CHROM. 3462

FORENSIC PROBLEMS IN THE GAS CHROMATOGRAPHY OF AMINES AND ALKALOIDS

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(Presented December 15th, 1967)

SUMMARY

Many forensic problems using GLC are similar to those of workers in other fields whose material is of biological origin. This paper is concerned with some of the problems encountered in the analysis of alkaloids by GLC. Because alkaloids are polar compounds, the GLC column used must display minimum adsorption. A method is described whereby adsorption of polar compounds by diatomaceous earth is reduced. It is suggested that this method is superior (especially at column temperatures from 250° to 340°) to methods employing conventional "silanising" procedures.

For identification purposes, on-column derivative formation of some of the alkaloids is described. Reagents used for this purpose are acid anhydrides, ketones and aldehydes. Reactive compounds are characterised by alteration of retention time consequent upon formation of derivative.

Reference is made to the formation of ion-pairs of certain alkaloids and the danger of these ion-pairs not being extracted by certain dilute aqueous acids (*e.g.* HCl) from certain organic solvents (*e.g.* $CHCl_3$).

When columns are prepared as described in this paper, they can be used successfully to analyse *sub-microgram* quantities of a large number of alkaloids ranging in mol.wt. from amphetamine to brucine and including such "difficult" alkaloids as morphine.

I think it is rather unfortunate that the meaning of the word "alkaloid" seems to depend to some extent on the particular scientific discipline to which one belongs. To the pharmacologist for example, an alkaloid is a naturally occurring basic nitrogen-containing compound, whereas to the forensic toxicologist an alkaloid is generally taken to denote any basic nitrogenous compound which can be extracted from aqueous alkaline solution by an organic solvent. This latter definition includes both the "classical" alkaloids of natural origin and the new synthetic compounds. It is this meaning to which I refer when I use the term "alkaloid" in this paper. I should, however, point out that this definition leaves out the quaternary ammonium compounds. It also means that all alkaloids are amines. Not all amines, of course, are alkaloids, because compounds such as adrenaline are water-soluble and are not extracted by immiscible organic solvents.

Many of the problems encountered in forensic work are similar to the problems of workers in other fields who use biological material as their starting point and who are trying to solve their difficulties by employing gas chromatography. One problem which is peculiar to the forensic toxicologist is where the examination of post-mortem samples may be complicated by autolysis when substances may be produced which interfere with the analysis. For example, after gross tissue decomposition, tissues may show the presence of β-phenylethylamine and pyridine. β-Phenylethylamine interferes with the assay of amphetamine by ultraviolet–spectrophotometry but by choosing the correct conditions these compounds can be separated by gas chromatography. Other problems include those associated with a limited amount of sample where one may be searching for submicrogram amounts of alkaloid in gram amounts of material. Sometimes complications arise due to the presence of "other" drugs having been administered in treatment just prior to death. An example of this type is seen in the use of nalorphine as an antidote in morphine poisoning and it is pertinent to mention here that about 4 % of an ingested dose of codeine is excreted as morphine. Gas–liquid chromatography provides an excellent solution to this complication by resolving all three drugs cleanly and quickly.

Whilst on the subject of morphine it will be relevant to refer to what I like to call "amphoteric" alkaloids, of which morphine is an example. Compounds in this group of alkaloids possess both a basic amine and an acidic (usually phenolic) group. Besides morphine, the group includes those alkaloids which have undergone metabolic transformation by microsomal enzymes into phenolic compounds.

These phenolic alkaloids usually undergo further detoxification by forming conjugates with glucuronic acid. But from the analytical point of view the phenolic alkaloids present two problems. First, in order to be able to extract them into an organic solvent the pH of the aqueous phase must be adjusted to a value which lies between the pK_a and the pK_b of the compound, and even then the compound may partition more in favour of the aqueous phase. Second, the compounds are more polar than the parent drug which means that unless the column of the gas chromatograph has been carefully prepared, the compounds will show severe tailing.

However, the *main* problem of the forensic toxicologist is one of *identification* of the poisonous compound or compounds in the biological samples submitted to him for analysis. Only when he is certain what the foreign substances are, can he set about finding out how *much* is present: and this quantitative aspect is generally a simpler problem than the qualitative one. In the rest of this paper I shall describe some of the ways in which we have attempted to solve some of these problems.

By my previous definition, an alkaloid may consist of a primary, secondary or tertiary amine, or combinations of these. Also, drugs which are tertiary amines may be metabolised by demethylation to secondary and primary amines and the possibility exists that each of these in turn may be hydroxylated to phenols.

It is well known that, in most cases, the body's detoxifying mechanisms act on drugs to produce compounds which are more polar than the parent substance. From a gas chromatographic point of view this increase in polarity produces certain difficulties if one wishes to avoid using a multiplicity of columns. For example, using a liquid phase of silicone gum rubber such as SE-30 on a diatomaceous earth support which has been "silanised" with dichlorodimethylsilane, it is quite easy to obtain good peak shapes for imipramine, but the monodesmethyl derivative of this compound shows

some tailing and the didesmethyl compound shows considerable tailing. Our problem thus resolves itself into one of finding a way of reducing the adsorption of the alkaloid by the support material. Two ways of tackling this problem immediately spring to mind. On the one hand, the support material itself may be modified and its polarity reduced, or, on the other hand, the compound being analysed may be modified prior to chromatography by derivative formation. A common example of this latter type is the formation of the alkyl esters of fatty acids. However, in forensic work, it is not always known what the compound *is* which is being chromatographed and for this reason I think it is, in general, preferable to attempt to chromatograph the unmodified compound. Besides this, I believe that ideally one would use a non-adsorbing column and that it is towards this end that we should direct our efforts, even though we may never reach our goal. This leaves us then with our attention focussed on the support material.

It is generally considered that the main forces responsible for adsorption of solutes are the weak VAN DER WAAL's forces and the stronger forces due to hydrogen bonding. The VAN DER WAAL's forces are neutralised by all liquid phases and so need not be considered here. In the diatomaceous earth, there will be both Si–OH groups and Si–O–Si groups and, as PALFRAMAN AND WALKER[1] have pointed out these will give rise to two types of hydrogen bond, one where the Si–OH functions as the proton donor in the hydrogen bond and the other where the Si–O–Si group functions as the proton acceptor. From this it would seem that even when the Si–OH groups have been "silanised", there still remains the possibility of adsorption of certain types of compounds, *i.e.* those compounds which can donate a proton to the Si–O–Si group, *e.g.* amines, alcohols and water. According to OTTENSTEIN[2] the hydrogen bond formed from the Si–O–Si group is much stronger than that formed from the Si–OH group. Many workers have made use (probably quite unwittingly) of this fact by coating the silanised diatomaceous earth with a polyhydric alcohol which would hydrogen bond to the Si–O–Si groups and leave a number of C–OH groups exposed. These groups are then probably readily "silanised" by an injection of hexamethyldisilazane into the column. Theoretically then, this type of treatment should reduce adsorption of solutes very markedly and, indeed, with such columns in practice quite symmetrical peaks are obtained for low molecular weight amines. It must be remembered, however, that there still will be a few C–O–Si groups present following the use of the hexamethyldisilazane. Incidentally, in the analysis of amines, it is usual to treat the support with potassium hydroxide because this gives improved results and less destruction of solutes. The use of the alkali seems to be quite empirical and I have not yet heard of a satisfactory explanation of its action. However, when *high* molecular weight alkaloids have to be analysed, the temperature of the column has to be raised often above the temperature at which the polyhydric alcohol is stable—and thus the column is ruined. We have, therefore, in our laboratory concentrated our efforts on the use of more stable liquid phases such as the silicone gum rubbers SE-30 and SE-52. It must also be remembered that there may be adsorbing sites in the system other than those associated with the support material, *e.g.* the metal or glass of the column wall and the injector block.

Previous experiments carried out in my laboratory (MCMARTIN AND STREET[3]) have demonstrated that washing with concentrated hydrochloric acid *does* improve the performance of the diatomaceous earth and that best results are obtained by

boiling the earth-acid suspension. We have also confirmed that treatment of the acid-washed earth with the usual "silanising" agents *does* reduce adsorption. However, when thoroughly dried support material is treated with dichlorodimethylsilane, the results are worse than when *damp* material is used. In other words, the presence of a small amount of water appears to be necessary to produce a satisfactory reduction in the amount of adsorption. Further experiments have shown that diatomaceous earth which has been treated with dichlorodimethylsilane improves on heating. These experiments have been extended to heating of the acid-washed earth coated with silicone polymer SE-30. With temperatures above 300° in the absence of oxygen, improved results are obtained. Extension of this work has resulted in the use of packed stainless steel columns which show very little adsorption even of alkaloids such as morphine[4]. These columns are stable up to at least 320°; they display relatively little "bleeding" even at this temperature; and they are suitable for use with sub-microgram quantities of high molecular weight alkaloids; and they are also suitable, at lower temperatures, for compounds of lower molecular weight, *e.g.* amphetamine.

The gas chromatographs used in our studies were the Model 800, Perkin-Elmer and Model 801 F. & M. instruments, fitted with metal injection ports and employing flame-ionisation detectors. Oxygen-free nitrogen was used as carrier gas in all cases; 6 ft. × 1/8 in. O.D. stainless steel columns were used.

I feel that the preparation of the column packing is so vitally important that I shall describe its preparation in detail. Acid washing of the diatomaceous earth is carried out as follows:

The first stage consists of washing and coating the earth.

About 250 ml of Chromosorb G (100–120 mesh) are washed several times with about 1 l portions of concentrated hydrochloric acid and the powder is then boiled in the acid in a large conical flask for 10 min. The powder is rinsed several times with concentrated hydrochloric acid and then with water until the supernatant liquid is neutral to a pH paper. The suspension of the powder in the water is then boiled for 10 min, rinsed several times with about 10 l of water, the "fines" decanted after each rinsing and excess water removed by vacuum filtration. The powder is placed in flat glass dishes and dried, with frequent stirring, on a boiling water bath.

60 ml of this washed Chromosorb G are put into a 400 ml beaker, 200 ml of toluene are added and the suspension is stirred thoroughly with a glass rod. The powder is allowed to settle and the "fines" are decanted. The washing with toluene is then repeated and as much toluene removed as is possible by decantation. 50 ml of toluene are then added, followed by 100 ml of 10% water-saturated SE-52 solution and the mixture is thoroughly stirred. Excess toluene is removed by vacuum filtration, and the coated powder is dried in four separate portions with stirring on a hot-plate. The second stage consists of the heat treatment of the coated diatomaceous earth.

The SE-52-coated Chromosorb G is placed in a Pyrex glass tube (see Fig. 1) measuring 2.5 cm in diameter and 40 cm long and fitted at one end with a sintered glass disc. Oxygen-free nitrogen is passed (30 ml per min) through the powder in the glass tube first at room temperature for 5 min and then whilst the tube is heated in a furnace at 370° for 1.5 h. At the end of this period, the tube is removed from the furnace and allowed to cool down to room temperature with the nitrogen flowing. The powder is 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

is then packed into a stainless steel column and the column heated at 370° in a stream of oxygen-free nitrogen (50 ml per min) for 18 h. The column is then emptied, packed with fraction F3, fitted with a short pre-column and heated at 370° in oxygen-free nitrogen (30 ml per min) for 1 h. This packed column is then ready for use.

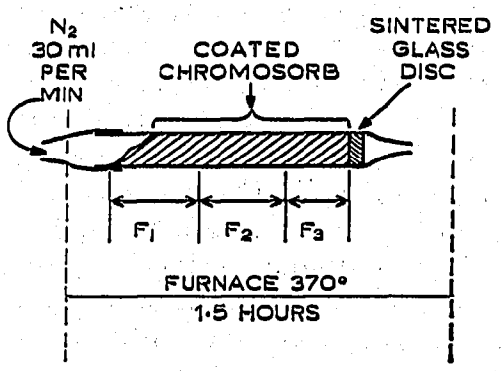

Fig. 1. Preparation of column packing. Heat treatment at 370° after silanisation.

We have also found that improved results are obtained by first heating the empty steel column in air to a temperature of about 800° (in a furnace) for 18 h.

A possible explanation of the results obtained with our procedure is that the water in the water-saturated SE-52 solution may be required for hydrolysis of the silicone polymer, and the breakdown products so produced, effectively "silanise" the Chromosorb G *at 370°*. It is also possible that a similar process may account for the silicone polymer treatment of the oxidised steel column. Here again, a thermal breakdown product of SE-52 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. This might then lead to less adsorption and/or less destruction of the compounds being analysed. Such a reaction might also account for the improvement in peak shape when stainless steel columns are heated *in air* prior to packing. The oxide or partial oxide so formed may facilitate subsequent reaction with the thermal decomposition product of the silicone polymer. It is important to note, however, that this thermal breakdown product *must* be formed in the *absence* of free oxygen.

We have also found that steel injector blocks can be treated in a similar manner by injecting a solution of SE-30 or SE-52 into the injector from which the column has been disconnected. The injector should be at about 380° to 400° and, of course nitrogen should be flowing through the block.

Metal systems prepared in this way have proved effective in reducing catalytic destruction of solute. For example, we have found it possible to chromatograph testosterone in amounts down to $5 \cdot 10^{-9}$ g in an all-metal system with a flame-ionisation detector.

Results obtained using one of these columns for a number of alkaloids have been described by STREET[4]. It is also pertinent to note here that the same column can be used for GLC analysis of acidic compounds such as the barbiturates (see MCMARTIN AND STREET[5]) and also of neutral drugs.

Having thus obtained such a column the problem of identification of peaks emerging from the gas chromatograph still remains. In many cases, use can be made of some other parameter such as ultra-violet or infra-red spectroscopy. These may be investigated by extraction of a duplicate sample of original material, or the sub-

stances may be trapped and collected as they emerge from the gas chromatograph. In recent times a lot of attention is being paid to the use of mass spectrometry combined with gas chromatography, and this procedure appears to be proving very successful, although very expensive.

We have been interested in devising a more simple and cheaper procedure for the identification of our peaks. The peak-shift technique, first described by ANDERS AND MANNERING[6] seems to be of value in solving *some* of our identification problems. These workers prepared derivatives on the column by following the injection of the parent compound with an injection of acetic or propionic anhydride.

This procedure enables one to acylate not only both primary and secondary amines, but also any -OH groups present in the alkaloid. We prefer to draw about 3 μl of the anhydride into the syringe, follow this with 2 or 3 μl of the alkaloid solution and to inject this mixture into the chromatograph. Fig. 2 shows the results of this procedure applied to bisnortriptyline.

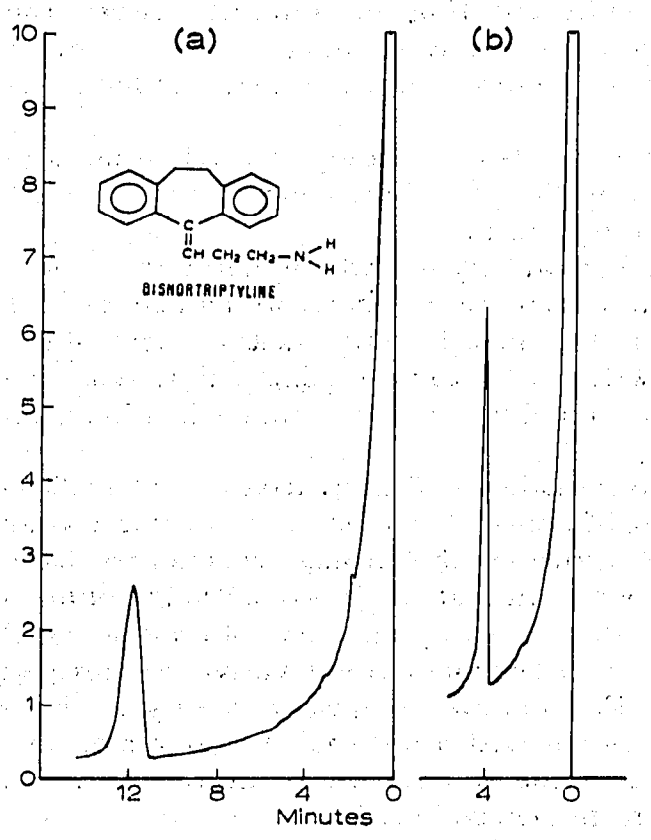

Fig. 2. Chromatograms showing the effect of on-column acetylation of bisnortriptyline, (a) 3 μl Ac_2O + 1 μg (1 μl) of a solution of bisnortriptyline, 250°, attenuation × 10^2; (b) 1 μl EtOH + 1 μg (1 μl) of a solution of bisnortriptyline, 250°, attenuation × 10^2.

In 1965, BECKETT AND ROWLAND[7] made use of a procedure originally suggested by BROCHMANN-HANSSEN AND SVENDSEN[8] in 1962 to identify amphetamine by alteration of its retention time by conversion to its acetone derivative. This they did by first dissolving their residues in acetone prior to injection. However, by using a minor modification, we have found that the residue can be dissolved in ethanol, a portion of this drawn into the syringe, followed by a few μl of acetone, and the acetone–ethanol

mixture injected to produce the same results. The advantage of our modification is that the retention time of the unreacted alkaloid is obtained by direct injection of an ethanolic solution of the residue, and can be followed by the acetone derivative without altering the bulk of the residue solution. This may be important if starting material is limited and if further tests are to be carried out on the residue.

One extension of the peak shift technique which we have found to be particularly useful for high molecular weight alkaloids involves the formation of Schiff's bases on the column. By injecting an ethanolic solution of the alkaloid together with 1 or 2 μl of benzaldehyde (in the same syringe) it is possible to distinguish most primary amines from secondary and tertiary amines. Although, it must be remembered that compounds, such as ephedrine, which display the grouping $>C(OH)-(CH_3 \cdot HN)C<$ will behave as primary amines under these conditions. We have further observed that with a primary amine such as bisnortriptyline, it is not possible to form the acetone derivative on the column at the temperature required to run the alkaloid. This may be because, at this temperature, there is insufficient time of contact between the acetone and the alkaloid to allow reaction to occur to any significant extent. This is supported by the fact that ketones of higher molecular weight *do* react under these conditions.

However, it will be obvious that by a successive use of anhydrides, ketones and aldehydes, much useful information can be obtained by comparing retention times of parent alkaloids and derivatives with reference data.

The condensation product formed from benzaldehyde and amphetamine shows, as expected, that the peak shape of the derivative is more symmetrical than that of the amine, and we are at present working on a method based on the measurement of the area of the peak displayed by the derivative with a view to producing a more sensitive procedure for the quantitative estimation of alkaloidal primary amines in biological material.

BECKETT, TUCKER AND MOFFAT[9] have also made use of derivatives for identification in urine of a number of stimulants. The differences between their work and ours are that their condensation derivatives are formed with ketones whereas we have used aldehydes as well as ketones; the upper temperature limit of their columns appeared to be not greater than 200° whereas we can operate on columns at 320° with only slight increase in background noise. Furthermore our derivatives are formed either in the injector block or on the column. Fig. 3 shows the peak given by the benzaldehyde derivative of bisnortriptyline compared with the peak of bisnortriptyline itself. One μg of bisnortriptyline was used and the column temperature was 270°. The two small peaks are due to an impurity in the benzaldehyde.

BECKETT and his colleagues[9] found that all the primary amines they studied formed condensation products with ketones with the exception of phentermine and chlorphentermine. They suggest that the reason for this may be that the two α-methyl groups in these compounds may hinder the reaction with ketones. We can confirm their findings but it is interesting to note that both phentermine and chlorphentermine *will* condense (on the column) with benzaldehyde—again producing a peak shift.

I would like to mention here the importance in forensic and clinical work of carrying out as extensive an analysis as possible. It may be that a patient is brought into hospital suspected of having taken an overdose of a particular barbiturate. This is mentioned to the analyst who then proceeds to confirm that this barbiturate is in

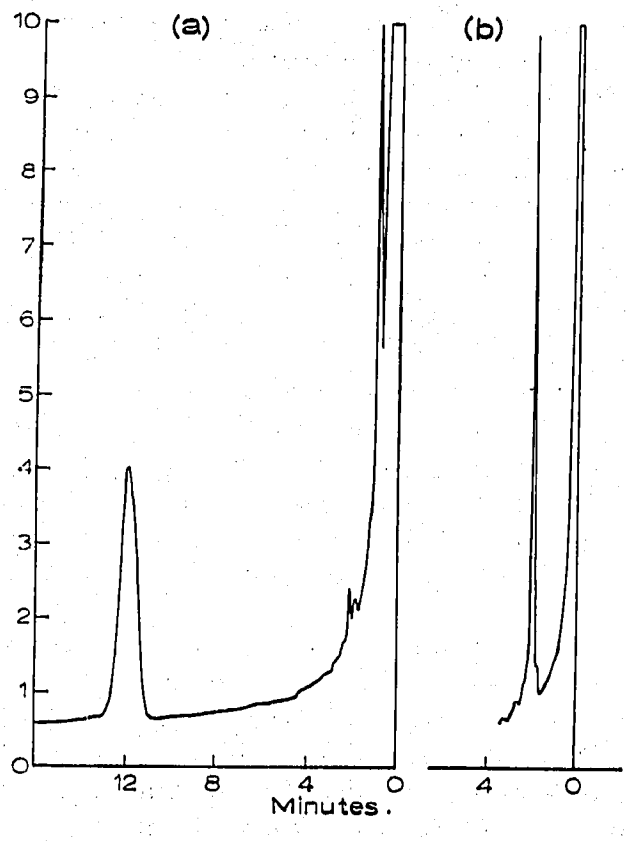

Fig. 3. Chromatograms showing the effect of on-column reaction of benzaldehyde with bisnortriptyline, (a) 1 μl C_6H_5CHO + 1 μg (1 μl) of a solution of bisnortriptyline, 270°, attenuation × 10^2; (b) 1 μg (1 μl) of a solution of bisnortriptyline, 270°, attenuation × 10^2.

fact present. But the blood level of the barbiturate may seem to him to be a little low to be responsible for the patient's condition and, if the analysis had been continued, other drugs may have been found to be present. Imipramine and amitriptyline are drugs which, because of their apparently low concentration, are likely to be dismissed as unimportant even if they *are* found in the blood. We have had post-mortem cases where the blood level of these alkaloids was of the order of 0.5 mg per 100 ml and yet liver levels in these cases were as high as 30 or 40 mg per 100 g.

I think it would not be out of place to refer here to some of the extraction procedures used in forensic toxicological analysis of alkaloids. Generally, one wants to use as rapid a procedure as possible, consistent, of course, with reliability and reproducibility. The usual techniques involve making the fluid or tissue-homogenate alkaline, and then extracting this mixture with an immiscible solvent. Chloroform and ether are the commonest solvents used for this purpose. It is often advantageous to extract the *acidified* tissue with solvent before addition of alkali. This will remove many substances which may interfere with the analysis, *e.g.* acidic and neutral compounds.

Direct extraction procedures of this type are not entirely satisfactory. This is particularly so when the alkaloid is markedly protein-bound. In such cases, direct extraction of protein-rich mixtures may fail completely to reveal the presence of the alkaloid. This failure also applies to procedures designed to remove the protein, by

precipitation or heat-coagulation, followed by extraction of the protein-free filtrate. Here, the bulk of the alkaloid may be left behind attached to the protein.

This difficulty was realised in 1953 by DUBOST AND PASCAL[10] who suggested that the alkaloid should first be released from the protein by brief treatment with hot concentrated hydrochloric acid. I would suggest, therefore, that in the analysis of alkaloids in protein-containing mixtures, this hot acid treatment should be applied. Of course, it must first of all be established by a control experiment that the alkaloid is stable under these conditions. This means that to be of value in an "unknown" analytical search, a list must be compiled of the behaviour under such conditions of all alkaloids likely to be encountered. Even if an alkaloid is *not* stable when treated in this way, the resulting pattern obtained by subjecting an extract of the acid-treated mixture to gas chromatography may be helpful in identification of the drug. As an example of this approach, I would mention that *d*-propoxyphene, which is a tertiary amine, is unstable in hot concentrated hydrochloric acid solution. Subsequent gas chromatography shows instead of the usual peak for the alkaloid, *two* peaks whose retention times might be used to suggest the presence of *d*-propoxyphene in the original mixture.

As regards the *type* of immiscible solvent to be used for extraction, we prefer diethyl ether to chloroform or other halogenated solvents. There are several reasons for this preference. Firstly, it is well-known that certain alkaloids, especially those of the phenothiazine and iminodibenzyl types, can form ion-pairs with a number of inorganic anions at low pH values. These ion-pairs in a chloroform/aqueous acid system, partition very markedly in favour of the chloroform. This means that a drug such as amitriptyline which has, say, been extracted into chloroform from aqueous alkaline solution, *cannot* be transferred from the chloroform by shaking with hydrochloric acid solution. It is very important to note this point. There is at least one recent publication (see FORBES, WEIR, SMITH AND BOGAN[11]) where the authors describe such a procedure for the analysis of amitriptyline in biological material. This is just not possible for amitriptyline itself, and I would suggest that what was actually extracted was probably the mono- or di-desmethyl derivative, *i.e.* one of the metabolites of amitriptyline. We have found that the *primary* amine derivatives do not form ion-pairs under these conditions. A simple change from hydrochloric to sulphuric acid will ensure that the drug *is* extracted from the chloroform. However, we prefer to use *ether* instead of chloroform because the ion-pairs are not extracted by ether.

The *second* reason for preferring ether to chloroform is that ether can be kept more easily in a purified state; it also shows less tendency to emulsion formation; and it is more volatile and therefore more easily removed.

Finally, I want to say a few words about the reporting of results with reference to the amount of drug introduced into the gas chromatograph. Many published articles dealing with gas–liquid chromatography do not give the actual weight of compound introduced into the gas chromatograph. In some cases, it is stated, for example, that 1–3 μl of a 0.5–1.0 % 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; with polar compounds 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 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 high molecular weight alkaloids can be "run" satisfactorily in amounts less than 2 μg. With our columns, a large number of high molecular weight alkaloids can be successfully chromatographed in the sub-microgram region.

ACKNOWLEDGEMENT

This work is part of a research programme which was supported by a grant from the Scottish Hospital Endowments Research Trust.

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