up to 1 l with water.

Citrate/phosphate, pH 5.0, Na^+ 0.05 *M*: 8.95 g $Na_2HPO_4 \cdot 12\ H_2O$ and 2.52 g citric acid monohydrate made up to 1 l with water.

Citrate/phosphate, pH 7.0, Na^+ 0.05 *M*: 8.95 g $Na_2HPO_4 \cdot 12\ H_2O$ and 0.563 g citric acid monohydrate made up to 1 l with water.

Borate, pH 8.5, Na^+ 0.05 *M*: 9.54 g borax and 5.27 g boric acid made up to 1 l with water.

Borate, pH 10.0, Na^+ 0.05 *M*: 2 g NaOH and 3.53 g boric acid made up to 1 l with water.

Borate/chloride, pH 10, Na^+ 0.10 *M*: 2 g NaOH, 3.53 g boric acid, and 2.62 g NaCl made up to 1 l with water.

Dye-extraction procedure 1

The lay-out of the apparatus and the details of the reagents used are shown in

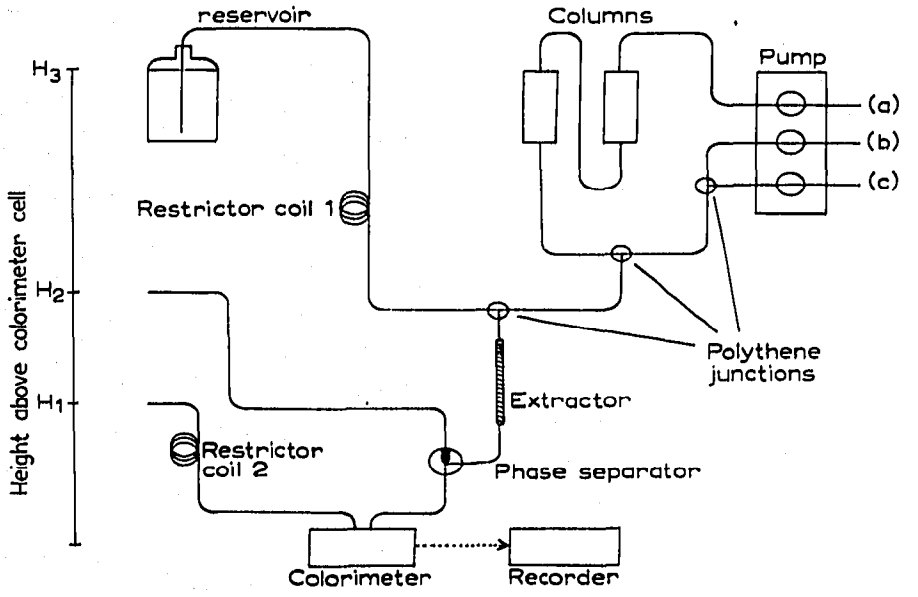

Fig. 1. Apparatus used for CM-cellulose chromatography combined with dye-extraction assay procedure 1 (see METHODS). Reagents and their flow rates were as follows: (a) Buffer used to elute columns at 0.33 ml/min. (b) Dye solution. 1% Bromocresol Green in 0.032 *M* NaOH at 0.07 ml/min. (c) 1 *M* citrate buffer, pH 3.7, to control pH in extractor, at 0.07 ml/min. Resistance coils 1 and 2 were 232 cm and 198 cm lengths of P.P. 25 polythene cannula. The connections to and from the pump to the columns, from the columns to the extractor and from the separator to the colorimeter were P.P. 25 polythene cannula. All other connections were P.P. 100 polythene cannula.

Fig. 1. The CM-cellulose columns were prepared as previously described[7]. The polythene junctions were made by drilling holes in soft polythene with a No. 53 drill at high speed to give holes into which polythene cannula (P.P. 100) could be fitted directly. The phase separator junction was made similarly, but the vertical hole leading aqueous phase and excess chloroform upwards was larger so that the phases could separate, and was made with a No. 32 drill. This hole was fitted with a short length of P.P. 220 polythene cannula into which was inserted a length of P.P. 100 cannula leading to waste. The extractor was made by inserting the spiral nylon outer binding of a guitar G string into 15 cm of P.P. 220 polythene cannula. This helix caused rotation of the two phases and was designed to accelerate the attainment of equilibrium between them.

The flow rate of chloroform into the extraction system and the flow rate of chloroform through the flow cell are controlled by the heights of the chloroform reservoir and outflows 1 and 2. Since most resistance to flow occurs in the restrictor coils 1 and 2, the flow rate of chloroform into the extractor will be related to $H_3 - H_2$. It was found that H_2 should be placed above the level of the mixing junctions and the colorimeter, since the positive pressure thus introduced suppressed the formation of air bubbles in the colorimeter cell. The flow rate of chloroform through the colorimeter cell can be adjusted by changing $H_2 - H_1$. This flow rate must be less than the total flow rate of chloroform into the extractor as it is necessary to reject some of the chloroform through outlet 2 in order to ensure that no aqueous phase passes through the colorimeter cell.

A 15-mm path length cell in a Technicon colorimeter fitted with 420-mμ filters

was used. Connections to the flow cell were made using acidflex (Technicon). Typical running conditions with H_1 27 cm, H_2 39 cm, H_3 228 cm, gave flow rates of 0.45 ml/min of chloroform into the system and 0.11 ml/min of chloroform through the cell.

When starting and stopping the procedure it is essential that the highly coloured aqueous phase should not pass through the colorimeter cell, since it may adhere to the glass wall. A satisfactory starting procedure is to lower outflow 2 and pass only chloroform into the system with the peristaltic pump stopped but set so that it clamps the pump tubes, thus preventing back-flow of chloroform. Outflow 2 can then be raised to its operating level, and when flow through the cell is established and all air bubbles are displaced, the pump can be started and aqueous phase introduced into the extractor.

To stop the assay, outflow 2 should be lowered and the pump stopped. When all aqueous phase has been cleared from the system, the outflow from the column and the leads carrying dye and buffer can be disconnected, the chloroform flow stopped and the system allowed to drain. It was found to be most convenient to run the apparatus continuously day and night for five-day periods. The level in the chloroform reservoir fell only a few cm during the day, and it was sufficient to refill the reservoir twice a day. About every five days the pump tubes were changed. Before connecting new pump tubes to the column or extraction system they were allowed to pump their individual reagents to waste for several hours, so that interfering compounds present in the tubing could be removed.

Samples were applied by stopping the pump, transferring the input leading to the column to the sample, and pumping the sample onto the column for a fixed time. The pump was then stopped, the input transferred back to buffer, and elution started immediately.

Dye-extraction procedure 2

For more sensitive work the procedure was modified in the following ways:

(1) The extraction helix was replaced with 1 m of P.T.F.E. capillary tubing, since this was found to reduce baseline wandering.

(2) This P.T.F.E. coil required higher chloroform pressures than could be readily achieved by gravity in the laboratory, and chloroform was pumped at 0.56 ml/min into the extraction system. Chloroform is difficult to pump directly because it attacks the tubing used in peristaltic pumps, so it was pumped by displacement from an aspirator into which water was pumped at 0.56 ml/min. To achieve uniform flow, the aspirator should not contain air. A simple head which can be fitted to a Winchester of chloroform, filled to the top with water, is shown in Fig. 2. Restrictor coil No. 1 was removed.

(3) As a result of the high concentration of dye in extraction procedure 1, a considerable amount of coloured material was extracted into the chloroform in the absence of samples and raised the baseline. In procedure 2, the concentration of dye was reduced, and the dye solution and citrate buffer (Fig. 1) were replaced by a 0.2% solution of Bromocresol Green in 1 *M* citrate buffer, pH 3.7, which was pumped at 0.1 ml/min into a junction where it mixed with the column effluent.

(4) The colour in the chloroform phase was measured using a 3-cm path length flow cell in a Beckman DB-UV spectrophotometer. The cell was constructed of a cylindrical P.T.F.E. body and siliconised microscope slides as shown in Fig. 3. The

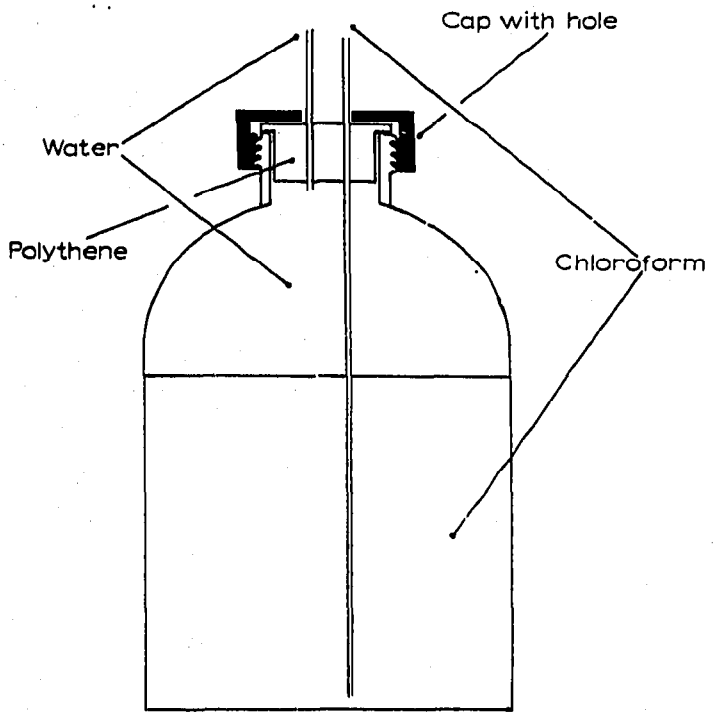

Fig. 2. Aspirator used to pump chloroform by displacement with water used in extraction procedure 2 (see METHODS).

P.T.F.E. faces were carefully finished on a lathe so that they would form a good seal with the glass on tightening the screws shown in Fig. 3. The polythene leads to and from the cell were fixed tightly by stretching a piece of cannula, cutting the narrow part and threading this through the hole in the P.T.F.E. from the outside to the inside of the cell. The cannula could then be pulled through the hole until tight,

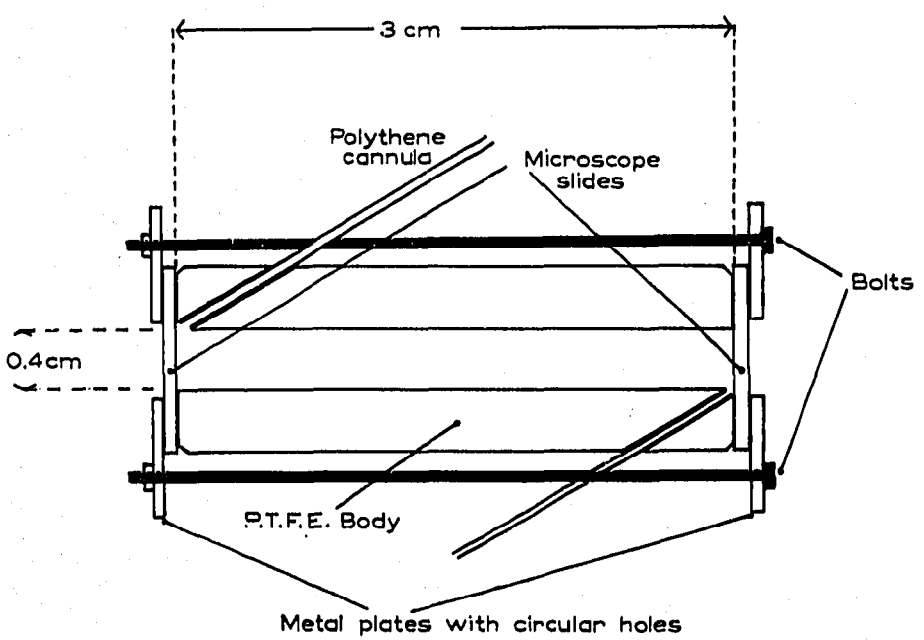

Fig. 3. 3-cm path length P.T.F.E. flow cell with glass windows.

the excess cannula coming from inside the cell cut and the projecting end pulled gently back until it was slightly recessed inside the hole in the P.T.F.E. body. The spectrophotometer was connected through a scale expander to a Servoscribe recorder. Full slit width and a wavelength of 432 mμ were used. To achieve a good baseline at × 10 scale expansion the system was left running overnight.

In this procedure, the pump cannot be stopped to apply sample because this stops the flow of chloroform through the system and can lead to aqueous phase passing into the colorimeter flow cell. Samples were applied to the column by clamping the input side of the pump tube leading to the column using artery forceps, rapidly transferring the line to sample, unclamping to allow this to be pumped into the column for a measured time and then reclamping to switch back to buffer.

RESULTS AND DISCUSSION

Colorimetric assay procedures

Bromocresol Green was chosen for dye extraction because it was found to give a better response for guanethidine than Methyl Orange or a number of other dyes. To establish optimum assay conditions, the effects of varying several parameters were tested using pulses of guanethidine applied directly to the assay procedure. It was found that increasing the length of the extraction helix did not significantly change this response, showing that extraction had reached equilibrium. It was also found that the extraction helix could be replaced by 1 m of P.T.F.E. cannula without impairing the efficiency of extraction. Although this gave a better baseline than the

TABLE I

RETENTION TIMES (min) OF NORMALLY OCCURRING HUMAN URINARY COMPONENTS AND OF A NUMBER OF DRUGS

Two 1.2 × 6 cm CM-cellulose columns were eluted with various buffers at 0.33 ml/min. Molarities refer to Na^+ concentrations.

Drug or urinary component	*Buffer*				
	Citrate/phosphate pH 5, 0.05 M	*Citrate/phosphate pH 7 0.05 M*	*Borate pH 8.5, 0.05 M*	*Borate pH 10, 0.05 M*	*Borate/chloride pH 10, 0.1 M*
U_1	34	32–40	30–50	34–47	30–40
U_2	48				
U_3	63				
U_4	94	102	107	112	70
2-(6-Carboxyhexylamino)-ethylguanidine	>180	105	—	50	42
Guanethidine N-oxide	>180	116	—	126	77
Guanethidine	>180	—	—	—	114
Cyclizine	—	—	69	—	—
Morphine	—	—	72	—	—
Pethidine	—	—	87	—	—
Strychnine	—	—	110	52	—
Methadone	—	> 360	120	93	—
Ephedrine	—	—	123	69	—
Bethanidine	—	122	127	—	—
Amphetamine	—	—	136	94	—

helix, a higher pressure was required to achieve the desired flow rate of chloroform, and the P.T.F.E. cannula was used only for the sensitive assay.

With the flow rates reported for procedure 2, 0.1% and 0.2% of Bromocresol Green were found to give the same response for guanethidine, so that under these conditions it seems likely that the drug is being completely extracted. These conditions, however, gave a poor response to 2-(6-carboxyhexylamino)ethylguanidine and in order to increase the sensitivity of the method for poorly extracted compounds, procedure 1 as described in METHODS, which allowed the dye to be introduced at high concentration, was used. The response to the metabolite still suggested that extraction of this compound was incomplete, but it was strong enough to allow the metabolite to be estimated in urine.

Behaviour of drugs and urinary components on CM-cellulose columns

The retention times of components found in normal human urine and of a number of drugs eluted from two 6-cm CM-cellulose columns connected in series using several buffers at 0.33 ml/min are shown in Table I. It can be seen that at pH 5, normal urine gives rise to four resolved peaks (U_1, U_2, U_3 and U_4) and that with buffers of pH 7 or higher, the retention times of U_2 and U_3 shorten, and these components merge with U_1 into a single, virtually unresolved peak. The retention time of the fourth component, rather than diminishing, increases slightly as the pH is raised. The retention times of the drug tested are such that all the drugs are eluted after U_1, U_2 and U_3, so that these components do not interfere with the assay of the drugs.

At pH 8.5 cyclizine, pethidine, morphine and amphetamine are separated from U_4. Fig. 4a, b and c shows chromatograms obtained with drug-free human urine and with urine to which these compounds had been added. From the available literature[10–12] it can be estimated that the sensitivity of the method should be adequate for the assay of pethidine, morphine and amphetamine in the urine of patients receiving therapeutic doses of the drugs. Pethidine, for instance, was isolated from pooled human urine by BURNS *et al.*[10], and it was shown that 5% of the dose was excreted unchanged. This proportion of a 60-mg dose in a 1 500-ml 24-hour urine sample would give a concentration of 2 μg/ml.

At pH 8.5 methadone, strychnine and ephedrine are not resolved from U_4. However, these compounds can be separated from U_4 by using a buffer at pH 10, since raising the pH does not markedly alter the retention time of U_4 but shortens the retention times of the drugs. This effect is almost certainly connected with the reduced degree of ionisation of the drugs at the higher pH. By changing the pH of the buffer, it should therefore be possible to adjust the retention of primary, secondary and tertiary amines relative to U_4. The insensitivity of U_4 to pH change suggests that this compound is a strong base such as a quaternary ammonium compound.

It can be seen from Table I that bethanidine, which is a strong base, does not shift relatively to U_4 when the pH is raised from 7 to 8.5. The retention time of bethanidine is slightly longer than that of U_4. Assay of this compound could probably be achieved by using a longer column to increase resolution and by reducing the concentration of the dye entering the extraction system so that the ratio of the responses of bethanidine, which is well extracted, and U_4, which is poorly extracted, can be increased.

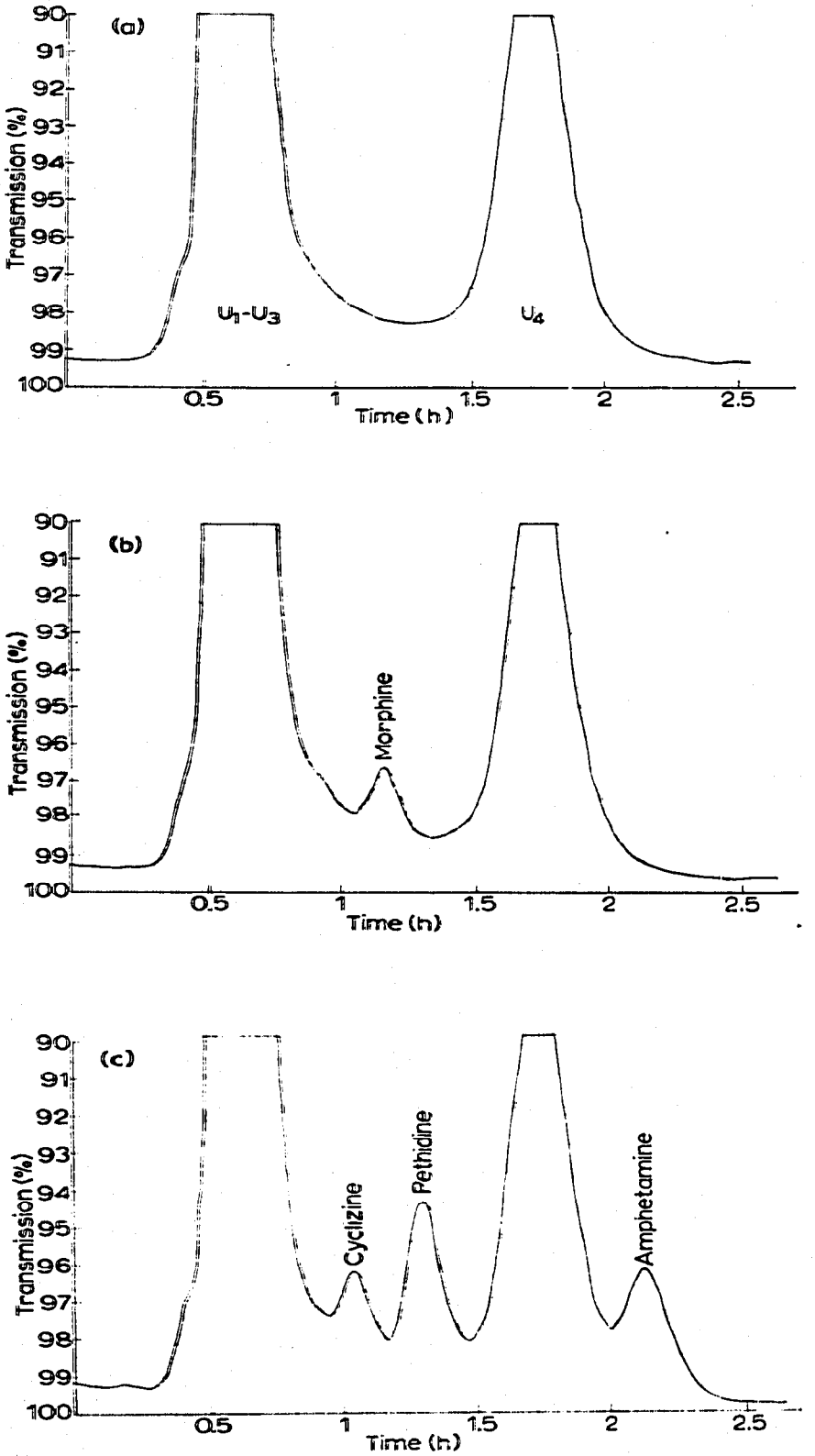

Fig. 4. Assay of human urine samples by CM-cellulose chromatography using extraction procedure 2. 2-min pulses (0.66 ml) of samples were applied and the column was eluted with borate buffer, pH 8.5, Na^+ 0.05 *M*. (a) Blank urine. (b) Blank urine to which morphine had been added to give a concentration of 2 μg/ml. (c) Blank urine to which cyclizine, pethidine and amphetamine had been added to give. concentrations of 1 μg/ml for each compound.

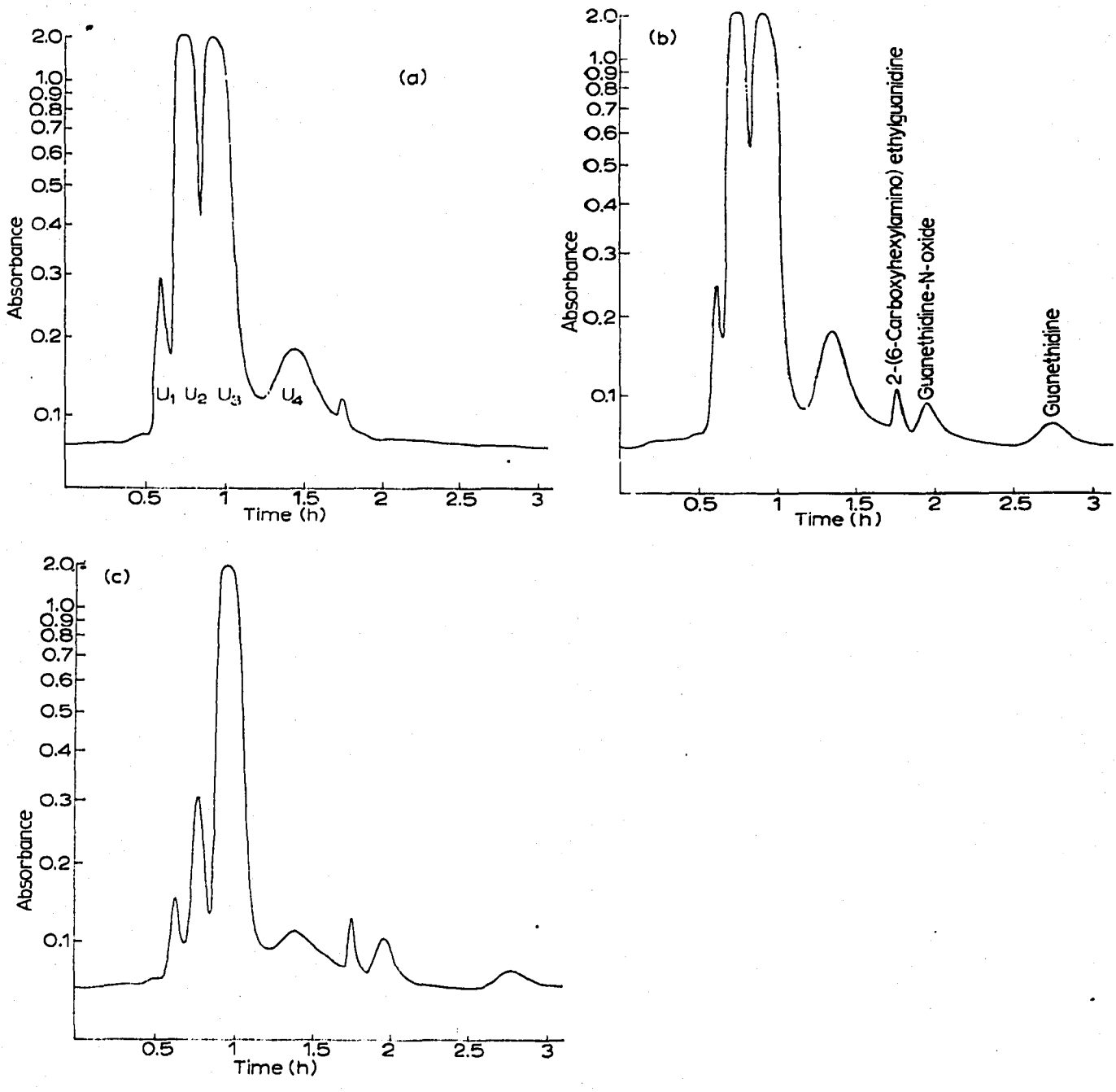

Fig. 5. Assay of guanethidine and its metabolites in human urine using CM-cellulose chromatography and extraction procedure 1. 2-min pulses (0.66 ml) of sample were eluted as described in RESULTS. (a) Blank urine. The small peak following U_4 is due to buffer change (see RESULTS). (b) Blank urine to which 2-(6-carboxyhexylamino)ethylguanidine sulphate monohydrate, guanethidine N-oxide sulphate, and guanethidine sulphate had been added to give concentrations of 2.5, 5 and 5 μg/ml respectively. (c) Urine from a patient receiving 40 mg of guanethidine sulphate per day.

The complete separation of guanethidine and its metabolites from each other and from the urinary components requires a buffer change during elution and is described in the following section in which the application of the method to the quantitative assay of these compounds is also shown.

Application to the quantitative assay of these compounds and its metabolites in human urine

The resolution of guanethidine and its metabolites from urinary components can be achieved by making use of the fact that at pH 5 the drug and its metabolites all exist to some extent in a form carrying two positive charges and are retained on the column at this pH for a longer time than the urinary components. At this pH, however, the retention time of guanethidine is very long, and in addition, guanethidine N-oxide and 2-(6-carboxyhexylamino)ethylguanidine are not resolved. The buffer was therefore changed during the run to pH 10, Na^+ 0.1 *M*. The column was left running in this buffer and eluted with pH 5, Na^+ 0.05 *M* buffer exactly 30 min before sample application.

The sample was applied to the column and elution was continued at pH 5, Na^+ 0.05 *M*. After 53 min the buffer was changed to pH 10, Na^+ 0.1 *M*. Under these conditions all the components are resolved as shown in Fig. 5a and b. If the buffer was changed sooner than this, 2-(6-carboxyhexylamino)ethylguanidine was not fully resolved from U_4, whereas if the buffer was changed later, this metabolite ran close to guanethidine N-oxide.

To find out whether the method could be applied quantitatively, normal urine samples, to which known amounts of guanethidine and its metabolites had been added, were applied to the column. The peak areas corresponding to the drug and the metabolites were estimated by multiplying the peak height in absorbance units by the peak width at half the height. Fig. 6 shows reasonable linearity and reproducible response for guanethidine, guanethidine N-oxide and 2-(6-carboxyhexylamino)ethylguanidine in the range 1–20 μg/ml of urine. Correction of the area of the 2-(6-carboxyhexylamino)ethylguanidine peak was necessary because a small peak due to the buffers alone occurred in the same position in the chromatogram as that of the metabolite. Provided the time of regeneration of the columns at pH 5 and the timing of the buffer change remained constant, this peak gave a constant area,

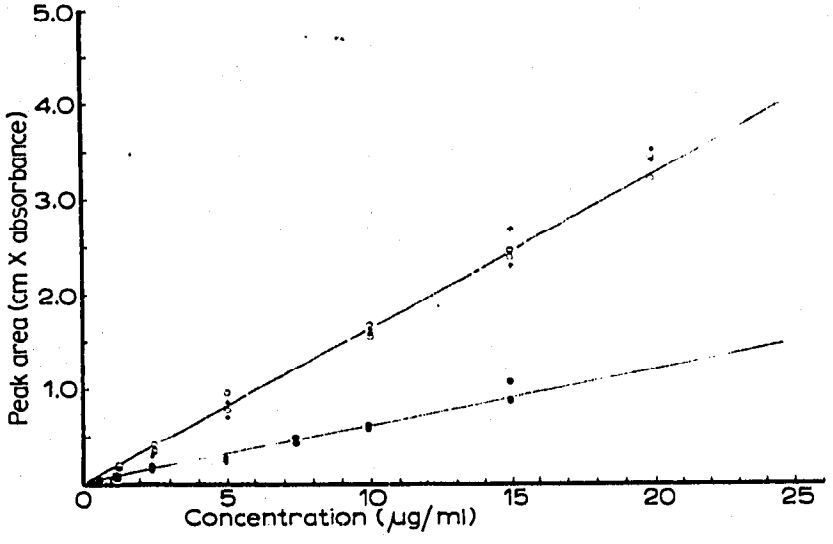

Fig. 6. Calibration curves showing the peak areas obtained when urine samples to which different qualities of guanethidine and its metabolites had been added were assayed by CM-cellulose chromatography as described in results. ○, guanethidine sulphate; +, guanethidine N-oxide sulphate; ●, 2-(6-carboxyhexylamino)ethylguanidine sulphate monohydrate.

equivalent to 1 μg/ml of the metabolite. This constant value was subtracted from the peak area of 2-(6-carboxyhexylamino)ethylguanidine to give the results shown in Fig. 6.

Studies with [^{3}H]guanethidine in man[13] have shown that 2-(6-carboxyhexylamino)ethylguanidine and guanethidine N-oxide are the only metabolites excreted in the urine in significant amounts. Fig. 5c shows a chromatogram obtained with urine from a patient receiving 40 mg guanethidine sulphate daily. The concentrations found in this sample were: guanethidine, 1.5 μg/ml; 2-(6-carboxylhexylamino)ethylguanidine, 2.9 μg/ml; guanethidine N-oxide, 2.2 μg/ml.

CONCLUSION

The results reported above indicate that CM-cellulose chromatography followed by continuous dye extraction can be used for the assay of a number of basic drugs in human urine at concentrations which would be expected following administration of therapeutic doses. The method can be used for quantitative assay of guanethidine and its metabolites, and it is reasonable to suppose that quantitative assay will be possible also for other drugs.

Since the assay will detect even polar compounds such as 2-(6-carboxyhexylamino)ethylguanidine, which contains a free carboxylic acid group, it is likely to be useful for the detection of unknown metabolites or the aglycones of metabolites of other basic drugs.

It may also be possible to apply this type of procedure to the assay of certain drugs in plasma samples. The amount of normally occurring dye-extractable material is much less in plasma than in urine, but the concentration of drug is also likely to be less. In the Methyl Orange assay described for pethidine in plasma, blank values of about 0.1 μg/ml were found and levels of pethidine 0–7 h after a therapeutic dose were 0.2–1.5 μg/ml. Increased sensitivity of the assay for compounds in plasma might be achieved by solvent extraction prior to chromatography. If the drug were extracted from a 5-ml plasma sample with an organic solvent and the whole extract applied to a column, it should be possible to obtain a response similar to that shown in Fig. 4, with 0.13 μg/ml of pethidine in plasma, and this response is probably at least four or five times above the limit of sensitivity of the method.

In the case of morphine, although the response to dye-extraction assay is weak, the chromatographic behaviour of the drug might be useful because morphine is a compound which is extremely difficult to isolate by solvent extraction from biological samples prior to sensitive assay. If the fluorescence assay described by TAKEMORI[14] were automated it might be possible to combine this with CM-cellulose chromatography to provide a sensitive and specific assay for this compound.

ACKNOWLEDGEMENT

This work was performed under joint Contract with the United States National Institutes of General Medical Sciences, Contract No. PH 43-66-1167.

REFERENCES

1 B. B. BRODIE, S. UDENFRIEND AND J. E. BAER, *J. Biol. Chem.*, 168 (1947) 299.
2 B. B. BRODIE, S. UDENFRIEND, W. DILL AND G. DOWNING *J. Biol. Chem.*, 168 (1947) 311.
3 B. B. BRODIE, S. UDENFRIEND, W. DILL AND T. CHENKIN, *J. Biol. Chem.*, 168 (1947) 319.
4 B. B. BRODIE, S. UDENFRIEND AND J. V. TAGGART, *J. Biol. Chem.*, 168 (1947) 327.
5 B. B. BRODIE, S. UDENFRIEND AND W. DILL, *J. Biol. Chem.*, 168 (1947) 338.
6 G. CRONHEIM AND P. A. WARE, *J. Pharmacol. Exptl. Therap.*, 92 (1948) 98.
7 C. MCMARTIN AND J. VINTER, *J. Chromatog.*, 41 (1969) 188.
8 N. R. KURZEL, *Technicon Symposium, 1, 1966*, Mediad, New York, 1967, p. 218.
9 F. B. ABRAMSON, C. FURST, C. MCMARTIN AND R. WADE, *Biochem. J.*, 113 (1969) 143.
10 J. J. BURNS, B. L. BERGER, P. A. LIEF, A. WOLLACK, E. M. PAPPER AND B. B. BRODIE, *J. Pharmacol. Exptl. Therap.*, 114 (1955) 289.
11 E. L. WAY AND T. K. ADLER, *Pharmacol. Rev.*, 12 (1960) 383.
12 L. G. DRING, R. L. SMITH AND R. T. WILLIAMS, *J. Pharm. Pharmacol.*, 18 (1966) 402.
13 A. W. D. LEISHMAN, C. MCMARTIN, G. SANDLER AND J. VINTER, in preparation.
14 A. E. TAKEMORI, *Biochem. Pharmacol.*, 17 (1968) 1627.