structure) can be performed by this method but only in combination with other, mainly spectroscopic, analytical techniques.

See Colour Plates 69, 70.


Further Reading


of automation are not only desirable but essential. Most chemistries used are either wet chemistry or dry-film technology. The nature of certain analytes may make them less amenable to the high volume chemistries or the volumes of the rarer analytes make the development of automated chemistries more expensive or time consuming. Chromatography fits this last scenario in clinical laboratories. An illustrative workload pattern is presented in Table 1.

Clearly chromatography is utilized for those tests that are low volume and for which, generally, rapid turnaround is not required. Table 2 illustrates the analytes, the mode of chromatography and reasons for use.

Table 1  Analytes, methodology, volume and cost: Illustration of a ‘typical’ clinical biochemistry laboratory workload, ranked in order of cost

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Tests per day (approx)</th>
<th>Method</th>
<th>Cost/test* (approx)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea and electrolytes (Na⁺, K⁻)</td>
<td>4000</td>
<td>Automated wet chemistries and ion-selective electrodes</td>
<td>£1.00 each, i.e. £4.00 profile</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>50</td>
<td>Automated nephelometry</td>
<td>£4.00</td>
</tr>
<tr>
<td>Thyroid function (thyroid stimulating hormone, thyroxine)</td>
<td>200</td>
<td>Automated non-isotopic immunoassay</td>
<td>£5.00</td>
</tr>
<tr>
<td>Bedside device for a single drug of abuse</td>
<td>1</td>
<td>Immunochromatography</td>
<td>£5.00</td>
</tr>
<tr>
<td>Serum proteins</td>
<td>30</td>
<td>Electrophoresis</td>
<td>£15.00</td>
</tr>
<tr>
<td>Drugs of abuse</td>
<td>30</td>
<td>Automated immunoassay and chromatography</td>
<td>£15.00</td>
</tr>
<tr>
<td>Haemoglobin A,c</td>
<td>50</td>
<td>Automated chromatography</td>
<td>£10.00</td>
</tr>
</tbody>
</table>

*Cost includes consumables, reagents, labour overheads and equipment depreciation.

Table 2  Common current clinical chromatographic applications

<table>
<thead>
<tr>
<th>Analyte class</th>
<th>Chromatographic mode*</th>
<th>Reason for use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin A,c</td>
<td>LC</td>
<td>Most specific, rapid</td>
</tr>
<tr>
<td>Haemoglobin variants</td>
<td>LC</td>
<td>Specific for certain variants</td>
</tr>
<tr>
<td>Metabolites associated with inborn errors of metabolism</td>
<td>LC/GCMS, LC-MS-MS</td>
<td>Specificity/flexibility</td>
</tr>
<tr>
<td>Drugs</td>
<td></td>
<td>Most specific, rapid</td>
</tr>
<tr>
<td>Therapeutic drug monitoring</td>
<td>LC/GC</td>
<td>Cost/flexibility</td>
</tr>
<tr>
<td>Drugs of abuse</td>
<td></td>
<td>Most specific, rapid</td>
</tr>
<tr>
<td>Screening</td>
<td>TLC; HPTLC; GC</td>
<td>Specificity/flexibility/cost</td>
</tr>
<tr>
<td>Confirmation</td>
<td>GC; GCMS; LC-MS</td>
<td>Specificity/flexibility</td>
</tr>
<tr>
<td>Toxicology</td>
<td>TLC; GC; LC</td>
<td>Flexibility</td>
</tr>
<tr>
<td>Markers of bone turnover</td>
<td>LC</td>
<td>Specificity, being superseded by immunoassay</td>
</tr>
<tr>
<td>Near patient testing devices, e.g. drugs of abuse</td>
<td>Immunochromatography</td>
<td>Convenience</td>
</tr>
<tr>
<td></td>
<td>‘stick’ tests</td>
<td></td>
</tr>
</tbody>
</table>

*LC, liquid chromatography; GC, gas chromatography; MS, linked mass spectrometer; TLC, thin layer chromatography; HPTLC, high performance thin layer chromatography.
agents, meant that for the first time quantitative TLC with performance equivalent to GC or LC was possible. While some laboratories, particularly in Germany, enthusiastically adopted this technique it gained very few adherents elsewhere, particularly in clinical laboratories where the demand and skill base were inadequate.

Since the mid-1980s there has been an increasing problem of drug abuse. The initial assays developed for screening in the late 1970s used enzyme-multiplied immunoassay technique (EMIT), followed a little later by fluorescence polarization immunoassay (FPIA). These techniques were good for screening large numbers of individuals for a range of substances or classes of substances, e.g. cannabis, cocaine, opiates, benzodiazepines, barbiturates. The test for opiates was designed to detect heroin abuse but in fact it detects other opiates, i.e. codeine and dihydrocodeine, which are legitimately available. Clearly, therefore, any positive for this assay system, and also for others, requires confirmation. Immunoassay-based methods inherently depend on the specificity of their antibody and may not detect subtle structural differences between compounds leading to false positives. This clearly has implications for the subject tested, whether for clinical, forensic or employment purposes. It is now a firmly established principle, regretfully not always adhered to, that before taking action confirmation with a non-correlated technique should be performed. Chromatography is the technique of choice.

In clinical testing, HPTLC with appropriate location reagents and visual inspection is adequate as a confirmation technique, although this would usually be supported by other chromatographic modes. HPTLC also enables screening for many of these compounds not detected by the immunoassay screens; consequently HPTLC is a dynamically utilized method in clinical laboratories performing substance abuse testing (Figure 1).

However, the other development in TLC was the development of a cellulose-based commercial system with a stylized Marquis reaction with reference to $R_f$ and sequential colour changes collected in a compendium. This system was developed for clinical toxicology work allowing it to be performed by the non-specialist. In overdoses it works well despite its lack of chromatographic efficiency. Unfortunately the system was inappropriately applied to drugs of abuse testing which has greater sensitivity and specificity requirements and external quality assurance data consistently demonstrate poor performance.

In clinical practice, knowledge of which drug has been taken as determined by laboratory studies is useful for only a very few drugs and the concept of screening, usually using chromatography, is no longer commonly performed in the UK; in difficult cases, however, this is the preferred method of investigation.

TLC has a further unique property in clinical investigation. The plate complete with separation can be sent from the original investigating laboratory to a reference centre which will be able to investigate directly (perhaps using TLC-MS-MS) (Figure 2) any difficulty to identify/confirm spots. Currently this type of approach is a neglected area.

Gas Chromatography (GC)

The heyday of GC in clinical laboratories was in the 1970s. Biological fluids contain proteins in high concentration ($\sim 75 \text{ g L}^{-1}$) in a complex mixture of endogenous and exogenous metabolites and the compound of interest may be nonvolatile and present in low concentrations. The trick therefore was to extract the compound of interest quantitatively, make it vol-

---

Figure 1  HPTLC of extracted urine from drug abusers. Morphine, the principal metabolite of heroin, is indicated with an arrow.
atil and chromatograph it. Method development could take many man-hours and while some automation pre- and post-chromatography could be performed, these were labour-intensive methods best suited to low molecular weight metabolites. In the 1980s with increasingly better LC systems, GC usage in clinical laboratories went into decline.

Continued use of GC is necessary for a few analytes, however. Methanol, ethylene glycol, propanol and ethanol poisoning present overlapping clinical pictures; knowledge of which alcohol has been taken and how much is vital. GC with flame ionization detection is the ideal method giving rapid, reliable results and can be utilized in an emergency situation. Some laboratories still use GC for drug analysis.

Capillary GC with flame ionization and/or nitrogen-phosphorus detectors offers high sensitivity and in the latter case good specificity for determining biological analytes. Applications cover substance abuse, therapeutic drug monitoring and intermediate endogenous metabolites. However, GC-MS as benchtop analysers, either ion-trap or quadrupole, are becoming more common, though they are still rare, in clinical laboratories. The main reasons for the poor uptake of this combination are cost, demand and staff skills required.

As noted in Table 2 intermediate metabolites found in inborn errors of metabolism which can lead to significant morbidity and mortality, are currently detected by capillary GC. Early detection is vital, and although family screening can now be performed by molecular biology, many cases are sporadic mutations requiring resolution for the optimal care of that individual. Failure to achieve this results not only in incapacity for the individuals and their family, but also in significant costs to the health service.

Capillary GC is satisfactory for a clinical service for drugs of abuse although GC-MS is essential in any forensic or employment issues, in which some clinical laboratories are involved.
Liquid Chromatography (LC)

Liquid chromatography, with its wide range of modes and better biocompatibility than other chromatographic methods, has been utilized in clinical laboratories. Classical column chromatography was used in sample preparation of many of the standard wet chemistry methods in the past. Protein isolation using affinity chromatography enabled isolation of antibodies and subsequent labelled antibodies to provide the first radioimmunoassays in the 1950s and 1960s. Moore and Stein in 1954 developed an amino acid analyser which was refined over the years. Although primitive by today’s standards, it enabled the first reliable measurement of the common amino acids allowing investigation into their metabolism, role in nutrition and relevance in inborn errors of metabolism. Amino acid analysers were automated with post-column reaction for detection and to a limited extent specificity. Much of the basic knowledge learnt through this pioneering work formed the basis for subsequent biological applications of LC.

Ten to fifteen years ago LC was making a significant impact in research-orientated clinical laboratories. Initially work focused on reversed-phase materials with many publications on the separation of a wide variety of drugs, endogenous metabolites and steroid hormones. A major difficulty was sensitivity and there was no true universal detector, nonetheless therapeutic drug monitoring already expanding due to the availability of EMIT technology consolidated using LC procedures for previously difficult analytes. A classic example was the common anticonvulsant carbamazepine which suffered thermal degradation on GC leading to significant imprecision. This was resolved by using LC. Additionally, it became readily possible to measure several drugs simultaneously. While this had been done using temperature-programmed GC it was frequently necessary to derivatize compounds to obtain satisfactory volatility and polarity and this could affect the selectivity. LC enabled the same approach, initially using solvent gradient programming on undervatized samples, often with minimal sample preparation. This approach enabled separation of a full range of anticonvulsants: ethosuximide, primidone, phenytoin, phenobarbitone and carbamazepine and their metabolites (Figure 4).

This meant that efficient processing, all samples followed the same analytical track, reduced costs per analyte and the occasional detection of inappropriate medication. In this particular area the wide and increasing range of nonisotopic immunoassays compatible with automation has meant many clinical laboratories find it more organizationally efficient to perform these assays by automated immunoassay; a hard core still use LC, though whenever possible using an automated system. Surveys through the UK National External Quality Assurance Programme have consistently shown LC methods to have the best accuracy with acceptable imprecision. The introduction of new anticonvulsants, e.g. lamotrigine, has led to a demand for analysis. This has meant development of LC assays demonstrating the role of chromatography in development of clinical investigations.

This is a common scenario in method development in that research or reference centres develop an LC assay for a new analyte to detect or monitor disease. As the utility of this determinand is demonstrated, demand rises, causing processing difficulties for the reference centre, parallel to an increasing demand from less specialist centres. Some will invest in an LC solution but the reagent manufacturers are alerted to the developing interest in the analyte. Knowing that clinical laboratories have a predilection for rapid, automated large single or multiple similar medium analysers, they develop wet chemistry or immunoassay methods. This approach will be reagent expensive but require minimal labour utilizing pre-existing equipment. Once this is marketed and adopted the LC procedure, albeit more accurate, declines and may be dropped altogether.

A further illustration of this is the measurement of bone turnover; the original analyte hydroxyproline was determined by a cumbersome, manual, wet chemistry assay. As interest grew in hormone replacement therapy effects in osteoporosis much effort was expended in developing a gold standard LC assay for pyridinoline and deoxypyridinoline. There were
particular problems, eventually resolved, with obtaining a satisfactory internal standard. This has become established as the reference method, but is now being supplanted by immunoassay measurement of deoxypyridinoline.

Screening methods using LC have suffered from the lack of reproducibility between reversed phases. The REMEDI system for drug abuse screening is however an established screening technique linked to a linear diode array with spectral library and has been shown to provide satisfactory identifications on screening for drugs.

The detection of inborn errors of metabolism by reference paediatric biochemistry laboratories has hitherto relied heavily on GC-MS with all the inherent problems plus the need for separate assay condition for different compound classes, e.g. organic acids and amino acids. Recently the NHS Research and Development Programme – Health Technology Assessment – after an evidence-based medicine research exercise, has indicated that LC–MS–MS would deliver appreciably greater benefits than current systems for the detection of a variety of different forms of inborn error of metabolism. Hitherto, funding for such equipment had been stalled on the capital cost, but demonstration of the economies delivered by LC tandem MS argued in favour of the system which is now being introduced in selected centres. It is such economic analyses that may progress and sustain chromatographic methods in the face of a decreasing skill base and drive for consolidation in clinical laboratories.

An example of where the specificity of LC is valued and allied to an improvement in efficiency is the analysis of haemoglobin A\textsubscript{1c} (Hb A\textsubscript{1c}). Hb A\textsubscript{1c} is glycated haemoglobin A, and is a long-term measure of control in diabetes mellitus. Recent international advice has called for close control of Hb A\textsubscript{1c} in diabetics and requires an accurate and precise method. Early studies on Hb A\textsubscript{1c} used classical column chromatography on mini-columns, this was labour intensive and commercial ‘kit’ LC solutions began to be offered. Soon an automated wet chemistry analyser compatible methods were developed and embraced.

However, significant proportions of the population, depending on ethnic mix, do not have haemoglobin A as their sole haemoglobin and to assess glycaemic control one must look at the glycation of the variant haemoglobin; the wet chemistry method cannot do this. There has therefore been a resurgence of interest in the ‘kit’ LC solutions; popular ones use anion exchange chromatography which have run times of 4 min providing full sample automation for over 250 samples per day (Figure 5).

![Figure 5: Ion exchange chromatography of haemoglobin A\textsubscript{1c}, an indicator of glucose control in diabetics. Hb A\textsubscript{1c} retention 1.75 min.](image)

As diabetic assessment is performed regularly and many organ systems are examined, it is not uncommon for blood to be drawn from a patient and the Hb A\textsubscript{1c} to be measured in the clinic using LC while they wait. This provides the clinician with the result when seeing the patient and improves the patient throughput.

**Immunochromatography**

Immunochromatography is the preserve of research laboratories as a laboratory technique. Near-patient testing, however, utilizes commercially produced
positive and negative controls. When the antigen and antibody combine they develop a visible colour spot or band which confirms the presence of the compound of interest (Figure 6). While these devices are expensive and inaccurate they have the benefit of immediacy which may be clinically acceptable provided they are used appropriately.

### The Future

Chromatography has maintained its role in certain niches in clinical laboratories. Interest in manufacturer-supplied solutions for chromatography, particularly LC, exists and compensates for the lack of skill base. For difficult low throughput analyses this may be how developments will be consolidated. Capillary zone electrophoresis could impact on much current LC work but again skill and capital costs militate against this. If accuracy rather than imprecision becomes a major clinical laboratory issue, as it may, then the inherent accuracy of chromatography probably linked to mass spectrometry will provide a role for definitive methods and may provide a role for methods used in routine laboratories.


### Further Reading


---

**COAL: FLOTATION**

B.K. Parekh, University of Kentucky, Lexington, KY, USA

Copyright © 2000 Academic Press

**Introduction**

The process of froth flotation for upgrading the quality of coal by removing mineral matter (ash and pyrite) has received increased attention since the 1960s. The froth flotation process is typically used for treating <0.5-mm size coal and is currently the only technique both effective and economical to clean coal on a commercial scale. In the USA, the majority of coal preparation plants discard the <0.5-mm coal owing to the high cost of processing of the fine coal.