RECENT ADVANCES IN 3 CLINICAL PHARMACOLOGY

Edited by Paul Turner and David G. Shand

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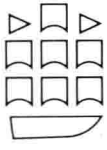

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Recent Advances in

CLINICAL PHARMACOLOGY

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Preface

In the preface to the first volume of this series we drew attention to the 'rapid changes taking place in our understanding of the pharmacological basis of normal human body function, of disease and of its treatment.' We hope that the contents of this third volume will demonstrate that the rapid growth in clinical pharmacology continues, in analytical methodology, pharmacokinetics, drug development, and in our understanding of drug action at receptor and molecular levels.

We have, once again, been encouraged by the willingness, indeed the eagerness, of our colleagues to collaborate with us, and hope that the infectious excitement of involvement in clinical pharmacological research will be evident to all our readers.

1983

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1. Analytical techniques in clinical pharmacology

S. Murray D. S. Davies I. A. Blair

The constant requirement for more specific and sensitive analytical techniques has resulted in many new developments. While existing methods have been substantially refined, analytical techniques based on completely new principles have also been devised. This chapter will discuss recent advances in methods which involve chromatographic resolution of the components of a mixture prior to qualitative or quantitative analysis. Because of the large number of endogenous compounds present in a biological matrix, chromatographic separation of the component of interest is often essential before further analysis can be carried out. These methods include gas liquid chromatography (GLC), combined gas chromatography mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC).

GAS LIQUID CHROMATOGRAPHY

A suggestion by Martin & Synge led to the development of GLC by James & Martin in 1952. This is a technique for separating a mixture of volatile substances in the vapour phase by percolating an inert gas stream (the carrier gas) over a stationary liquid. The latter is spread as a thin film over a finely powdered inert support and packed into a glass column. Compounds are selectively retarded, depending on how they partition between the carrier gas and the stationary phase, until they form separate bands in the carrier gas. These component bands leave the column in the gas stream and are recorded as a function of time by a detector. The column is enclosed in a variable temperature oven and so strongly retarded compounds can be eluted more rapidly by raising the column temperature.

Applications

The sensitivity, speed, accuracy and simplicity of this method for the separation, identification and determination of volatile compounds has resulted in a phenomenal growth of the technique.

Qualitative analysis

The retention time of a compound is the time from sample injection to the detector registering the peak maximum. This property is characteristic of the compound and the liquid phase at a given temperature and carrier flow rate. Under stable operating conditions it can be reproduced to within one per cent and used to identify each peak registered by the detector. While several compounds can have close or even identical retention times, each compound has only one retention time and this is not influenced by the presence of other components. To show that an unknown sample is the same compound as a known standard, several columns with different stationary phases can

be used in turn. If the unknown sample has the same retention time as the standard on each of the different columns, it is highly likely that they are the same compound. The GLC column can also be linked directly to instrumentation which can give structural information on a component as it is eluted from the column. By far the most sensitive and specific is a mass spectrometer and combined GC/MS will be discussed in detail later.

Quantitative analysis

The primary application of GLC in clinical pharmacology is in quantitative analysis and, provided the proper techniques are employed, high accuracy can be obtained. In order to determine amounts of compound present, the detector response must be calibrated and there are two ways of doing this.

ABSOLUTE CALIBRATION

Exact amounts of solutions of pure compound are injected onto the GLC column and the areas or heights of the peaks registered by the detector are plotted against the known weights injected. This is called a calibration curve which should be linear and pass through the origin. An exact amount of the sample to be analysed is now injected and the peak area or height measured. From the calibration curve, the amount of compound present in the sample can then be calculated. Disadvantages of the absolute calibration method are that firstly it is difficult to inject microlitre quantities accurately and reproducibly. Secondly, if a prior extraction procedure is required to purify the sample, there is no way of allowing for losses which occur during this process. Finally, the detector response must remain constant.

INTERNAL STANDARDISATION

This method, also known as relative or indirect calibration, is preferred to the absolute calibration method. Known weight ratios of the compound to be analysed and a standard are prepared and chromatographed. The peak areas or heights are measured and ratios of these are plotted against weight ratios to obtain a standard curve. An accurately known amount of the internal standard is then added to the unknown sample and the mixture chromatographed. Area or height ratios are measured and from the standard curve the weight ratio of the compound to the standard is read. Since the amount of standard added is known, it is a simple calculation to determine the amount of compound present in the unknown sample.

The advantages of this method are that quantities injected need not be accurately measured and detector response need not be known or remain constant. The compound chosen as internal standard should have a retention time close to the compound of interest but not so close that the peaks overlap. Also, it should be structurally similar to the compound being analysed so that losses of compound and standard during extraction and purification are similar.

Recent advances

Since the initial development of GLC, the method has been substantially refined. Major advances have been made in column design resulting in greatly increased resolving power. Also specific detectors have been developed which are extremely sensitive to molecules containing certain atoms and functional groups. This has meant

that limits of detection have been markedly improved and now certain compounds can be detected down to the subpicogram level.

Capillary columns

One of the main aims when using GLC is to ensure that the components of a mixture are completely separated from one another when they leave the column. If the mixture is complex with many different components, this can only be achieved if the column has high resolving power. The latter property is a function of column length and packed columns are limited in their practical length to a few metres.

Capillary, or open tubular, columns were developed by Golay in 1956 in the course of theoretical considerations of packed column behaviour. While packed columns have internal diameters of two to four millimetres, capillary columns have internal diameters of a quarter to a half millimetre and the liquid phase is spread as a thin film on the inside wall. The resolving power per unit length of a capillary column is comparable with a packed column but much greater lengths (up to one hundred metres) can be used because capillary columns have higher permeabilities, i.e. they are 'open tubes' with smaller resistance to gas flow. Initially, capillary columns were made of stainless steel because glass columns of such narrow bore were very fragile. However, fused silica columns coated on the outside with silicone rubber or polyamide have been introduced and these have proved quite robust.

For routine drug analysis, where a biological sample undergoes substantial extraction and purification prior to GLC analysis, the high resolving power of a capillary column is often unnecessary. However, when studying a group of drug metabolites or endogenous compounds which are structurally similar to one another, a capillary column is often needed to separate the various components. Consequently, capillary columns are being used more and more widely in pharmacological analysis (Novotny et al, 1976; Bailey et al, 1977; Fitzpatrick, 1978).

Specific detectors

The chromatographic detector is a device which responds to the various separated components in the carrier gas. There are several different types in common use and the one universally used in drug analysis is the flame ionisation detector. Effluent gas from the column is mixed with hydrogen and burned in air or oxygen. Organic material in the gas is ionised and the ions and electrons formed in the flame enter an electrode gap, decrease the gap resistance and permit a current to flow. This current, after suitable amplification, is recorded as a peak by a pen recorder.

In order to be useful, a detector should be sensitive and have a large linear range, i.e. there should be a linear relationship between concentration of component and detector response over a wide range of concentration. The flame ionisation detector has both these characteristics and responds to virtually all compounds except the inert gases, air, water, carbon disulphide and a few others. Recently, however, greater use has been made of specific detectors which respond selectively to certain classes of compound. The specificity of these detectors can be a great advantage since extracts of biological origin, even after substantial purification, often contain many endogenous compounds which would mask the presence of the compound of interest if a universal detector were used.

ELECTRON CAPTURE DETECTOR

This detector measures a loss of signal rather than a positively produced electrical current. As the carrier gas flows through the detector, a tritium or nickel 63 source ionises the gas molecules and slow electrons are formed. These slow electrons migrate to an anode under a fixed voltage and produce a steady current amplified by an electrometer. If a sample containing electron absorbing molecules is then introduced, this current will be reduced. The loss of current is a measure of the amount and electron affinity of the compound.

The electron capture detector is extremely sensitive to halogenated compounds, conjugated carbonyls, nitriles and nitrates but is virtually insensitive to hydrocarbons, alcohols and ketones. This selective sensitivity makes the detector valuable for the analysis of compounds containing the requisite electron capturing atoms and groups. Even if the latter are not initially present, they can often be introduced into the molecule by suitable derivatisation. A disadvantage is that all electron capture detectors suffer from a narrow linear range and care must be taken that the detector is operating within this range.

Examples of the use of the electron capture detector in drug analysis are given in Table 1.1.

Table 1.1 Some examples of the use of the electron capture detector in drug analysis

Compound	Biological fluid	Limit of assay (ng/ml)	References
Thiamylal	Plasma	1.0	Smith et al, 1977
Thiopental	Plasma	0.1	Smith et al, 1977
Oxycodone	Plasma	1.0	Weinstein & Gaylord, 1979
Mefloquine	Blood	10.0	Nakagawa et al, 1979
Metoclopramide	Plasma	7.0	Tam et al, 1979
Diazepam	Plasma	50.0	Wallace et al, 1979
Valproic acid	Serum	1.0	Chan, 1980

ALKALI FLAME DETECTOR

This detector consists of a normal flame ionisation detector plus the addition of a small alkali salt pellet (either caesium bromide or rubidium sulphate) placed on the burner jet. With precise control of the hydrogen and air flow rates, the detector can be made to give an enhanced response to phosphorous containing compounds (a selectivity of 5000:1 for phosphorous over normal hydrocarbons) and nitrogen containing compounds (a selectivity of 50:1 for nitrogen over normal hydrocarbons).

The alkali flame detector has found wide application in drug analysis since many therapeutic agents have nitrogen atoms in their structure. Examples are given in Table 1.2.

GAS CHROMATOGRAPHY MASS SPECTROMETRY

During the past decade, integrated GC/MS has become recognised as one of the most versatile and powerful instrumental systems available to the pharmacologist and has been the subject of a number of reviews (Jenden & Cho, 1979; Draffan et al, 1980; McCamish, 1980; Garland & Powell, 1981; Baillie, 1981). The principles of GLC and

Table 1.2 Some examples of the use of the alkali flame detector in drug analysis

Compound	Biological fluid	Limit of assay (ng/ml)	References
Amitriptyline	Plasma	10.0	Hucker & Stauffer, 1977
Amitriptyline	Plasma	10.0	Dawling & Braithwaite, 1978
Diazepam	Plasma	10.0	Dhar & Kutt, 1979
Chlorpromazine	Serum	5.0	Bailey & Guba, 1979
Phencyclidine	Plasma	5.0	Bailey & Guba, 1980

of several different types of GLC detector have already been discussed. By coupling a mass spectrometer to a GLC column so that effluent from the column enters the mass spectrometer, the latter is in fact acting as a GLC detector. However, it is by far the most sophisticated and versatile detector available, offering great sensitivity and specificity.

When a compound is introduced into a mass spectrometer, the sample molecules are firstly ionised, usually by a beam of electrons emitted from a heated metal filament. This process, called electron impact ionisation, is the most commonly used technique although other methods (to be discussed later) are becoming more popular. The positively ionised molecules formed under these conditions are not necessarily stable and normally some or all will break apart to give fragment ions. The mixture of molecular ions and fragment ions then pass through a mass filter which separates them according to mass. Mass spectrometers used in the biomedical sciences fall into two main categories depending on the nature of the mass filter. One type separates ions of different mass with a magnetic field generated by an electromagnet while the other uses an electric field located at the centre of a quadrupole rode assembly. Finally a mass analyser, located at the end of the mass filter, measures the abundance of the various ions. The profile of ions formed and the relative abundance of these ions is called the mass spectrum of a compound.

One of the fundamental problems encountered when linking a GLC column to a mass spectrometer is how to interface the two units. While the gas leaving the GLC column is at one atmosphere, the pressure inside the ion source of a mass spectrometer must be roughly 10^{-10} atmospheres in order that a mass spectrum can be obtained. With packed GLC columns, where a carrier gas flow rate of 20–30 ml/min is normal, a direct coupling to the mass spectrometer is not possible. The vacuum pumps which maintain the requisite low pressure in the mass spectrometer ion source are not capable of handling such a high influx of gas. Consequently, molecule separators which allow passage of sample molecules while eliminating a large proportion of the carrier gas had to be developed. Recently there has been an increased use of capillary columns linked to mass spectrometers. Because of the narrow bore of these columns, carrier gas flow tends to be as low as 0.5–2.0 ml/min. Molecule separators are not usually required when capillary columns are used and column effluent can be introduced directly into the mass spectrometer ion source.

Applications

The mass spectrum of a compound can be likened to a fingerprint in that it is highly characteristic of that compound. The chances of two different substrates having the

same molecular ions and fragment ions in their mass spectra are remote and even if this does occur, such as when two compounds are structurally very similar, the relative abundances of the various ions are usually different. With modern instrumentation a full mass spectrum can be obtained using nanogram quantities of material and much smaller amounts can be detected if only specific ions are monitored. This combination of specificity and sensitivity means that the technique has important qualitative and quantitative applications.

Qualitative analysis

As has been mentioned previously, GLC can be used for compound identification on the basis of chromatographic retention time. However, GC/MS is much less ambiguous in this role since it will give a mass spectrum as well as a retention time for every component of a mixture and it is frequently possible to identify specific compounds in body fluids in amounts smaller than with any other technique.

In general, rigorous identification requires a reference sample of authentic material and comparison of mass spectra under identical conditions. However, comparison of a spectrum with a reference file may provide strong evidence of identity and extensive libraries of mass spectra are available for this purpose. In some cases, drugs and metabolites isolated from biological fluids have been present at such low concentrations that full mass spectra could not be recorded. In these situations, identification was based on the presence of just a few prominent, characteristic ions at the appropriate retention time (Wright et al, 1977).

When screening for the presence of unknown and unsuspected drug metabolites, much use has recently been made of the so-called isotope cluster, ion doublet or twin ion technique. Firstly the substrate under investigation is enriched at a level of approximately 50% with one or more atoms of a suitable heavy isotope. The mass spectrum of the substrate, and all metabolites derived from it, will thus exhibit artificially generated 'twin' peaks for the molecular ion and all fragment ions retaining the labelled atoms. By this approach, metabolites may still be detected against a complex background of endogenous compounds by virtue of this conspicuous feature of their mass spectra (Knapp et al, 1976; Vose et al, 1978).

Quantitative analysis

Combined GC/MS was originally used in the biomedical sciences almost exclusively for the identification and structural analysis of unknown compounds. However there has been a shifting emphasis over the last decade towards the quantitative applications of GC/MS. The reasons for this are that firstly a mass spectrometer provides a uniquely sensitive detector. Various examples of the greater sensitivity of certain GLC detectors can be cited but such are exceptional. Secondly, a mass spectrometer is a very specific detector. The technique of selected ion monitoring gives assays great specificity and precision. Finally, there is now a much greater selection of chemical precursors incorporating stable isotopes. This allows the ready synthesis of stable isotope labelled analogues which can be used as internal standards in quantitative analysis.

When a mass spectrometer is used for quantitative analysis, it can be adjusted to rapidly and repetitively scan the entire mass spectrum of the effluent from the GLC column. The mass spectrometer is then acting as a universal detector and is

monitoring all ions formed (the total ion current). However, biological extracts are often very impure and, even after passage down a GLC column, compounds of interest can be masked by endogenous material which leaves the column at the same time. But if the mass spectrometer is only set to monitor ions which are known to be prominent, intense ions in the mass spectrum of the compound of interest, then the chances of interference from other material are very much reduced. This is called selected ion monitoring; as well as giving much greater specificity, it also increases the detection limit by up to three orders of magnitude. The output of the technique, a plot of ion abundance versus time, is called a selected ion current profile.

Because compounds have to be in the vapour phase in order to pass down a GLC column, it is often necessary to derivatise samples either to improve volatility or to chemically modify polar groups which would otherwise lead to poor chromatographic behaviour. For GLC analysis alone, derivatives are chosen for good peak shape and a convenient retention time at a given column temperature. However, for GC/MS analysis, the nature of the derivative will dictate the mass spectrum obtained. For selected ion monitoring work it is best to have a few, intense, high mass ions with little fragmentation. Consequently this will often decide the type of derivative which can be used.

Just as with analysis by GLC alone, in order to have good accuracy and precision a GC/MS quantitative assay must incorporate an internal standard. There are three types of internal standard that can be used in selected ion monitoring assays. Type one is a compound that has similar properties to the drug being measured but whose mass spectrum has no significant ions in common with the mass spectrum of the drug. This compound will undergo similar extractive losses to the drug and the mass spectrometer monitors two separate ions, one from each species. An example of this technique is found in the measurement of norphenazone, a metabolite of antipyrine, in urine using 4'-methylnorphenazone as internal standard (Murray, 1980). Type two is a homologous compound whose mass spectrum contains a reasonably intense ion at the same mass as the ion being monitored for the drug but has a different GC retention time. Hence the mass spectrometer can detect both species by remaining permanently focused on this one ion and, because the instrument is at its most stable, this method represents the most sensitive of all mass spectrometric means of detection. The analysis of terbutaline in biological fluids (Clare et al, 1979) provides an example.

Type three is a compound which is a stable isotope labelled analogue of the drug to be quantitated. Here the internal standard matches as closely as is possible the physical properties of the drug and the two species have very similar if not identical retention times. However the mass spectrometer can distinguish between the two because of mass spectral differences. Even though the mass spectrometer is monitoring two or more ions, this method (termed stable isotope dilution) in general is the most accurate and precise. Also the difficulty of synthesising compounds labelled with deuterium, carbon-13 and nitrogen-15 has been greatly lessened in the last few years by the increasing commercial availability of suitable precursors and reagents. These factors have meant that a large proportion of the assays published recently use an internal standard of this type (Biggs et al, 1976; Cole et al, 1977; Jindal & Vestergaard, 1978; Bjorkhem et al, 1979; Baba et al, 1980; Koyama et al, 1980).

Quantitative analysis by GC/MS suffers from the disadvantages of requiring expensive equipment and of often involving slow processing of samples. However, the