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Analytical Survey

Metabolic and drug profiling

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Abstract: Over recent years, advances in analytical technology have greatly improved our ability to study the metabolism of compounds from either endogenous or exogenous sources. The application of gas–liquid chromatography, mass spectrometry, high-performance liquid chromatography and immunological approaches are discussed in relation to the analysis of steroids, bile acids, organic acids, prostaglandins, porphyrins and bile pigments, amino acids, proteins, nucleotides, catecholamines, vitamins and drugs.

Keywords: *Bile acids; profiling; steroids; mass spectrometry; prostaglandins; therapeutic monitoring.*

Introduction

The dictionary definition of a profile mentions an outline or a contour, but under the present title it is generally accepted to be the demonstration and measurement of a medium to a large group of metabolites of compounds from either endogenous or exogenous sources including drugs. Modern analytical technology has enabled considerable advances to be made in this area and it is the intention here to review the techniques currently available for detecting and measuring the groups of compounds most commonly purified in clinical chemistry.

The principles most appropriately applied are those involved in gas–liquid chromatography (GLC), mass spectrometry (MS) (separately and together as GLC–MS), high-performance liquid chromatography (HPLC) and electrophoresis. Immunological techniques are useful for screening purposes involving the detection and measurement of single compounds at any one time, but they are difficult to adapt to profiling.

It is interesting to look back in history to see how, for example in the study of steroid metabolism, earlier techniques have been replaced to considerable effect, exemplifying the thesis that advances in technology largely precede advances in the understanding of

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medicine. In the late 1950s the Bush type of paper chromatography with its broad, poorly resolved bands was the best which was available for practical use in the profiling of urinary steroids. This was replaced in the 1960s by thin-layer chromatography (TLC) and this in turn later in that decade by gas chromatography, firstly using packed columns and then in the mid-1970s using capillary columns with their high resolution. Thin-layer chromatography can still be used to considerable effect, as shown in Fig. 1.

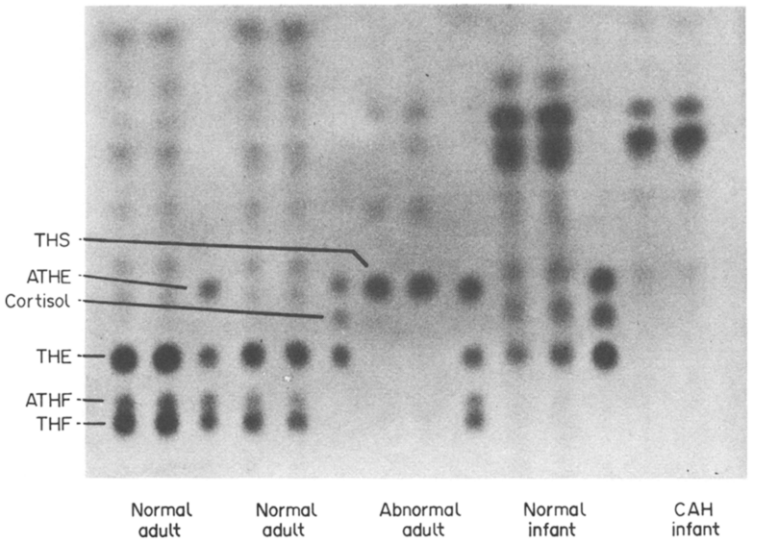

Figure 1
TLC separation of polar, blue tetrazolium-reacting steroids from urine samples of normal adults, an adult with adrenal hyperplasia (CAH) due to an 11β-hydroxylase defect, one normal infant and one with CAH due to 21-hydroxylase defect. The following steroids are identified: THF, tetrahydrocortisol; THE, tetrahydrocortisone; THS, tetrahydro-11-deoxycorticol; ATHF, 5α-THF; ATHE, 5α-THE.

Inevitably, the large number of compounds separated by exploiting the new technology led to problems of identification, which were fortunately solved by the timely availability of mass spectrometry. Since the mid-1970s, advances in steroid profiling have been largely in the area of eliminating tedious preliminary solvent extraction and purification procedures by using ion-exchange chromatography. Soft ionization techniques now coming into use in MS are opening up new applications both in the steroid and other fields and are greatly extending the analysable molecular mass range. More details of the clinical application of steroid analyses in general are given below.

HPLC has been applied to steroid analysis, but not with the dramatic effects of its introduction into other areas. It does not have the disadvantage of gas chromatography, where the destructive conditions on the column often necessitate the preliminary formation of more stable conjugates. Since its successful connection on-line to a mass spectrometer has not yet been satisfactorily achieved, the many advantages of mass spectrometry which have so enhanced gas chromatography have not yet accrued to HPLC. Nevertheless, it rivals gas chromatography for being the most powerful analytical tool currently available to medicine.

The laboratory facilities currently available for profiling will be discussed under the headings of various groups of compounds.

Steroids

Steroid hormones are produced from cholesterol both by the adrenal cortex and the gonads. Structurally they are C_{18}, C_{19} or C_{21} derivatives of the cyclopentanoperhydrophenanthrene nucleus, substituted with 2 to 6 oxygen functions. Their individual biological functions differ, but include the regulation of glucose, salt and water metabolism, and the development and maintenance of reproductive function. The major active hormones have either a 3-oxo-4-ene configuration or a phenolic A-ring, but after circulating in plasma a number of transformations take place, both in the liver and peripherally, to reduce the biological activity and to assist elimination from the body, usually as conjugates with glucuronic or sulphuric acids. Thus in blood or urine samples there is always a wide range of steroids with variations in structure and amount. In many clinical situations, mostly clearly circumscribed, valuable information can be obtained from precise measurements of a few individual hormones or metabolites; often, however, a clinical situation demands profiling. Chromatographic analysis of steroids in urine was first used to establish the nature of compounds interfering in the laboratory assessment of adrenocortical function of newborn infants [1]. After separation using paper or thin-layer chromatography, the steroids were located by their ability to form coloured products with group selective reagents to give, for example, the blue tetrazolium or Zimmerman reactions. In the investigation of infants, C_{19} steroids with the 3β-hydroxy-5-ene configuration were found to predominate and were later shown to originate from a zone of the adrenal glands which is unique to the foetus and neonate. During pregnancy many of these steroids are metabolized to oestrogens by the placenta, but the occasional absence of the ability to carry out this conversion seems to have little effect on the outcome of a pregnancy. Several of the major steroids involved were identified in urine by mass spectrometry after purification using TLC [2], but these techniques lack the resolution to separate all the steroids in neonatal urine.

Gas chromatographic steroid profile analysis was first described by Gardiner and Horning in 1966 [3], but the possibilities for resolution have been extended since 1970 when capillary columns suitable for steroid analysis became available. Temperature programming is required to separate all the steroids isolated from urine; conjugates have first to be hydrolysed and the free steroids then made stable for high temperature (180–260°C) column operation. Methyloxime and trimethylsilyl ether derivatives [4, 5] are the most commonly used. Steroids can be partly identified by their retention characteristics relative to a homologous series of co-injected *n*-alkanes with linear chain length of C_{22} to C_{34}. Confirmation of the steroid structures is carried out by GLC–MS, in which MS replaces the conventional GLC flame ionization detector. This approach has proved useful for establishing the steroid excretion profiles in most metabolic disorders of steroid synthesis and catabolism; profiles for each disorder have been published as an atlas with a clinical comment for each example [6].

Most of the steroids in urine are represented by a single peak in the GLC elution profile, but the steroid hormones in plasma are present in lower concentrations and give rise to more than one product, largely due to the formation of syn- and anti-oxime derivatives of the 3-oxo-4-ene groups [5]; LC profiles of the steroids found in plasma or tissue extracts would therefore be most useful since derivatization would not be necessary. Mixtures of the reference compounds have been separated by straight-phase or reversed-phase liquid chromatography, with bonded phases being most widely used. Resolution of the multiple components in steroid mixtures is only attained using

microparticulate column packings and for some compounds, notably the C_{18}-hydroxylated C_{21} steroids, it is desirable that the support be fully capped [7]. The mobile phases employed are very polar and typically comprise mixtures of methanol–water or acetonitrile–water, used in gradient elution mode. Steroids are usually detected by their UV absorption at 240 nm and therefore sensitivity is limited to about 1–10 ng. A refractive index detector requires an isocratic elution system and the sensitivity may be only 50 ng. Oestrogens can be monitored at their peak UV absorption of 280 nm or with an electrochemical detector. The detection limits in all these systems are too high for the amounts of endogenous steroids usually available in clinical specimens, so that it becomes necessary to use some form of GLC–MS or radio-immunoassay (RIA) [8].

Sufficient steroids are present in 0.1–0.2 mg of adrenal tissue to allow measurement using HPLC profile analysis [9]; the steroids produced by *in vitro* preparations of living (tumour) cells can be detected as the radioactive products from labelled precursors [10]. HPLC profile analysis has also been used for monitoring the circulating levels of synthetic adreno-corticosteroids administered therapeutically [11], and for polar cortisol metabolites in neonatal urine [12].

The separation of urinary steroids after hydrolysis, using GLC with capillary columns, is at present the most reliable means of obtaining comprehensive information on steroid excretion, but there are new developments which make possible the direct analysis of steroid conjugates without prior hydrolysis. Steroid glucuronides as combined methyl ester, methyloxime and trimethylsilyl ether derivatives were first separated using GLC in 1967 [13]. The stability of the packed columns then available permitted temperature programming to about 300°C, so that only the glucuronides of androsterone, aetiocholanolone and pregnanediol could be measured directly in urine. Glass capillary columns can now be prepared in which the usual siloxane stationary phase is stable at 350–400°C [14], enabling corticosteroid glucuronides with molecular weights of over 1000 Daltons — tetrahydrocortisol glucuronide Me-Mo-TMSE is 1017 Daltons, for example — to be analysed. When suitably derivatized, the steroid glucuronides excreted in urine have GLC retention times equivalent to 36–44 methylene units [15] and each of the conjugates so far analysed gives a single chromatographic peak. Steroids with glycol or dihydroxyacetone side chains, however, give poor peak height responses relative to equivalent amounts of added internal standards and therefore correction factors are required for methodological losses at present.

In the formation of combined derivatives of the steroid conjugates, the sulphated steroids of the 3β-hydroxy-5-ene and phenolic series are converted to TMSE derivatives of the parent steroid alcohols. A GLC profile of all the steroid conjugates in urine without hydrolysis thus distinguishes the two conjugate groups (Fig. 2). Sulphated steroids have the same GLC retention times as the free steroid derivatives and the glucuronides elute much later. In most cases, therefore, when conjugate-type separation is required, it is not necessary to carry out a preliminary liquid chromatographic separation. However, the method has yet to be optimized, in view of the fact that steroid sulphates need a 16 h reaction with trimethylsilylimidazole for optimum exchange between sulphate and TMSE groups. Moreover, some losses are involved because the measured amounts of the final derivatives are less than the amounts formed when conjugates are hydrolysed before derivative formation [16].

Fast atom bombardment (FAB) is a new ionization technique in mass spectrometry [17] which offers exciting possibilities for the profile analysis of polar charged molecules. The analysis of steroids in urine using this method has been one of the first reported

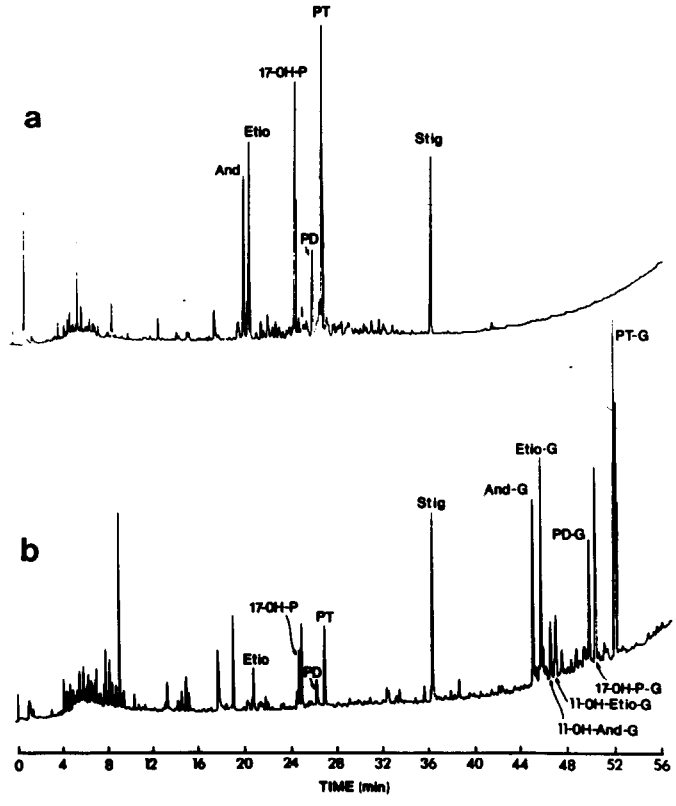

Figure 2
Temperature-programmed GLC profile analyses of steroids in the urine of a child with CAH due to 21-hydroxylase defect. (a) Steroid (MO-TMS) derivatives after enzymic hydrolysis of conjugates; (b) steroid conjugates as methyl ester-MO-TMS derivatives; sulphated steroids chromatograph as the MO-TMS derivative of the free steroid and the glucuronide derivatives elute later. Full chromatographic details can be found in refs [4] and [16]. The following abbreviations are used: And, androsterone; Etio, etiocholanolone; 17-OH-P, 17α-hydroxy-pregnanolone; PD, pregnanediol; PT, pregnane-3α, 17α, 20α triol; Stig, stigmasterol (internal) standard); 11-OH-And (-Etio), 11β-hydroxyandrosterone (etiocholanolone). The suffix G refers to the intact glucuronide derivatives. (This figure is reproduced by kind permission of Dr C. H. L. Shackleton.)

applications to biological specimens [18]. The sample (ca. 10 μg), dissolved in a small amount (5 μl) of a viscous liquid on a probe, is bombarded with a stream of neutral atoms (argon or xenon at 2 to 5 keV) and the positive or negative ions produced are analysed by the mass spectrometer. Glycerol has proved to be the most effective targeting matrix medium, although ions in the spectrum may be produced from interaction of the glycerol with the analyte. A caesium ion beam is also effective for the ionization of samples [19], and gives rise to secondary ion mass spectra (SIMS) which are frequently indistinguishable from FAB–MS. In all cases the spectrum from each steroid is dominated by a pseudomolecular ion with minimal fragmentation. Positive ion spectra of steroid conjugates from biological specimens are variable because the ions generated by SIMS or FAB–MS include the cations, and the spectrum for the isolated compound can have species from $[M + Na]^+$, $[M + 2\,Na]^+$, $[M + K]^+$ etc., where M is the anion of the steroid sulphate or glucuronide. Negative ion spectra $[M - H]^-$ reflect the loss of the

cations and are easier to interpret (Fig. 3). In the mass spectrum of mixtures of steroids each compound is represented largely by one fragment ion (Fig. 4). The technique does not distinguish isomers and for this purpose it may be necessary to form derivatives or to use further refinements of mass spectrometry; a recent publication has addressed this problem [20]. Characteristic profiles of steroid conjugates from the urine of patients with adrenal metabolic disorders have been published [16, 18] and the technique has also been used for steroids in umbilical cord plasma [16].

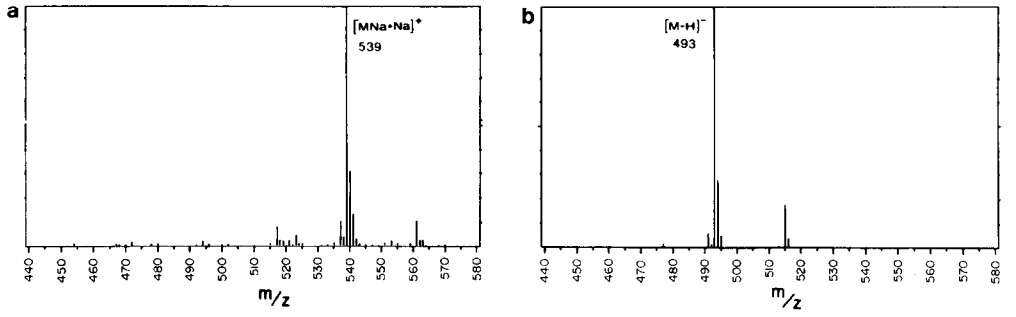

Figure 3
Secondary ion mass spectrometry of reference pregnanolone glucuronide (sodium salt) using caesium ion beam: (a) positive ion spectrum; (b) negative ion spectrum. (This figure is reproduced by kind permission of Dr C. H. L. Shackleton.)

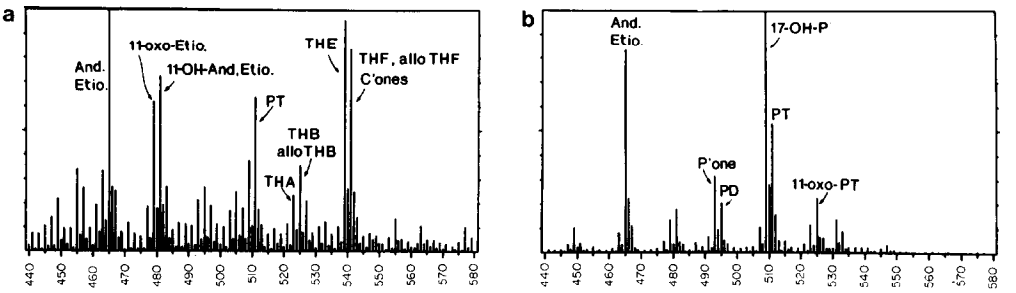

Figure 4
Profiles of steroids in human urine obtained as negative ion SIMS spectra of extracts from (a) normal adult urine and (b) a patient with CAH due to 21-hydroxylase defect. For key to abbreviations see Figs 1 and 2, in addition: THB, tetrahydrocorticosterone; allo THB, 5α-THB; THA, tetrahydro-11-dehydrocorticosterone; 11-oxo-etio, 11-oxo-etiocholanolonc; C'ones, cortolone (hexahydrocortisone). (This figure is reproduced by kind permission of Dr C. H. L. Shackleton.)

Bile Acids

Cholic acid is synthesized from cholesterol in the liver and excreted as taurine and glycine conjugates via the bile into the intestine, to aid the emulsification and absorption of lipids. Conjugated bile acids persist in the intestinal lumen as far as the ileum, where conjugates are hydrolysed by enzymes of intestinal bacteria to several metabolites of which lithocholic acid predominates. Some of these are excreted in the faeces and the

remainder are actively or passively absorbed and circulate in the enterohepatic circulation. Profile analysis of bile acids in blood, urine, bile and faeces has provided information for the diagnosis of hepatobiliary and intestinal disease in which there may be altered metabolic transformations of primary and secondary bile acids; these products could be implicated in the aetiology of colonic cancer [21], although this is not proven. Bile salts are used therapeutically to dissolve cholesterol gall-stones.

Conjugated bile acids are of more clinical interest than those occurring free and can be separated by reversed-phase HPLC systems similar to those used for fatty acids. UV-detection is at 195 nm [22], but other methods have been devised to permit detection at the levels found in blood and urine. *p*-Nitrobenzyl esters of the bile acids can be prepared prior to their separation and detection at 254 nm [23]. Bile acids in the eluates from a separation column can be passed with NAD into a second column containing immobilized 3α-hydroxysteroid dehydrogenase, where the NAD reduced in this reaction is proportional to the bile acid concentration. The resultant $NADH^+$ is mixed with phenazide methosulphate to give a final product which is monitored with an electrochemical detector [24], to give a reported sensitivity of 20 pmol for each bile acid.

Analysis of bile acids by GLC requires lengthy extraction followed by separation of the conjugates before hydrolysis to liberate the free acids. Combined derivatization is required to stabilize the functional groups before high temperature GLC analysis [25]. The need for deconjugation is overcome when using derivative formation with heptafluorobutyric acid in pyridine, when both the free acids and the glycine and taurine conjugates form the same derivatives [26].

Organic Acids

The detailed analysis of organic acids in biological fluids has provided profiles which reflect the nature and amounts of metabolic precursors due to a number of inborn errors of metabolism. These disorders frequently present with acute clinical symptoms in early life, so rapid diagnosis is desired. Since paper and thin-layer chromatography lack the resolution required to separate the range of homologues and isomers involved, they are now only useful for initial screening.

GLC with flame ionization detection is the best available technique for profiling organic acids once they are in the form of stable derivatives. Extraction, preparation of derivatives and analysis by GLC and GLC–MS have been fully reviewed and the data interpreted for diagnostic purposes [27, 28]. Prenatal diagnosis has been achieved for several of the organic acidurias from the GLC pattern of organic acids in amniotic fluid [28]. Since about 1977 capillary columns with split, on-column sample injection have been used for improved separation of the organic acids (see ref. [28], p. 89, for a comparison of packed and capillary column analysis of the same sample), giving greater specificity for the measurement of certain compounds; identification by GLC retention time can be highly reliable.

HPLC techniques for organic acids in biological samples are limited by the sensitivity of detectors currently available. Absolute identification and measurement thus require further analysis by immunoassay, GLC or GLC–MS.

The GLC profiles of short chain (C_1–C_6) carboxylic acids provide information towards the identification of bacteria, either when in laboratory culture or directly in clinical specimens, as for example in fluid taken from wounds [29]. Bacteria are grown in culture media supplemented with glucose and then the volatile fatty acids are injected on to a

GLC column by using either a head-space sampling technique or direct ether extract. Profiles of the metabolites thus reflect the enzyme complements of the bacteria and act as an index which can be used in their classification [30].

Prostaglandins

The prostaglandins are a group of oxygenated, polyunsaturated C_{20} fatty acids with a cyclopentane ring from C_8 to C_{12} (prostanoic acid). The presence in semen of smooth muscle stimulant materials was recognized in 1935, but was not fully characterized until 1962 when the structures of PGE and PGF were elucidated by using mass spectrometry [31]. The isolation and purification of prostaglandins is increasingly important for the elucidation of their physiological and pharmacological effects, as the number of compounds and the variety of their action increases. Thromboxanes (with a six-membered ring), prostacyclins (with a second ring) and leukotrienes are newly discovered relatives with actions on blood vessel muscles and on platelet aggregation.

TLC has been commonly used for prostaglandin separation but lacks resolution. The recovery of prostaglandins is seldom optimal, partly because of their chemical instability. The thromboxanes and prostaglandins co-chromatograph on a silicic acid column when used in HPLC systems, so that reversed-phase columns are now preferred for the separation of prostaglandins, thromboxanes and prostacyclins from various tissues [32, 33]. Detection of microgram amounts is achieved by UV-absorption, but quantitative determination in biological specimens requires more sensitive methods, such as selected ion-monitoring MS [34] or radioimmunoassay (RIA) [35].

Capillary column GLC with MS has been used for the rapid profiling of the major prostaglandin metabolites in the cyclo-oxygenase pathway of arachidonic acid, after combined derivative formation as used for steroid glucuronides and bile acids. This method has improved sensitivity when butylboronate formation is included in the stabilization of the functional groups of the prostaglandins before GLC analysis [36].

Porphyrins and Bile Pigments

Porphyrins are intermediates in the biosynthesis of haem, and abnormal porphyrin metabolism is associated with diseases such as the porphyrias and various types of porphyrinurias. The differential diagnosis of the porphyrias often depends on the biochemical estimation of porphyrins or their precursors in urine, faeces and blood. Since compounds are non-volatile and thermally labile, they are unsuitable for GLC analysis. TLC has been extensively used for porphyrin profiling and is adequate for the majority of cases. However, resolution, speed and sensitivity are greatly increased by the use of HPLC and it has become the accepted method for obtaining porphyrin profiles in the porphyrias [37] and in other diseases associated with abnormal haem synthesis.

HPLC is particularly useful when separation of the porphyrin isomers is required. For example, coproporphyrin I and III [38] and uroporphyrin I and III isomers [39] can easily be separated by reversed-phase chromatography on an ODS-Hypersil column with acetonitrile in 1 M ammonium acetate buffer (pH 5.18) as the mobile phase (Fig. 5).

Bilirubin and its conjugates are the most clinically important bile pigments and their estimation in plasma is essential for the diagnosis of various diseases associated with jaundice. The classical diazo reaction, widely used in the determination of conjugated and unconjugated bilirubins, can produce misleading results with differing amounts of the various forms of bilirubin present in blood. HPLC profiles from human sera have

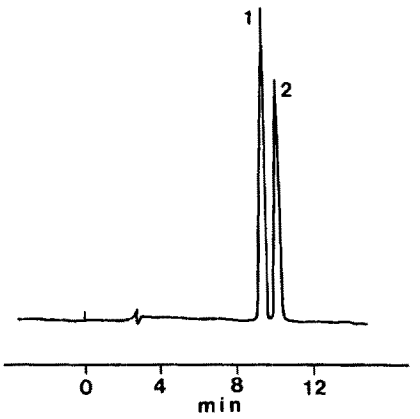

Figure 5
Separation of uroporphyrin I (1) and III (2) isomers by HPLC. Column, ODS-Hypersil; eluent, 13% acetonitrile in 1M ammonium acetate buffer pH 5.18, flow rate 1 ml/min.

shown the presence of four different bilirubin species; unconjugated bilirubin, bilirubin mono- and di-glucuronide and bilirubin covalently bonded to albumin [40]. The last of these is only observed in certain liver diseases [40] and is present in negligible amounts in neonatal plasma. In the absence of the covalently bonded bilirubin, the transesterification-HPLC method [41, 42] for the separation and estimation of bilirubin and its conjugates (Fig. 6) can be used. HPLC of bile pigments has been recently reviewed [43].

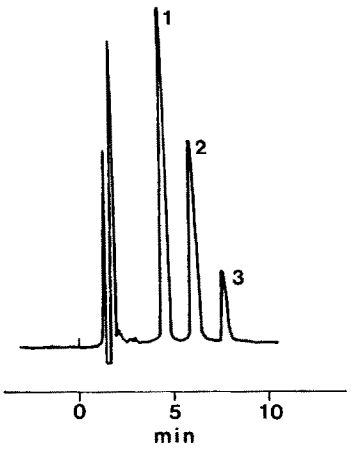

Figure 6
HPLC separation of bilirubin monomethyl ester (1), unconjugated bilirubin (2) and bilirubin dimethyl ester (3) after transesterification of bilirubin conjugates. Column, SAS-Hypersil; eluent, acetonitrile–dimethyl sulphoxide–water (34:34:32, v/v/v); flow rate, 1 ml/min.

Amino Acids, Peptides and Proteins

Amino acid analysers have been available for many years and have been widely used for amino acid profiles, but are gradually being replaced by rapid and efficient HPLC procedures. The majority of HPLC systems separate and detect the amino acids as the phenylthiohydantoin [44, 45], dansyl [46] or dimethylaminoazobenzene sulphonyl derivatives [47], but separation of underivatized amino acids followed by on-line post-column detection has also been successfully used for profiling [48, 49].

HPLC is a versatile technique capable of operating in a number of modes, including reversed-phase, reversed-phase ion-pairing, ion-exchange, affinity and size-exclusion. All these systems have been exploited for the separation of peptides and proteins [50–56]. The increased interest in the pharmacologically active peptides such as the

neuro- and gastrointestinal-peptides is largely due to the development of efficient HPLC systems for their separation [57]. Reversed-phase chromatography, which utilizes the hydrophobic interaction of peptides with the column packing, is the most widely used technique and excellent resolution can be achieved. However, the presence of residual silanol groups on the column may result in poor recovery of the peptides; this problem can often be solved by adding an ionic component to the mobile phase or by using a high molar concentration of buffer. The potential of HPLC for the clinical profiling of proteins in body fluids has been clearly demonstrated by its use in the separation and analysis of isoenzymes [56, 58], urinary proteins [59] and serum proteins including the lipoproteins [54, 60].

The use of HPLC for the screening of haemoglobin disorders in neonates is another important application. The complete separation of haemoglobins A, S, C and F has been achieved and has made possible the diagnosis of conditions associated with various haemoglobin disorders [61–64]. With improvements in column technology, HPLC may well rival the two-dimensional electrophoresis technique [65]. More recently the FAB mass spectrometry technique [66] has been applied to the analysis of peptides and this, when used in combination with HPLC, may well provide a new and powerful approach to peptide analysis.

Nucleotides, Nucleosides and Bases

These important constituents of nucleic acids and enzyme cofactors are essential for the normal function of cells, tissues and organs. Defects in purine and/or pyrimidine metabolism are associated with cardiovascular diseases, renal failure, gout, toxaemia and mental retardation.

The nucleotides, nucleosides and bases were one of the first groups of compounds to be analysed by HPLC because of their strong UV-absorption at 254 nm. Ion-exchange, reversed-phase and reversed-phase ion-pair systems are available for their separation in samples from cells, tissues, blood and urine [67–71]. The profiling of these compounds by HPLC, and its application in the clinical diagnosis of diseases, have been reviewed [72].

Catecholamines and Metabolites

Metabolic profiles of catecholamines and their metabolites are used in the diagnosis of hypertension, Parkinson's disease, cardiac disorders and neural crest tumours. Apart from some urinary metabolites the catecholamines may present in quantities such that a highly sensitive and specific detection system is essential for their analysis. The radioenzymic method involving catechol-*o*-methyl transferase [73] has the necessary sensitivity and specificity, but is complex, tedious and expensive. GLC and GLC–MS procedures are available, but require complicated and time-consuming sample preparation and derivatization steps.

HPLC with electrochemical detection [74–82] has been established as a technique of choice because of its simplicity, sensitivity and specificity. Most HPLC methods require preconcentration by elution of the catecholamines from alumina prior to chromatography. However, Kraak and coworkers [83] have developed a method for the extraction of catecholamines into organic solvents as the diphenyl or phenyl borate complexes; a recovery of over 90% is reported. This approach marks a considerable improvement in the isolation of catecholamines for chromatographic separation.

On-line post-column fluorescence detection is an alternative to electrochemistry

[84–86]. A detection limit of 1 pg for adrenaline and noradrenaline has been achieved using the trihydroxyindole reaction, compared with 10–20 pg for electrochemical detection. The HPLC analysis of catecholamines and metabolites has been extensively reviewed [87, 88].

Vitamins

GLC methods are generally unsuitable for the analysis of vitamins because of their instability to derivatization, and the formation of multiple thermally labile derivatives. Of the fat-soluble vitamins much attention has been paid to the analysis of vitamin D and its metabolites. These compounds are important in calcium metabolism and have been used for the treatment of bone diseases.

The HPLC separation and detection (by UV-absorption) of vitamins D_2, D_3, 25-hydroxy-D_2 and 25-hydroxy-D_3 in serum is relatively easy, with separation usually being carried out by adsorption chromatography on silica, using hexane–dichloromethane–methanol or a similar solvent as mobile phase; reversed-phase chromatography of the hydroxylated metabolites is also possible [89–91]. The normal amounts of the dihydroxylated metabolites, especially 1,25-dihydroxy-D_2 and 1,25-dihydroxy-D_3 are, however, below the UV detection limit; their determination is important since these are the most potent and rapidly acting metabolites. Sensitive RIA, radioreceptor binding assay and bioassay [92, 93] have been developed for the quantitation of 1,25-dihydroxy-D in serum or plasma, but there is severe interference from other metabolites. The only sensitive and specific approach for assaying the dihydroxy metabolites seems therefore to be a combination of methods, involving an effective HPLC separation with a sensitive detection system like RIA, radio-receptor assay or bioassay.

Vitamin A and its metabolites are also often assayed in clinical chemistry. Retinol, retinyl acetate and retinoic acids have all been satisfactorily analysed by reversed-phase chromatography with UV or fluorescence detection [94–96].

HPLC profiles may be obtained for other vitamins in body fluids including ascorbic acid [97–99], folates [100], vitamin B complex [101–104], tocopherols, nicotinamide and metabolites [105] and vitamin K [106].

Drug Metabolism and Therapeutic Monitoring

The analysis of drugs is mainly required for studying their pharmacokinetics and metabolism, and in the therapeutic monitoring of patients. Therapeutic drug monitoring is carried out after establishing the therapeutic range, usually for drugs with low therapeutic ratios (toxic/effective dose) or where serious side effects are known to occur.

The principal techniques available for the analysis of drugs in body fluids are GLC, GLC–MS, HPLC, RIA, enzyme immunoassay (EIA) and spectrophotometry.

Spectrophotometric techniques usually have low specificity and are gradually being replaced by those involving chromatography and immunoassays; they are, however, still sometimes useful for preliminary screening purposes.

Immunoassay techniques have considerable advantages, due to their high sensitivity and the ease with which a large number of samples may be dealt with. A major problem, however, arises due to cross-reactivity with non-active metabolites and other compounds with related structures. Immunoassay methods are only useful for the analysis of individual drugs and are unsuitable for metabolic profiling.

Chromatographic techniques, especially GLC and HPLC, are more specific and are

able to measure drug metabolites or even different groups of drugs simultaneously. GLC–MS provides one of the most powerful techniques for the study of drug metabolism; HPLC–MS has also been used [107, 108], but it is generally less sensitive and is more difficult to operate due to the problem of removing relatively large volumes of solvents. With the recent development of micro- and capillary-column HPLC, the problems of interfacing MS should be simplified, so that HPLC–MS can be expected to become another powerful technique for drug analysis.

The anti-epileptics, theophylline, anti-arrhythmics, tricyclic antidepressants, antibiotics, cardiac glycosides and antineoplastics are all examples of drugs commonly analysed in biological fluids in clinical laboratories.

The anti-epileptic or anti-convulsant drugs represent the most widely monitored groups. Kits are available commercially for the common anti-epileptics using enzyme multiplied immunoassay technique (EMIT) and substrate-labelled fluorescence immunoassay (SLFIA) [109, 110]. The accepted procedure for these drugs is GLC [111–113], but versatile HPLC methods are being used increasingly [114–117]. Since the drugs are sometimes administered in combination and may produce active metabolites, methods involving chromatography are usually used in preference to immunoassay.

Theophylline is commonly analysed, and many GLC and HPLC methods have been described for monitoring serum and plasma samples [118–120]. Immunoassays are to be preferred for batch analysis, because of their speed and the ease with which large numbers of samples may be handled.

Immunoassays are also the methods of choice for digoxin and related glycosides; both RIA and EIA kits are available commercially. The EIA procedures are usually the more tedious to perform. GLC and HPLC separations of the cardiac glycosides have been developed [121], but the methods are insensitive and too long and tedious for routine use.

Spectrofluorometric procedures have been used for many anti-arrhythmic drugs, but since they are non-specific and unable to differentiate active from non-active metabolites, they are largely being replaced by other techniques. EMIT kits are now available for procainamide, *N*-acetylprocainamide, quinidine, propranolol, lidocaine and disopyramide. Of the chromatographic techniques, GLC is generally unsatisfactory for anti-arrhythmics but rapid and specific HPLC methods are available both for the drugs and their metabolites [122–125]. The separation of verapamil from its active metabolite norverapamil is an example (Fig. 7).

Until recently, monitoring the tricyclic anti-depressants has been a problem, since spectrophotometric and fluorometric methods do not provide the necessary specificity, while TLC and GLC with flame ionization detector lack the required sensitivity. However, the recent development of reliable nitrogen-specific detectors provides adequate sensitivity and has led to the development of GLC methods which are now widely used [126]. The fully automated HPLC system, Technicon FAST-LC [127], was reliable and relatively fast for the monitoring of tricyclic antidepressants, but is now no longer commercially available. Immunoassays have been developed for these drugs, but have not shown sufficient reliability.

There are four basic techniques for assaying the antibiotics — microbiological, immunoassay, radioenzymic (transferase) and HPLC. Microbiological assays are inexpensive, but are relatively non-specific and slow. Immunoassays are available for aminoglycosides, particularly gentamicin, and are rapid, sensitive and specific. Radioenzymic assays are also rapid and sensitive, but are less specific compared with

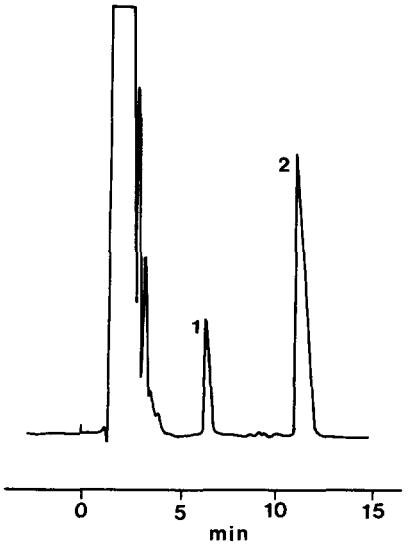

Figure 7
Separation of norverapamil (1) and verapamil (2) in serum by HPLC. Column, MOS-Hypersil; eluent, 55% methanol in 0.1M ammonium acetate; flow rate, 1 ml/min.

immunoassays. Very simple HPLC assays are available for chloramphenicol [128] and antifungal agents [129]. HPLC is less useful for the aminoglycosides because derivatization is necessary for detection [130].

Immunoassays and HPLC techniques are the methods of choice for the determination of antineoplastics. A combination of HPLC and RIA is sometimes used to give improved sensitivity for drugs that cannot be detected satisfactorily with a normal HPLC detector. It is anticipated that drug analysis and profiling will increase considerably in the future and immunoassays, GLC and HPLC will continue to play important roles in the field of metabolic and drug profiling.

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