GAS–LIQUID CHROMATOGRAPHY OF SUBMICROGRAM AMOUNTS OF DRUGS

III. ANALYSIS OF ALKALOIDS IN BIOLOGICAL MEDIA

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In this paper, the term "alkaloid" is used to denote any basic nitrogenous compound which is extractable from aqueous alkaline solution by an organic solvent. Not only are there a large number of drugs included under "alkaloids" by this classification but also these drugs themselves display a wide variety of functional groups and cover an extensive range of molecular weight. This is precisely where gas–liquid chromatography (GLC) is of inestimable value owing to its capability of dealing with the separation of compounds possessing almost any thermostable non-ionic functional group encountered in drugs. Furthermore, GLC can handle drugs varying in molecular weight from less than 100, *e.g.* ethanol, to about 500, *e.g.* emetine, and it may be that future improvements in the thermal stability of liquid phases and in the sensitivity of detectors will permit alkaloids of molecular weight greater than 500 to be successfully separated by GLC. In this respect it is interesting to note that SUBBARAM[1] claims to have separated a mixture of cholesteryl palmitate and cholesteryl stearate (mol. wt. 624 and 652, respectively) on a column of Anakrome ABS coated with 2 % SE 30 programmed from 200° to 340° at 3° per min. Even metallo-porphyrins have been separated in a high pressure (1830 p.s.i.) column by KLESPER, CORWIN AND TURNER[2] using dichlorodifluoromethane as carrier gas although, in this case, the compounds were not eluted but were located within the column after the "run". These examples serve to illustrate that ultimately the limiting factor may not be the molecular weights but may be the thermal stability in nitrogen (or other carrier gas) of the compounds being separated. However, it is perhaps fortunate for the analytical toxicologist that the majority of alkaloids in current therapeutic use have a molecular weight below 500.

In 1960, LLOYD *et al.*[3] described the separation of alkaloids by gas chromatography using 2–3/100 SE 30 at 160°–222° but no mention was made of acid-washing or of silanising the Chromosorb W. A chromatogram was shown displaying the separation of six alkaloids of the papaveraceae group but the exact weight of alkaloid injected was not stated. The peaks also showed marked tailing. Working on the quantitative determination of morphine in opium by gas–liquid chromatography, BROCHMANN-HANSSEN AND SVENDSEN[4] in 1963 found that "phenolic alkaloids, such as morphine, are difficult to gas chromatograph even on a treated support". They overcame their difficulty by reacting the phenolic-OH group with hexamethyldisilazane (HMDS). The trimethylsilyl ethers so formed showed no tailing at 183° on

their column of acid- and base-washed Gas Chrom P, HMDS-treated, and coated first with 0.1 % polyethylene glycol 9000 and then with 4 % SE 30. Even so, the weight of morphine injected corresponded to between 20 and 30 μg.

Many other papers have appeared in the literature dealing with gas–liquid chromatography of alkaloids but in may cases. e.g. ANDERS AND MANNERING[5]; SHAW[6]; BROCHMANN-HANSSEN AND SVENDSEN[7]; LINDSTEDT[8], it has been found necessary to form a derivative in order to obtain a satisfactory "run" of the compound. ANDERS AND MANNERING[9] in another article, described the separation of a number of pheno-thiazine derivatives. These were "run" as free bases but the amounts of drugs used were from 5 to 10 μg. BROCHMANN-HANSSEN AND FONTAN[10,11] have also published two articles dealing with the gas chromatography of alkaloids with polar stationary liquids. In neither of these articles is there any mention of the amount of alkaloid injected. It seems quite probable that the tailing which they would encounter with their polar phases would inevitably lead to dependence of retention time on amount of drug injected. Quite recently, MASSINGILL AND HODGKINS[12] have tabulated their results of GLC of alkaloids using four packed columns. The liquid phases used were Epon 1001 Resin, XE 60, SE-52 and JXR. Again, amounts of drugs injected did not go below 1 μg; furthermore, no peak shapes were illustrated.

McMARTIN AND STREET[13] have recently described their work on the preparation of columns displaying reduced amounts of adsorption and have discussed the scope and limitations of such columns. In a further paper, McMARTIN AND STREET[14] showed that these columns could be applied successfully, in the sub-microgram region, to the analysis of barbiturates and related compounds in biological material.

The present paper describes the analysis of sub-microgram amounts of alkaloids by the application of columns prepared in a similar manner, with slight but important modifications, to that given by McMARTIN AND STREET[13].

EXPERIMENTAL

Gas chromatography details

Gas–liquid chromatography was carried out with a Perkin-Elmer Model 800 or with an F. & M. Model 810. In each case, an all-metal system fitted with a flame ionisation detector was used. Injector temperature was generally about 50° above column temperature; detector temperature was usually about the same as column temperature. The flow rate of the carrier gas (oxygen-free nitrogen) was adjusted to give the shortest retention time consistent with good peak shape and resolution of various mixtures, but was generally between 50 and 60 ml per min. The flow rates of air and hydrogen were optimised. Detector signals were recorded on a Honeywell recorder (−0.25 to +2.5 mV). Chart speed was 1 in. in 4 min. unless otherwise stated. The procedures of McMARTIN AND STREET[13,14] for preparation of steel columns and for treatment and coating of diatomaceous earth have been modified in several ways. To avoid confusion, the complete details incorporating the modifications are given below.

Preparation of metal column. A 6 ft. length of stainless steel tube $^1/_8$ in. O.D. and 0.085 in. I.D. was coiled into a helix about 3 in. in diameter and 18 in. long. A steel sintered plug was pressed into one end of the tube. The column was heated in air in a furnace at 800° for 18 h, removed from the furnace and allowed to cool in air. Swage-lock ferrules and compression nuts were then fitted on the steel tubing and air was

drawn through the column for several minutes. The inside surface was then washed by drawing about 50 ml of toluene through the column. The first 10 ml or so of toluene washings were distinctly yellow as they emerged from the column. Traces of toluene were removed by drawing air through the column and then by heating the column in a stream (60 ml per min) of oxygen-free nitrogen at 360° for 1 h.

Preparation of SE 30 solution. Dissolve 50 g of silicone gum rubber (SE 30) in 500 ml of hot toluene, shaking the mixture frequently over a period of several hours. Allow the liquid to cool to room temperature, and make up the volume to 500 ml. Shake 110 ml of the clear, viscous solution with 5 ml of water. (The shaking should be quite vigorous for about 5 min.) Allow the water-saturated SE 30 solution to stand overnight in a separating funnel. Remove and discard the excess water which has separated out. The "cloudy" SE 30 solution is then ready for coating the diatomaceous earth.

Preparation of column packing. About 250 ml of Chromosorb W (100–120 mesh) were washed several times with concentrated hydrochloric acid and the powder was then boiled in the acid in a conical flask for 10 min. The powder was rinsed several times with concentrated hydrochloric acid and then with water until the supernatant liquid was neutral to a pH paper. The suspension of the powder in water was then boiled for 10 min, rinsed several times with water, the "fines" decanted after each rinsing and excess water removed by vacuum filtration. The powder was placed in flat glass dishes and dried, with frequent stirring, on a boiling water bath.

60 ml of this washed Chromosorb W were put into a 400 ml beaker. 200 ml of toluene were added and the suspension was stirred thoroughly with a glass rod. The powder was allowed to settle and the "fines" were decanted. The washing with toluene was then repeated and as much toluene removed as was possible by decantation. 50 ml of toluene were then added, followed by 100 ml of 10 % water-saturated SE 30 solution (prepared as described above) and the mixture was thoroughly stirred. Excess toluene was removed by vacuum filtration, and the coated powder was dried in four separate portions with stirring on a hot-plate. The four portions were placed in a Pyrex glass tube measuring 2.5 cm in diameter and 40 cm long and fitted at one end with a sintered glass disc. Oxygen-free nitrogen was passed (30 ml per min) through the powder in the glass tube first at room temperature for 5 min, and then whilst the tube was heated in a furnace at 370° for 1.5 h. At the end of this period, the tube was removed from the furnace and allowed to cool down to room temperature with the nitrogen flowing. The powder was removed by suction in three separate fractions. These will be referred to as F1, F2 and F3 where fraction F1 is nearest to the nitrogen input. Fraction F1 was packed into the stainless steel column (prepared as described above) using the packing procedure described by MCMARTIN AND STREET[13], and the column heated at 340° in a stream of oxygen-free nitrogen (50 ml per min) for 18 h. The column was then emptied, packed with fraction F3 and heated at 370° in oxygen-free nitrogen (30 ml per min) for 1 h. This packed column was then ready for use.

Compounds studied

The drugs listed in Table 1 were subjected to GLC as pure solutions in ethanol or chloroform. Several of these drugs were added to blood and/or urine and then taken through one of the extraction procedures described below. Reagent blanks were carried through each procedure by starting with water in place of biological sample. Where possible, the 6th edition of Merck Index was used for drug nomenclature.

TABLE I

RETENTION TIME (IN MINUTES) OF ALKALOIDS SUBJECTED TO GAS–LIQUID CHROMATOGRAPHY AT VARIOUS COLUMN TEMPERATURES

With the exceptions noted below, the amount of alkaloid injected was 0.1 μg in 1 μl of solution at attenuation × 20. This is not the minimum detectable amount (MDA) but represents the amount necessary to produce a peak height of at least 1 in. at *attenuation × 20*, which is a practical attenuation limit for the extraction procedures described (see text). Exceptions—MDA at attenuation × 20 are: a = 0.2 μg; b = 0.3 μg; c = 0.5 μg.

Alkaloid	Mol. wt.	Column temperature									
		130°	140°	160°	180°	205°	215°	235°	270°	290°	310°
Amphetamine	135	1.9	1.5								
Phentermine	149		1.7								
Methylamphetamine	149	2.45	1.85								
Mephentermine	163		2.65	1.5							
Chlorphentermine	184		4.1	2.25	1.25						
Ephedrine[b]	165	6.4	4.4	2.3	1.3						
Phenmetrazine	177		6.0	2.65	1.7						
Hordenine[b]	165			3.2							
Meclophenoxate	242			4.4	2.3	1.2					
Metaraminol[c]	167					1.6					
Pheniramine	240					2.85					
Metyrapone	250					3.5	2.65				
Orphenadrine	269				9.1		2.85				
Aribine	182				10.0	4.25	3.2				
Chlorpheniramine	275						3.9	2.1			
Adiphenine	311						6.85	3.5			
Nortriptyline[a]	263							3.7			
Chlorcyclizine	301						7.6	3.9			
Diazepam	285							6.6	2.4		
Chlorpromazine	319							7.9	2.75		
Pentaquine[a]	301							9.3	2.95		
Acetylpromazine	326							13.5	4.3		
Quinine	324							16.4	5.05		
Papaverine	339							18.4	5.15		
Proclorperazine[b]	374								7.6	4.0	
Octaverine	397							33.2	8.3	4.05	
Prolixin[c]	421								8.8	4.5	
Strychnine[a]	334										3.1
Brucine[a]	394										5.7

Purification of reagents

Ether. Anaesthetic ether (diethyl ether) was shaken with 10 % sodium hydroxide solution to remove anti-oxidant additives from the ether. This ether was then used to wash a fresh solution of 10 % sodium hydroxide to remove impurities from the alkali. The purified alkali was then used to wash a fresh batch of untreated anaesthetic ether. This washed ether was then shaken with several portions of water until the washings were neutral.

Anhydrous sodium sulphate. 1 kg of anhydrous "Analar" Na_2SO_4 was washed with three separate 2-litre portions of absolute ethanol. After the ethanol washings had been decanted, the ethanol-wet Na_2SO_4 was blotted with filter paper and then heated in a furnace at 600° for 20 h. When cold, the purified Na_2SO_4 was stored in a suitable dry container. This purification of Na_2SO_4 is absolutely essential if "peak-free" blanks are to be obtained.

Sodium hydrogen carbonate. Solid $NaHCO_3$ is purified by washing with liberal amounts of purified ether.

TREATMENT OF URINE SAMPLES

Basic alkaloids

5 ml of urine are acidified by addition of 0.1 ml of $2N$ sulphuric acid solution. The mixture is extracted with 30 ml of washed ether. The layers are allowed to separate and the organic solvent is discarded. 0.2 ml of $2N$ sodium hydroxide solution is added and the mixture is tested with an indicator paper to make sure the pH is not less than 10 units and then extracted with two separate 30 ml portions of washed ether. The aqueous phase is kept for extraction of amphoteric alkaloids (see below).

The combined ether extracts are shaken with about 1 g of purified anhydrous sodium sulphate. The dried ether extract is carefully evaporated to dryness in a 15 ml conical centrifuge tube at about 20° using a stream of dry nitrogen. If the analysis is not to include the more volatile alkaloids, the ether may then be carefully removed by holding the tube in the steam from a boiling-water bath.

Amphoteric alkaloids

Morphine and N-allylnormorphine are the chief drugs in this group. Other substances which may be present include those basic drugs containing an aromatic ring which may have become hydroxylated by microsomal enzymes thus giving rise to an (acidic) phenolic group.

"Free" (*i.e.* unconjugated) morphine is extracted from the aqueous phase remaining after extraction of basic alkaloids (see above). 0.1 ml of $2N$ sulphuric acid is added first and then 1 ml of a saturated solution of purified sodium hydrogen carbonate. The mixture is extracted with two separate 30 ml portions of washed ether. The ether is dried by shaking with about 1 g of purified anhydrous sodium sulphate and evaporated carefully to dryness in a 15 ml conical centrifuge tube held in the steam from a boiling-water bath.

TREATMENT OF BLOOD AND LIVER SAMPLES

In the following description of the analysis of blood and liver, the procedure given is for *basic alkaloids only*. If other fractions are required a procedure similar to that described for urine (*q.v.*) may be applied after preliminary treatment.

The first part of the extraction procedure for blood and for liver is based on CURRY's[15] modification of the method described by DUBOST AND PASCAL[16] for analysis of phenothiazine derivatives. As CURRY[15] has pointed out, the treatment with hot hydrochloric acid gives increased yields of alkaloids other than the phenothiazines. This is, presumably, due to liberation of more protein-bound drug. It is suggested, therefore, that, *provided the alkaloid is stable under such conditions*, the hot HCl procedure may be used for analysis of all alkaloids in protein-containing fluids and tissues.

Procedure for blood

1 ml of water and 4 ml of concentrated hydrochloric acid are added to 5 ml of

blood. The mixture is placed in a boiling water bath for 5 min, then cooled in ice-water. 6 ml of ether-washed 60 % potassium hydroxide solution is added making certain that the mixture is at least pH 10. The mixture is then shaken with two 30 ml portions of washed ether, the combined ether extracts are washed successively with 5 ml of 2.5 % ether-washed sodium hydroxide solution and two 5 ml portions of water. The washed ether extract is shaken with 5 ml of 0.1 N sulphuric acid; the two phases are separated and the aqueous phase is made alkaline (again to about pH 10) by dropwise addition of 60 % ether-washed potassium hydroxide solution and extracted with 20 ml of washed ether. The ether phase is dried with purified anhydrous sodium sulphate and evaporated to dryness in a conical centrifuge tube held in the steam from a boiling water bath.

Procedure for liver

20 g of liver is macerated with 20 ml of water and 27 ml of concentrated hydrochloric acid and the mixture is heated in a boiling water bath for 5 min and then cooled in ice-water. 24 ml of ether-washed 60 % potassium hydroxide solution is added slowly with stirring, making sure that the mixture is at least pH 10, cooled in ice-water and then shaken with two 100 ml portions of washed ether. The combined ether extracts are washed first with 20 ml of 2.5 % sodium hydroxide solution, then with 20 ml of water and finally with 10 ml of water. The washed ether extract is shaken with 10 ml of 0.1 N sulphuric acid and the aqueous phase is made alkaline (again, pH not less than 10 units) with ether-washed 60 % potassium hydroxide solution and extracted with 20 ml of washed ether. The ether is shaken with about 1 g of purified anhydrous sodium sulphate and evaporated to dryness in a conical centrifuge tube over a boiling water bath.

GLC ANALYSIS OF THE SEPARATED FRACTIONS

The residue from each of the above fractions is dissolved in 100 μl of ethanol and 5 μl of this solution is injected into the GLC apparatus. For quantitative results, an internal standard may be included in the ethanol. If there is a relatively large amount of residue in the conical tube, it may be necessary to use more than 100 μl of ethanol and then to *inject* a larger volume of the solution.

RESULTS AND DISCUSSION

Amount of drug introduced into gas chromatograph

Many published articles dealing with gas–liquid chromatography do not give the actual weight of compound introduced into the chromatograph. In some cases, it is stated, for example, that 1–3 μl of a 0.5 to 1.0 % solution of a drug was injected. A tracing of the chromatogram is shown but the reader does not know whether the peak he sees was obtained with 1 μl of a 0.5 % solution or 3 μl of a 1.0 % solution (or any other combination), *i.e.* it is not clear whether 5 μg or 30 μg of drug were responsible for the peak shown. Certain manufacturers too have a habit of showing very tall thin peaks displaying very little tailing and of stating that these peaks were obtained by injection of "*x*" μl of solution but without stating the *concentration* of drug in the solution. The reporting of results in this form is most unsatisfactory; in the sub-

microgram region it may be completely misleading. For instance, there is no difficulty at all in obtaining reasonably good peak shapes when 30 μg of morphine are used on some *conventional* columns but 1 μg of morphine usually fails to emerge at all from such columns.

When the weight of compound injected is not stated it becomes very difficult, and in some cases impossible, to assess the value of other workers' results. This is especially so when, in many cases, the exact details of column preparation are not given. Where amounts *are stated* for alkaloids, these are usually greater than 1 μg, and are often of the order of 20 μg. Also, because peak shapes are not shown in many cases, and where they *are* shown tailing may be quite marked, it is unwise to extrapolate to the sub-microgram region. It is my experience that, with *conventionally* "silanised" supports in metal columns, very few alkaloids can be "run" satisfactorily in amounts less than 2 μg.

Column preparation

In the preparation of packed stainless steel columns there are two very important factors to be considered. One is the treatment of the diatomaceous earth, the other is the treatment given to the metal column itself. McMARTIN AND STREET[13,14] incorporated tristearin in their procedure for coating the diatomaceous earth because the ester was found to increase peak heights without, apparently, affecting the tailing. Such columns are stable at temperatures up to 245°. However, after our papers had been published, I observed that when "tristearin-columns" are run for several hours at temperatures around 310°, such as may be required to run brucine in a "reasonable" time, they subsequently show very bad tailing not only at 245° with some of the alkaloids but also when the temperature is dropped to about 160° to "run" the barbituric acid derivatives. This difficulty has been overcome by omitting tristearin and modifying the "silanising" procedure in the manner described below.

We noted in our original article (McMARTIN AND STREET[13]) that we obtained effective "silanisation" using *wet* Chromosorb W and dichlorodimethylsilane (DDS). It is interesting to note that it has now been found that heating the diatomaceous earth (in the absence of air) with a *water-saturated* SE 30 solution produces powders which show remarkably little adsorption and which are stable at temperatures up to 320° with sub-microgram amounts of a wide range of drugs. The preparations are also reproducible. Similar results can be produced by heating *wet* diatomaceous earth with "dry" SE 30 solution in toluene. However, the final preparation in this case is not always reproducible, possibly because it is difficult to obtain an even distribution of water throughout the powder. It is possible that water may be required for hydrolysis of the SE 30 and that the breakdown products so produced, effectively "silanise" the diatomaceous earth *at 370°*.

The second important factor is the treatment of the steel column. For the production of low adsorption columns, it is *essential* that a new steel surface be heated with a silicone gum rubber in the absence of air. It is not sufficient to pour a solution of SE 30 down the tube and then to heat this, probably because the coating of the metal is thereby uneven. The simplest way is to pack the tube with one of the SE 30 coated fractions and to heat this in a stream of nitrogen. A possible explanation of the effect of this treatment is that a thermal breakdown product of SE 30 may react with the metal oxide on the inner surface of the metal column to produce a layer which is less

polar than the oxide itself. (N.B. that the thermal breakdown product *must* be produced in the *absence* of free oxygen.) This could then lead to less adsorption and/or less destruction of the compounds being "run". Such a reaction might also account for the fact that less "tailing" is observed when steel columns are heated *in air* prior to packing, thus forming the oxide or partial oxide necessary for reaction with the SE 30 product. Furthermore, it has been found that steel injector blocks can be treated in a similar manner to give improved over-all performance of the GLC apparatus. In fact, with this type of treatment, it has been found possible to run testosterone (Δ^4-androsten-17β-ol-3-one) in an "all-metal" system, using a liquid phase of SE 30, in amounts down to 5×10^{-9} g. However, it is intended in a future article to deal with the analysis of sub-microgram amounts of steroids using steel columns.

Alkaloids in pure solution

The results obtained with pure solutions of alkaloids are presented in Table I in terms of retention time at different column temperatures. With the exceptions noted in Table I, where the figures in brackets indicate minimum detectable amounts, all the alkaloids were injected into the GLC apparatus in 0.1 μg amounts in a volume of 1 μl of solution. However, 0.1 μg is not the smallest amount of alkaloid detectable by this procedure. Indeed, in may cases, the lower limit for pure solutions of drugs is around the 0.01 μg region.

Extraction procedures

In body fluids and tissues, it is usually analytically simpler to look for the *unchanged* drug rather than the metabolic transformation product(s) of the drug. This is because the body's detoxication mechanisms operate mainly to convert the (toxic) drug into more polar and more water-soluble compounds which can then be more readily excreted by the kidneys. Hence, in many cases, *e.g.* in conjugates with glucuronic acid, the metabolite cannot be extracted from aqueous solution by the usual organic solvents. For this reason, the procedures described in this paper have been deliberately aimed at identifying the unchanged drug. This is not an entirely satisfactory approach because, ideally, one should search for both unchanged drug *and* its metabolites. Although it is true that, in many cases, the metabolites are no longer toxic, there are cases where the metabolite is the compound actually responsible for the pharmacological action ascribed to the parent drug, *e.g.* imipramine and its metabolite mono-desmethylimipramine; phenacetin and *p*-acetamidophenol. A more complicated example is seen in the case of phenylbutazone. BURNS et al.[17], state that phenylbutazone is converted in man into two metabolic products. According to YÜ et al.[18], one of these metabolites, oxyphenbutazone, is responsible for the anti-rheumatic action attributed to phenylbutazone, whilst the second metabolite, in which the butyl side-chain of phenylbutazone is hydroxylated, is stated by BRODIE et al.[19] to account for the uricosuric action of the parent drug. However, until considerable improvements are made in analytical procedures, either in extraction or analysis *without* extraction, toxicologists will, in general, have to be content with a search for unchanged drugs.

With the extraction procedures described, quantitative experiments, based on peak height comparison, indicate that recovery varies with the particular drug being studied. In general, recoveries of alkaloids are about 80 % when the drugs are present

in the sample at a concentration of 0.5 μg per ml of sample. In some cases, *e.g.* trifluoperazine, recovery may be up to 90 % whilst in other cases *e.g.* morphine, recovery may be as low as 50 %. Morphine and other alkaloids possessing an acidic group in addition to their basic amino group represent a special case because of their amphoteric nature. Under the conditions described, using diethyl ether as the extracting solvent, the recovery of morphine is never greater than 60 %. Although mixtures of *n*-butanol and benzene, or isopropanol and chloroform are more efficient extracting solvents, ether has been deliberately chosen because ether is more easily purified than the other solvents referred to, it can also be kept in a purified state, and it is more convenient to remove, after extraction, because of its high volatility. Again, in the case of strychnine and brucine, ether is not the ideal extracting solvent but, in my opinion, solvent purity is especially important when working with microgram and sub-microgram amounts of drugs, relatively large volumes of solvent, and low attenuation settings on the gas chromatograph. It must also be remembered that we are dealing here with the transfer from glass vessel to glass vessel of *microgram* amounts of polar compounds and that adsorption of the drugs could be expected to occur on the glass surfaces. The adsorption might be even greater on *ground* glass surfaces. Indeed the surprising thing is that the recoveries are so *high*.

In the extraction procedures described above for blood and liver, it was suggested that the brief pre-treatment with hot concentrated hydrochloric acid could be used for those alkaloids which have been shown to be stable under such conditions. An extension of this procedure is to use it in cases where the alkaloid is *unstable* under such conditions but which is converted (preferably quantitatively) into a product or products which can be readily identified. In this connexion, we have observed that *d*-propoxyphene is converted by the hot HCl treatment into two compounds which are separable by GLC on one of our SE 30 columns. This fact was recently used by us in one of our routine cases as evidence to suggest that *d*-propoxyphene had been ingested. Neither direct extraction nor tungstic acid treatment revealed the presence of *d*-propoxyphene. We have not yet identified the compounds responsible for the two GLC peaks, but there is no evidence for the presence of unchanged *d*-propoxyphene following the acid treatment.

Alkaloids in biological media

In the section on alkaloids in pure solution, it was noted that the detection limit for many drugs is of the order of 0.01 μg. When these drugs are extracted from biological material, using one of the procedures described above, the "background" collected during the process usually prevents the use of the GLC apparatus at maximum sensitivity. Thus an attenuation of sensitivity of not less than 20 has been used and it is at this attenuation level (1/20 of maximum sensitivity) that 0.1 μg of the drugs is readily detected, *i.e.* give a recorder peak height of *at least* 1/10 of full scale deflection. The tracings shown in this paper are given merely as examples of what can be achieved with these columns, and anyone intending to use the procedure should establish his own conditions to suit his particular purpose.

For a given alkaloid, the retention times, in many cases, were identical for 1 μg and 0.1 μg. In *all* cases, the increase in retention time, as the injected amount was changed from 1 μg to 0.1 μg, was not greater than 5 %.

Although it is of great importance to display the *shapes* of the peaks obtained

and to illustrate that, for a given alkaloid, the retention time is independent of the amount of alkaloid injected, it is not practicable to show here the chromatograms obtained for *each* drug studied at *each* concentration because of lack of space. However, a few representative tracings are shown of chromatograms obtained from extracts of urine containing alkaloids.

Fig. 1 shows the results obtained from an ether extract of 5 ml of urine containing 1.0 μg each of brucine and strychnine per ml of urine. One twentieth (5 μl) of the final extract was injected into the GLC apparatus at a column temperature of 300°. Even at this temperature there is very little background interference. The "shoulder" on each of the peaks is probably due to impurity present in the original solid sample of alkaloid. Complete resolution is achieved within 10 min.

The results shown in Fig. 2 were obtained from an ether extract of 5 ml of urine containing 0.5 μg each of imipramine, promazine, chlorpromazine and mepazine per ml of urine. Again, one twentieth (5 μl) of the final extract was injected into the gas chromatograph but in this case the column temperature was 240°. Resolution of the four drugs is obtained within 9 min.
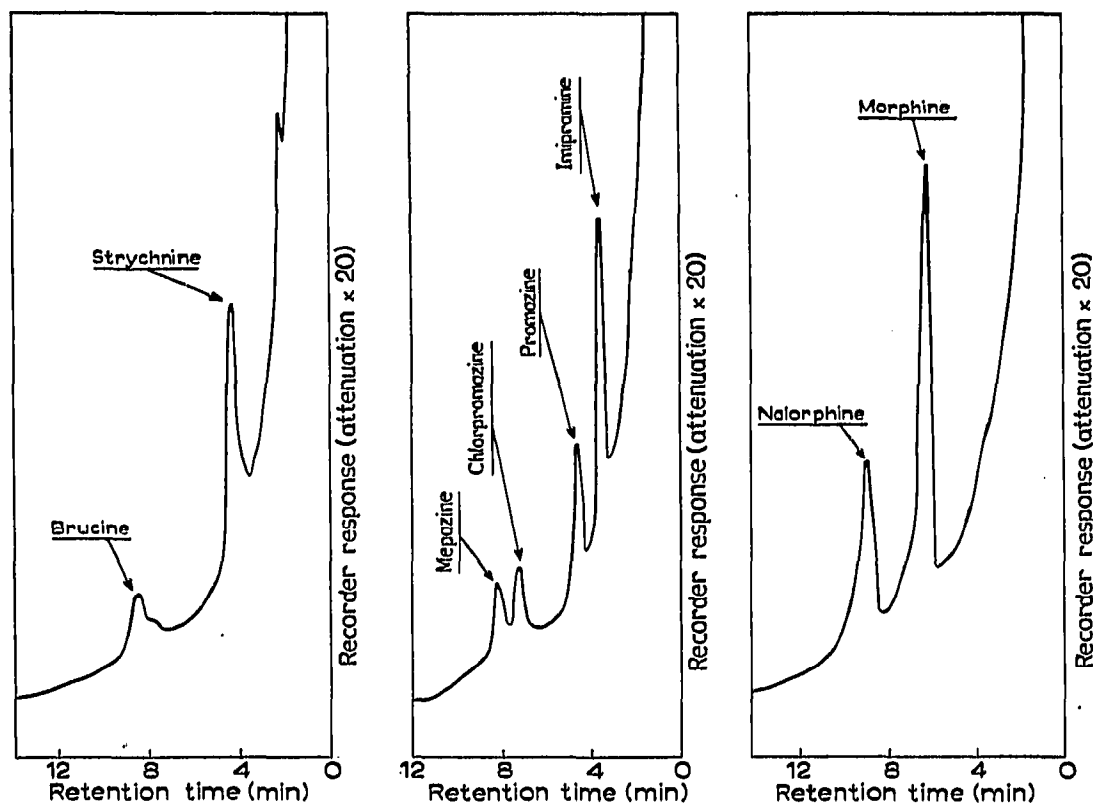

Fig. 1. A chromatogram of an ether extract (see text) of 5 ml of urine containing 1 μg each of strychnine and brucine per ml of urine. Residue was dissolved in 100 μl of ethanol; 5 μl of this solution was injected into the gas chromatograph. Column temperature: 300°; attenuation × 20.

Fig. 2. A chromatogram of an ether extract (see text) of 5 ml of urine containing 0.5 μg each of imipramine, promazine, chlorpromazine and mepazine per ml of urine. Residue was dissolved in 100 μl of ethanol; 5 μl of this solution was injected into the gas chromatograph. Column temperature: 240°; attenuation × 20.

Fig. 3. A chromatogram of an ether extract (see text) of 2.5 ml of urine containing 7.5 μg each of morphine and nalorphine per ml of urine. Residue was dissolved in 100 μl of ethanol; 5 μl of this solution was injected into the gas chromatograph. Column temperature: 240°; attenuation × 20.

Resolution of a mixture of morphine and nalorphine is seen in the chromatogram presented in Fig. 3. In this case, each drug was present in the urine at a concentration of 7.5 μg per ml of urine. 2.5 ml of urine was extracted with ether and one twentieth (5 μl) of the final solution was injected into the GLC apparatus, at a column temperature of 240°,

FELDSTEIN AND KLENDSHOJ[20] analysed the urine of hospital patients who had received from 1/4 to 1 grain of morphine during their stay in hospital and found, on average, about 1 mg of "free" (unconjugated) morphine per 100 ml of urine, i.e. 10 μg per ml. They also found the ratio of "bound" morphine to "free" morphine to vary from 1.6 to 2.2. Hence, the results shown in Fig. 3 illustrate that our GLC procedure could be used to detect morphine in the urine of patients receiving the drug in therapeutic amounts.

The procedure for alkaloids is intended mainly as a rapid screening test for the *presence* of drugs excreted in urine. However, it is probable that a measurement of the height of the GLC peak and reference to a calibration curve, prepared from peak heights obtained by injection of different concentrations of the appropriate standard drug solution, will provide a quantitative assessment which will be of acceptable accuracy in most cases. The recoveries referred to in the previous section should, of course, be taken into account. But it must be remembered that, in the interpretation of quantitative results of urine analysis, it is often difficult to decide whether a particular concentration of a drug in the urine represents the excretion of a recently administered therapeutic dose or the final stages of excretion of a large overdose taken some time previously.

It is suggested, however, that such a procedure could be adopted for the quantitative analysis of alkaloids *in plasma* although, here again, the *interpretation* of plasma alkaloid levels may be difficult in some cases. One such difficulty is illustrated in a paper by CURRY[21] where he showed that in a fatal case involving Tofranil, the level of imipramine in the liver was over sixty times greater than the level in the blood. This order of difference between blood and liver imipramine levels is in keeping with my own findings as described below.

Recently, a case involving ingestion of an overdose of Tofranil was encountered in our Department. Analysis by CURRY's[15] modification of the method described by DUBOST AND PASCAL[16] showed that the blood contained 0.5 mg of imipramine per 100 ml and that the liver contained 36.1 mg of imipramine per 100 g. Imipramine was identified by the blue colour formed on addition of dilute nitric acid solution, by the elevated temperature reversed-phase paper chromatography procedure described by STREET[22-25], and also by its retention time in one of the columns prepared as described in this paper. It is pertinent to note here that, using a liquid phase of nitrile silicone gum (XE 60) in place of SE 30 it has been found possible to resolve a mixture containing sub-microgram amounts of mono-desmethylimipramine and di-desmethylimipramine.

As I have already pointed out (see STREET[26]), the fundamental problem of the analytical chemical toxicologist is the *qualitative* identification of the poisons. Once the drugs have been identified, their quantitative estimation does not usually present great difficulties. Although resolution by GLC of *submicrogram quantities* of drugs extracted from gram amounts of biological material will assist the toxicologist in his identification of toxic agents in cases of poisoning, the question of *absolute* identifi-

cation still remains. It is suggested that a combination of GLC and mass spectrometry will provide the final answer to this question.

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SUMMARY

The surface properties of diatomaceous earth and of oxidised stainless steel can be modified to produce GLC columns which show very little adsorption and which can by used for GLC *analysis of submicrogram amounts of alkaloids.* The modification is effected by heating with a water-saturated silicone polymer at about 370° in a stream of nitrogen. GLC stainless steel columns containing such a preparation have been used to analyse 2.5 ml urine samples containing 7.5 μg morphine and nalorphine per ml; and 5 ml urine samples containing 1 μg brucine and strychnine per ml, and 0.5 μg of many other basic alkaloids, *e.g.* imipramine, promazine, chlorpromazine and mepazine per ml of urine. Details of extraction procedures for alkaloids in blood, urine and liver are described. Retention times are given of 29 alkaloids at various column temperatures.

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