Journal of Chromatography, 168 (1979) 117–124

CHROM. 11,281

GAS–LIQUID CHROMATOGRAPHY OF SUBMICROGRAM AMOUNTS OF DRUGS

V. PREPARATION OF LOW-ACTIVITY PACKED COLUMNS AND THEIR APPLICATION TO THE TOXICOLOGICAL ANALYSIS OF UNDERIVATIZED POLAR DRUGS IN THE LOW NANOGRAM RANGE

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(Received April 19th, 1978)

SUMMARY

When diatomaceous earth and glass columns are acylated prior to coating with a silicone liquid phase, then subjected to heat treatment in an atmosphere of nitrogen, gas chromatographic columns can be prepared that show a marked reduction in adsorption. These columns can be used with a nitrogen-specific detector to chromatograph unmodified polar compounds such as morphine and cyclobarbital in nanogram amounts. Virtually no alteration of peak shape and no variation of retention time are observed over the range 10^{-6}–10^{-9} g of polar drugs. This represents, for these polar drugs, an "improvement" in chromatographic capability of the order of about 1000-fold in comparison with the best conventional commercial columns. Application to toxicological analysis of morphine in urine is described.

INTRODUCTION

In 1966, McMartin and Street[1,2] discussed the preparation, scope and limitation of gas chromatographic (GC) columns. They showed that heat treatment of diatomaceous earth coated with a silicone gum rubber containing a small amount of tristearin could be used to produce steel GC columns for the sub-microgram determination of barbiturates and other polar drugs. A year later, Street[3] showed that these tristearin columns, when used for prolonged periods above about 250°, were unstable and he described[3], for the GC determination of alkaloids, the preparation of more stable steel columns that can be used up to about 330° without significant deterioration. However, although these columns can be used satisfactorily for many drugs in amounts less than 10^{-7} g, they generally show a working cut-off

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point for more polar drugs at about 0.1 μg of drug. In subsequent years, experimentation has been directed towards decreasing these detection limits by reducing the activity of the columns. These further experiments, with glass columns, have shown that acylation prior to applying a combination of coating the surface of both column and support material with SE-52 followed by the heat treatment procedure of Street[4] produces columns displaying much less activity, which has enabled the detection limits to be extended to the low nanogram range even for such polar compounds as morphine, cyclobarbital and paracetamol (in the free, underivatized, unmodified state) using a nitrogen-specific detector.

EXPERIMENTAL

The preparation of the diatomaceous earth consists of three stages: acylation; coating and heat-treatment; and column deactivation.

Acylation

A 300-g amount of Chromosorb G NAW (100–120 mesh) was washed with acid and water as described by Street[4], dried and then treated with 300 g of pyridine and 200 g of benzoyl chloride. The suspension was stirred thoroughly and then allowed to stand for 48 h. The treated Chromosorb G was then washed with acetone until it no longer smelled of pyridine and was dried until free-flowing.

Coating and heat treatment

A 150-ml volume of 10% (w/v) SE-52 silicone gum in toluene was added to 100 g of acylated support material. The suspension was stirred thoroughly and allowed to stand at room temperature for 48 h. The mixture was then filtered under nitrogen into a Pyrex tube (I.D. 4 cm) and dried until again free-flowing. Still under nitrogen, the product was heated at 410° for 1 h and, when cool, was divided into three equal portions (F1, F2 and F3) as previously described[4].

Preparation of the deactivated column

A Pyrex glass column (6 ft. × 1/8 in. O.D.) was cleaned with concentrated hydrochloric acid and then washed to remove acid with de-ionized water followed by acetone. The column was dried and filled with a mixture of pyridine and benzoyl chloride (3:2, v/v), making sure that all gas bubbles were excluded. The tube was stoppered and kept for 3 days, then emptied, rinsed thoroughly with toluene and dried in an oven at 100°. Two variations of deactivation were performed:

(a) A 5% (w/v) solution of SE-52 silicone gum in methylene chloride was drawn by suction into three windings of the column and slowly moved through it to the other end. This procedure was repeated and, after pouring out the excess liquid, the column was dried first at 100° and finally at 350° under nitrogen for 10 h. The glass column was then ready for filling.

(b) The glass column was filled with coated support material (fraction F1)[4] and heated at 400° under nitrogen for 1 h, then this powder was poured out. The column was re-filled with fraction F3 of coated support material[4] and heated at 350° under nitrogen in the gas chromatograph until a stable baseline was obtained.

Gas chromatography

The gas chromatograph used to test the prepared columns was a Perkin-Elmer Model F20B employing both a nitrogen–phosphorus-specific detector (NPD) and a flame-ionization detector (FID). Unless otherwise stated, the GC conditions were as follows: carrier gas (nitrogen) flow-rate, 30 ml·min^{-1}; hydrogen flow-rate, 2 ml·min^{-1}; air flow-rate, 100 ml·min^{-1}; column temperatures, 250° for morphine, amitriptyline and methaqualone and 220° for cyclobarbital, paracetamol and meprobamate; injector and detector temperatures, 300°.

The detection limit was taken to be three times the baseline noise. Decreasing amounts of cyclobarbital, morphine (or other drug) were injected on to the column and the corresponding peak heights were measured. The volume of the solutions injected was kept constant at 1 μl. Pure solvents were injected between drug injections to exclude any "memory effect".

In an attempt to characterize drug adsorption on the columns, equal amounts of cyclobarbital and C_{24} (tetracosane), or morphine and C_{24}, (each containing from 0.25 to 10 μg·μl^{-1}) were injected with the NP detector in the FID mode. Comparisons were also made using caffeine as the reference compound instead of the hydrocarbon, thus obviating the necessity to change from the NPD to the FID. In this case nanogram amounts of solute were injected.

Application to analyses of morphine in urine samples

To 10 ml of a putrid sample of post-mortem urine (pH 9.5) were added 10 μg of morphine. This mixture was saturated with ammonium sulphate and extracted with three 5-ml portions of ethyl acetate. The organic solvent phase was separated and re-extracted with 1 ml of 10% (w/v) tartaric acid solution and the acid phase was then adjusted to pH 9.5 and extracted with 100 μl of ethyl acetate. A 1-μl volume of this extract, containing less than 100 ng of morphine, was injected into the gas chromatograph.

RESULTS

Fig. 1 shows how the peak height varies when various amounts of cyclobarbital are chromatographed (a) on a conventional commercial OV-1 column and (b) on the SE-52 deactivated column. The graphs displayed in Fig. 2a and b were constructed in a similar manner but relate to the injection of various amounts of morphine.

The ratios of the peak heights of cyclobarbital (or morphine) to the peak heights of the hydrocarbon C_{24} plotted against amounts of drug injected are shown in Fig. 3, the glass column being deactivated by heating the coated support material for 1 h at 400° [deactivation procedure (b)]. Fig. 4 gives the results obtained in the same way as those in Fig. 3 except that the glass column was first coated with a solution of SE-52 [deactivation procedure (a)]. These results were obtained with the NPD in the FID mode.

Figs. 5 and 6 display the results obtained by substituting caffeine for the C_{24} hydrocarbon used in the experiments for Figs. 3 and 4 and using the NPD in the nitrogen-detector mode.

Fig. 7a and b shows the use of the proposed column when applied to the detection of morphine in urine. For comparison purposes, Fig. 7c gives the chromatogram obtained when 50 ng of morphine were injected.

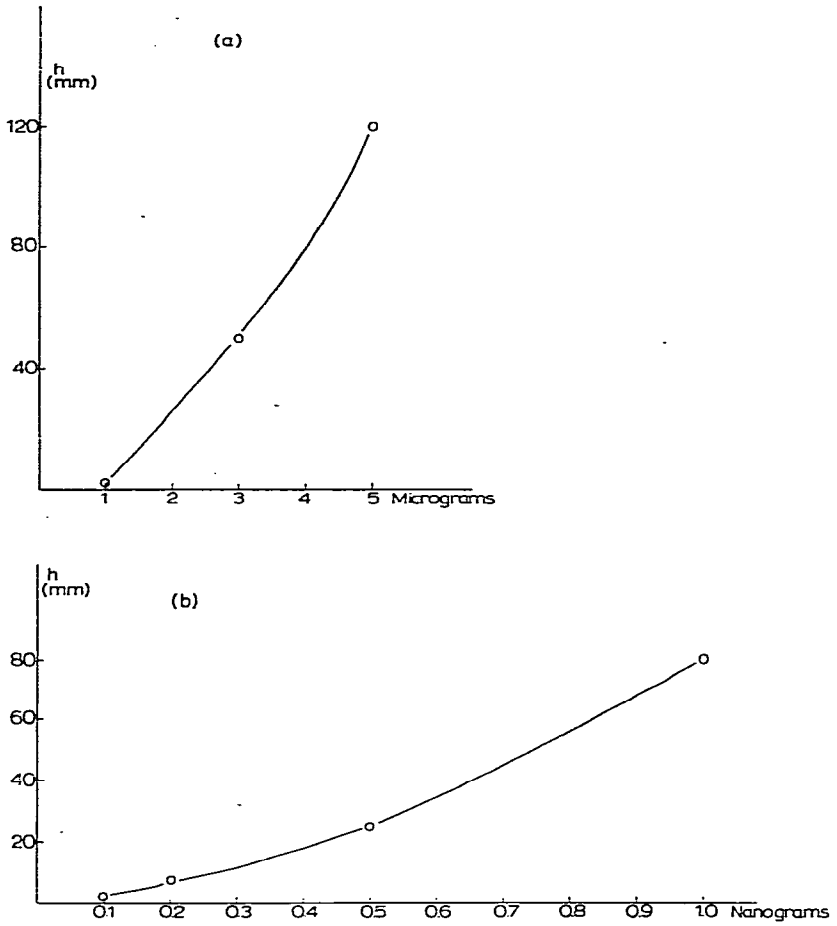

Fig. 1. (a) Graph showing relationship between peak height (h) and micrograms of cyclobarbital injected into a commercial OV-1, glass column (6 ft. × $\frac{1}{8}$ in. O.D.). Note that if less than 1 μg of drug is injected, no peak is observed. (b) As (a) but for nanograms of cyclobarbital injected into the proposed column. Note that a peak is still just discernible when only 0.1 ng of cyclobarbital is injected.

DISCUSSION

Comparison of Fig. 1a and b shows that when decreasing amounts of cyclobarbital are chromatographed, the commercial OV-1 column (Fig. 1a) gives no peak when less than 1 μg is injected, whereas with the deactivated column described in the text, a peak is still discernible when 0.2 ng of cyclobarbital is injected, the cut-off point here being at 0.1 ng. A similar comparison of Fig. 2a and b shows that the injection of decreasing amounts of morphine gives a cut-off point for the commercial column at 0.4 μg, whereas with our column the cut-off point is 0.8 ng. Hence, for these two polar drugs the proposed column shows an improvement in detection limit of the order of 1000-fold. Apart from the obvious results accruing from this improve-

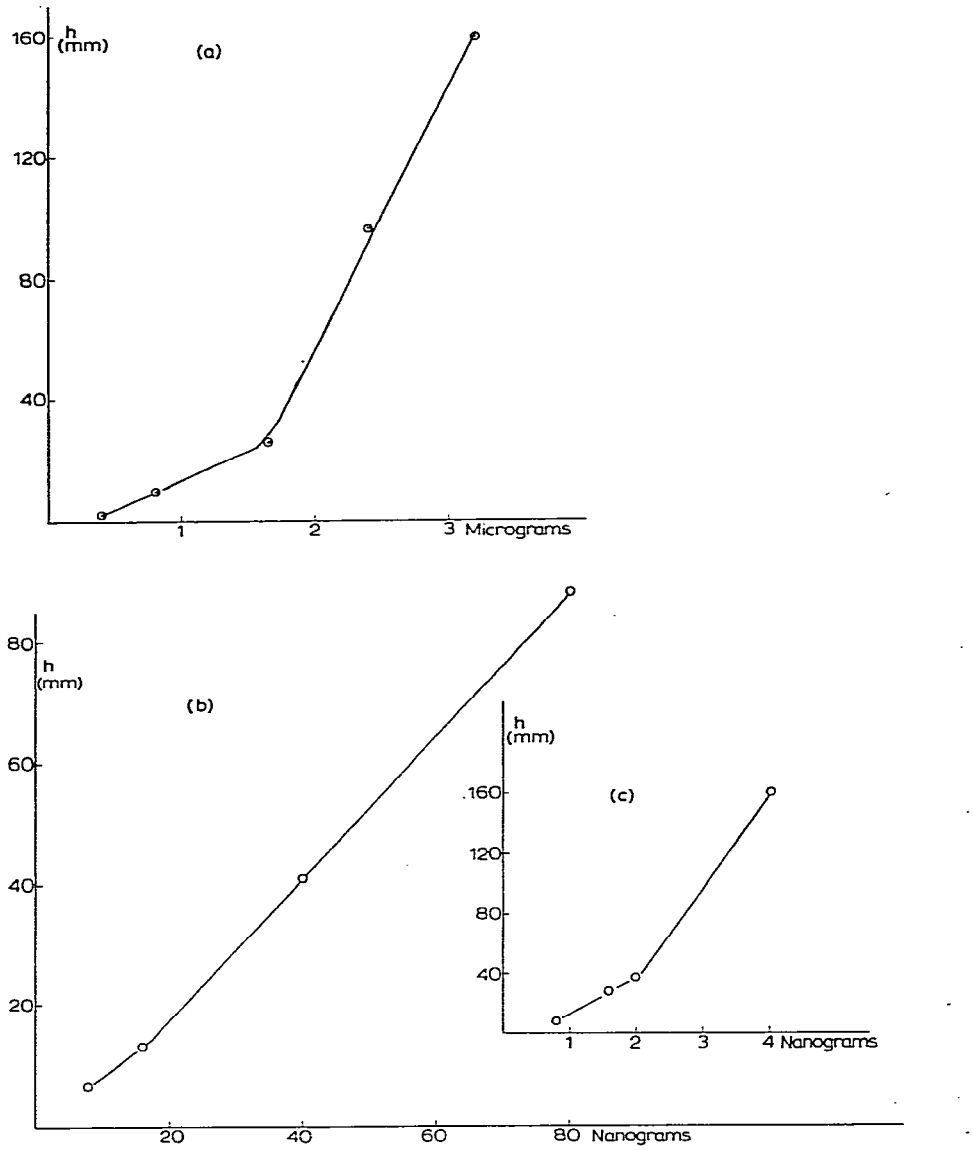

Fig. 2. (a) Graph showing relationship between peak height (h) and micrograms of morphine injected into a commercial OV-1 column. Note that the "cut-off" point is about 0.5 μg of drug. (b) and (c) as (a) but for nanograms of morphine injected into the proposed column. Note that the "cut-off" point is less than 1 ng.

ment, we have found in some preliminary experiments that such columns are of great value when coupled to a mass spectrometer.

Coating of the glass surface as well as the diatomaceous earth [procedure (a)] produces results for morphine that show less adsorption relative to the C_{24} hydrocarbon than when the diatomaceous earth only is treated. In this instance,

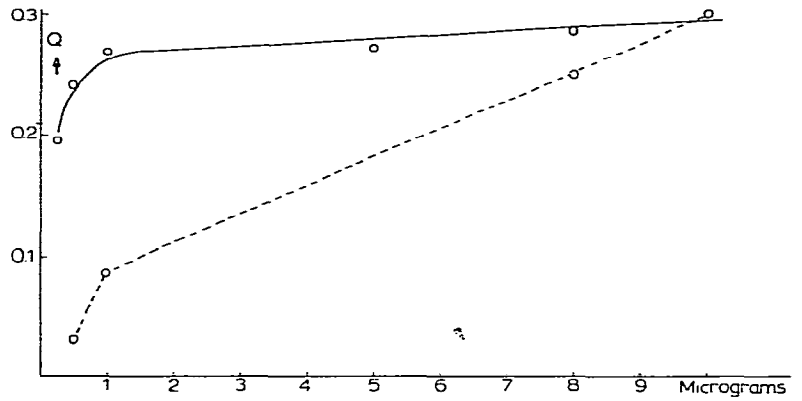

Fig. 3. Graphs showing how the relative adsorption (Q) varies with amount of cyclobarbital (solid line) and morphine (broken line) injected, using the treated support only. Q is defined as the ratio of the peak height of the drug to the peak height of C_{24}. Note that the detector was in the FID mode.

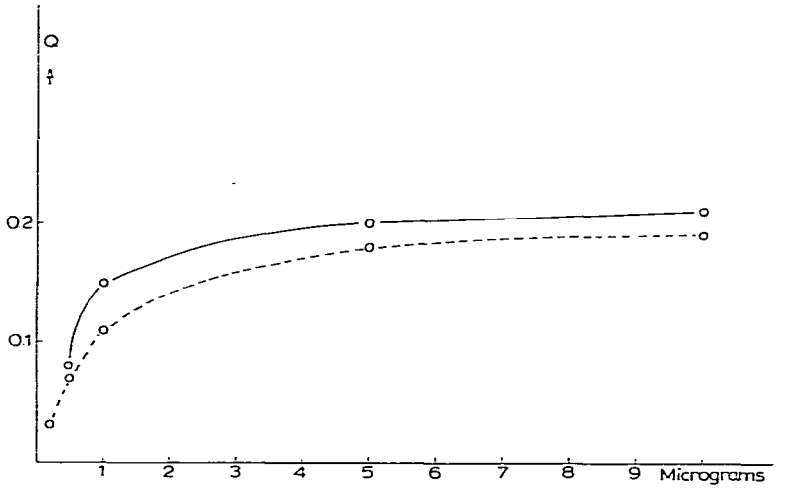

Fig. 4. As Fig. 3, but for treated glass column and support.

the FID had to be used in order to detect the hydrocarbon. This also meant that microgram amounts of morphine had to be used. By substituting caffeine for the C_{24} hydrocarbon, we were able to use the nitrogen-specific detector and could therefore attempt to quantitate the "relative adsorption" in the nanogram range. It is realised that factors other than adsorption may be contributing to peak-height differences between caffeine and the drug in question. For example, the NPD is more responsive to caffeine[5], perhaps because there are four nitrogen atoms in one molecule of caffeine and perhaps also because CN radicals may be more readily formed from the particular groupings in the caffeine molecule[6]. However, the results in Figs. 5 and 6 indicate that over the range of about 25–100 ng of morphine or cyclobarbital

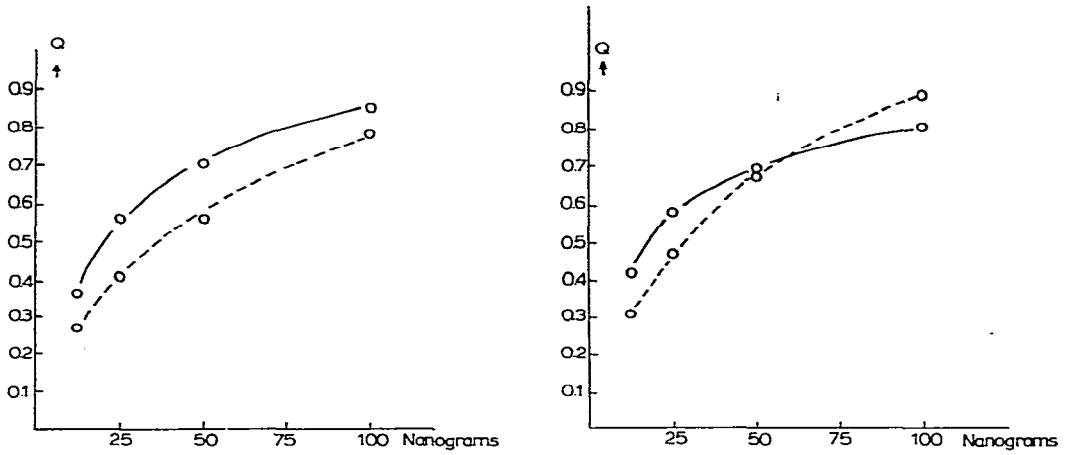

Fig. 5. Graphs showing how the relative adsorption (Q) varies with amount of cyclobarbital (solid line) and morphine (broken line) injected, using the treated support only. Q is defined as the ratio of the peak height of the drug to the peak height of caffeine. Note that a nitrogen-specific detector was used.

Fig. 6. As Fig. 5, but for treated glass column and support.

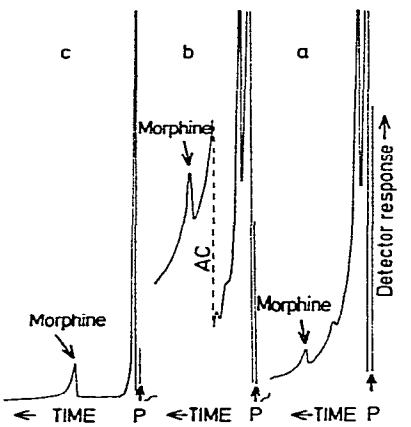

Fig. 7. Gas chromatograms obtained by injecting 1 μl of an extract (containing less than 50 ng of morphine) derived from a putrefied post-mortem urine sample. (a) Attenuation $\times 1024$; (b) attenuation $\times 256$; (c) injection of 1 μl (50 ng) of a pure solution of morphine, attentuation $\times 1024$, for comparison purposes. Column at 280°; injector at 290°; other GC conditions as in text. P = Point of injection; AC = attenuation change from $\times 1024$ to $\times 256$. Retention time of morphine is about 1.7 min.

there is only a slight difference between procedure (a), in which both glass and support are treated, and procedure (b), in which the support only is treated. It may be noted that deactivation procedure (a) is an extension to glass columns of the technique described by Street[7] for the treatment of metal surfaces.

In the application of these proposed columns to toxicological analysis. Fig, 7 shows the gas chromatograms obtained from an extract of a putrified post-mortem specimen of urine containing 1 $\mu g \cdot ml^{-1}$ of morphine. The extraction procedure

(described under Experimental) is one which is used routinely in the Vienna laboratory for qualitative thin-layer and gas chromatography, etc. Absolute recoveries by this method are probably not greater than 50%, so that the peaks shown in Fig. 7 represent less than 50 ng of morphine (*i.e.*, in the 1 μl injected). The conditions were chosen to illustrate that even when one hundredth of the final extract was injected, it was still possible to detect morphine. Obviously, these conditions could be varied to suit particular needs, *e.g.*, a greater volume could be injected, or a smaller volume of original urine sample could be taken.

As regards treatment of the support material, it is probable that the function of the acylation step may be to facilitate contact (*i.e.*, "wetting") between the hydrophobic silicone solution and the hydrophilic areas of the surface of the diatomaceous earth. In this way, a more efficient coating would be produced prior to possible reaction with the surface during the heat treatment stage at about 400°. It would also not be unreasonable to suggest that certain support materials other than diatomaceous earths might respond in a similar way to such a process of acylation and heat treatment. Work is currently in progress along these lines and it is hoped to publish the findings later.

It follows, of course, that as the described column can be used successfully with polar drugs, its performance with relatively non-polar drugs is at least as good. In many instances, results with non-polar drugs may show considerably greater peak heights and more nearly Gaussian peak shapes owing to the operation of factors other than adsorption, *e.g.*, diminished molecular association, which might affect the rate of initial transfer of solute to the gas phase.

Many other drugs have been subjected to GC using the proposed columns. Of interest in this connection are paracetamol, meprobamate, methaqualone and the so-called tricylic antidepressant drugs. The advantages of our columns lie in the decreased detection limits, in the much reduced "memory effect" and in the fact that the retention time of the drug remains virtually constant as the amount of injected drug is decreased. These points are of special significance in toxicological analysis, where polar substances may have to be identified (and often quantitated) by GC screening, or by GC–mass spectrometry using special detector settings, *e.g.*, multiple ion detection and selective ion detection.

In conclusion, it is noteworthy that the proposed columns have shown good thermal stability over the past 18 months under the routine conditions of a busy toxicology laboratory.

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