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Novel strategies for clinical drug analysis with new column technology in liquid chromatography

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Abstract: Clinical drug analysis has been the focus of much attention and controversy because of the increase in substance abuse testing of addicts, patients, employees and others, in addition to the rapidly growing fields of therapeutic drug monitoring and clinical toxicology. This review focuses on the latest methodological and technological advances in liquid chromatography (LC) as it is applied to clinical drug analysis. Based on the author's experience, key chromatographic parameters such as carbon load, functionalities and temperature are proposed for the separation of structurally similar metabolites and for resolving chromatographic interferences by other drugs and metabolites. Novel sample preparation for cyclosporine and gradient elution of its metabolites are reviewed, followed by an update on monitoring of 3′-azido-3′-deoxythymidine with emphasis on automated sample preparation. Various approaches of direct sample analysis are advocated for increased efficiency as a result of minimal sample preparation and potential advantages such as decreased exposure of personnel to infectious samples. An update of microbore LC indicates that 2-mm columns may be readily used for clinical paediatric and neonatal analysis without dedicated chromatographs. Potential applications of multidimensional–multimodal chromatography include analyses of a cocaine impurity, anticonvulsants, antidepressants, and five ingredients of a common cold medication.

Keywords: *Clinical drug analysis; column effects; liquid chromatography; AZT; cyclosporine; antidepressants; direct injection.*

Introduction

Drug analysis in the clinical laboratory is primarily concerned with the identification and quantitation of therapeutic and illicit agents — activities readily classified within the specialties of therapeutic-drug monitoring (TDM) and traditional toxicology. The rationale for TDM has been previously reviewed by Pippenger [1]. More recently in the United States, as a result of the epidemic abuse of illicit drugs such as cocaine, substance abuse testing has been recommended for various groups such as athletes, transportation workers, federal employees, and other professionals [2]. Consequently, clinical and forensic drug analysis has been expanding. In order to keep up with the increasing demand for drug testing, much of the drug monitoring is performed by automated immunoassay. However, for substance abuse testing, the National Institute of Drug Abuse, by the executive order of former President Reagan, requires drug confirmation analysis by gas chromatography (GC)/mass spectrometry (MS) [3]. Even though liquid chromatography (LC) offers many advantages, the following disadvantages have, thus far, limited its widespread application in the clinical laboratory: critics claim that LC is labour-intensive and difficult to automate totally, some sample preparation is always necessary, no universal detector is available, column stability is variable, and, when compared with immunoassay, LC may be less precise. However, immunoassay is an indirect measurement, and may suffer from cross-reactivity. Also, an immunoassay may be more costly than an LC assay. With the introduction of new column packings, a re-evaluation of the possible rôle of LC in clinical drug analysis seems highly desirable. This article reviews four areas of LC for clinical drug analysis:

(1) novel strategies for TDM of antidepressants, cyclosporine and 3′-azido-3′-deoxythymidine (AZT),
(2) the emerging rôles of direct sample analysis,
(3) microbore high-performance liquid

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chromatography (HPLC) for micro-sample analysis, and

(4) multidimensional/multimodal analysis.

Some Novel Strategies for Therapeutic Monitoring of Selected Agents

Antidepressants

Liquid chromatography is uniquely suited to the analysis of this drug class because of its ability to analyse parent drugs and metabolites simultaneously and to resolve potential interferences from other drugs and metabolites with similar structures. For the newly introduced antidepressants such as trazodone and fluoxetine, LC may be the only methodology currently available [4–7].

Based on the above considerations, a systematic approach of antidepressant monitoring by reversed-phase LC has been developed in this laboratory [4]. Several recent reviews have dealt with the clinical pharmacology, sampling considerations and current methodologies [4–7]. This present review focuses on the rôle of key chromatographic parameters in enhancing clinical analysis. The following parameters will be discussed: carbon load, functionality and multifunctional/multimodal separations and internal surface reversed-phase chromatography [8].

Prior to designing a strategy for LC analysis of antidepressants, knowledge of their metabolic pathways is helpful. In general, tricyclics and other antidepressants are metabolized via demethylation and oxidation. For example, imipramine is demethylated to desipramine, and both are hydroxylated at the 2-position to form pharmacologically active metabolites (Fig. 1) [9]. These hydoxylated metabolites are conjugated to glucuronides for urinary ex-

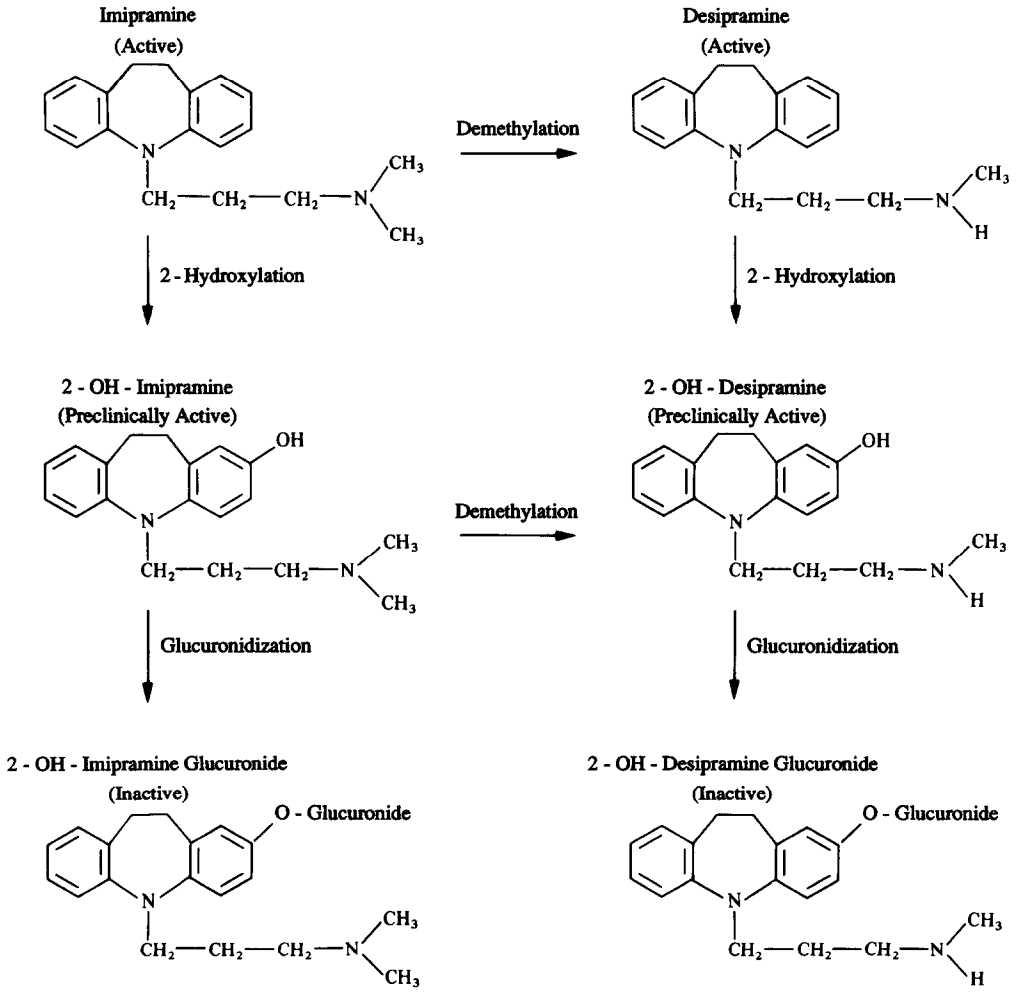

Figure 1
Major routes of metabolism of imipramine. (Adapted from ref. 9.)

cretion. Recently, Nelson *et al.* [10] demonstrated that the plasma concentrations of the sums of desipramine and 2-hydroxy-desipramine were significantly correlated with clinical response and their routine clinical measurements may be desirable [10].

A previous method for the measurement of antidepressants and metabolites, which employs a C_{18} column (Fig. 2), is widely used in many clinical laboratories, including this one [11]. Unfortunately, a significant interference has been observed in about 10% of clinical samples when using this method for the monitoring of imipramine, desipramine, amitriptyline, nortriptyline, doxepin and *n*-desmethyl doxepin. The majority (about 90%) of these interferences could be resolved by the use of a phenyl mini-column.

The rationale for using a phenyl column is based on the selectivity offered by the possible aromatic stacking interactions of the tricyclics with the phenyl groups of the stationary phase. Additionally, the proposed mini-column approach [11] offers the possibility of using two columns with the first mini-column as the guard column. Subsequent column changing involves discarding the first column and re-using the old second column as the new guard column. With the mini-column, nonylamine was used in the mobile phase to reduce the tricyclic interactions with the active silanol groups, effectively minimizing peak tailing. With this approach, the mobile phase contained 10% acetonitrile compared with 40% acetonitrile which was used in the previous method [5]. Furthermore, the column was operated at ambient temperature; rather than the 50°C that was needed for the previous procedure [5].

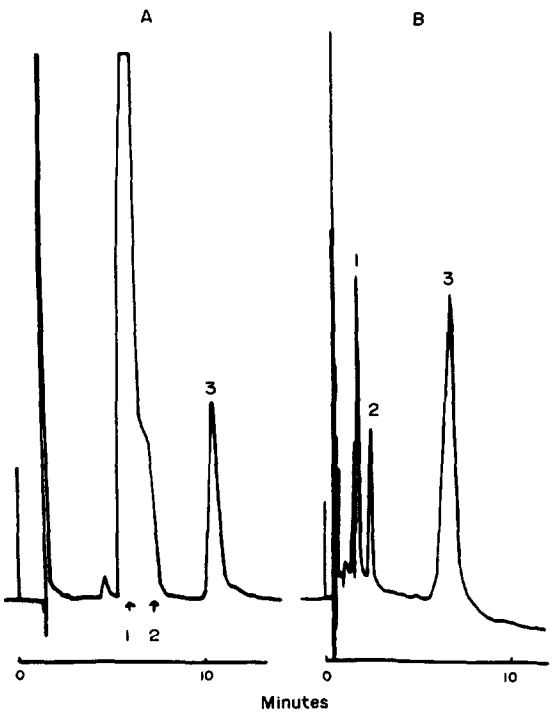

Figure 2
Chromatogram of plasma extract of a patient medicated with doxepin and other drug(s), (A) C_{18} and (B) phenyl. (Note that the interference was resolved by the phenyl column.) Peak identification: 1, N-DOX, 120 μg l^{-1}; 2, DOX, 113 L; 3, I.S.NOR for Fig. 2A and clomipramine for Fig. 2B. (Adapted from ref. 11).

Elevated column temperature is not popular in LC because of possible deterioration in column efficiency as a result of column voiding caused by the heating and cooling cycles. However, raising the column temperature can offer certain advantages, and in some separations (e.g. cyclosporine) [12, 13], it may be essential. Thus, the ambient temperature separation offered by the above phenyl column procedure is not that significant. Rather, elevated temperature should be considered as a viable adjunct for routine drug analysis. For example, elevated temperature has been used in combination with columns of high carbon loading for the separation of polar metabolites such as 2-hydroxy-desipramine, 7- and 8-hydroxy-amoxapines and 1-metachlorophenyl-piperazine (mCPP), a metabolite of trazodone [4, 5, 14, 15]. In methods using C_{18} or phenyl columns with a low carbon loading, the polar metabolites elute in the solvent front. For the analysis of 2-hydroxy-desipramine, a 15% carbon load column was needed to resolve it from 2-hydroxy-imipramine. For structurally similar metabolites such as 7- and 8-hydroxy-imipramine, an even higher carbon load column of 20% was needed, in combination with 0.2 M phosphate to suppress analyte interactions with the active silanol groups. An elevated temperature of 50°C was used to shorten the chromatographic run to about 22 min. Since the plasma concentration of 7-hydroxy-amoxapine, an active metabolite, is much lower than that of the pharmacologically active 8-hydroxy-amoxapine, the elution of the minor 7-hydroxy-amoxapine before 8-hydroxy-amoxapine ensured accurate quantitation. Another example of this approach is the resolution of a minor metabolite of trazodone, mCPP, from an unknown interference peak [15]. In a limited clinical study [15], mCPP

plasma concentrations ranged from 9 to 49 μg l^{-1}.

Recently, the separation of the optical isomers of fluoxetine has been achieved by forming mandelic acid derivatives which were then separated on a NH_2 column [17]. Other interesting developments in the clinical analysis of antidepressants involve the use of internal surface reversed-phase as part of the sample preparation step prior to analytical separation [16], and the availability of ceramic reversed phase for analysis of tricyclic antidepressants using alkaline mobile phase for minimized tailing.

Cyclosporine

The clinical monitoring of cyclosporine had been greatly facilitated by LC, allowing specific determination of cyclosporine in the presence of its many metabolites. More recently, however, monoclonal antibody-based immunoassays have been introduced, and the results compare favourably with those of LC [18]. According to the latest survey from the American Association for Clinical Chemistry [19], LC was used in 26 of 64 laboratories engaging in monitoring cyclosporine. Even with the availability of monoclonal antibody-based immunoassays, LC will most likely continue to play an important rôle in cyclosporine monitoring because it provides the opportunity to monitor the active metabolites [20, 21].

When developing the LC of cyclosporine, methodological considerations include its hydrophilicity, which renders extraction difficult; the lack of a good chromophore, which dictates detection at about 200 nm; the preferred use of whole blood to avoid temperature-dependent cyclosporine redistribution; and the occasional interference peak. Presently, either extraction into ether or solid-phase extraction is used for the preparation of clinical cyclosporine samples.

The procedure used in this laboratory [13] was adapted from that of Kabra *et al.* [12]. EDTA-preserved whole blood was haemolysed by mixing with acetonitrile–water. After rinsing a Bond-Elut (C_{18}) column with ethanol and water, aliquots of the clear supernatant were transferred onto the extraction columns, washed with acetonitrile–water and eluted with ethanol. The ethanol extract was then subjected to a hexane wash prior to the LC analysis. The column was a C_{18} cartridge column (4.6 × 30 mm) (Perkin–Elmer) and the mobile phase was acetonitrile–water (6:4, v/v) pumped at 1 ml min^{-1}. The separation temperature was 70°C. Detection wavelength was 210 nm, 0.005 AUFS.

More recently, Lensmeyer *et al.* [20] demonstrated the gradient elution LC of cyclosporine and nine metabolites (M1, M8, M17, M18, M21, M25, M26, M203–218 and MUNDF1) in whole blood samples. Solid-phase extraction columns were used, followed by chromatography on a CN-bonded phase column. The authors [20] suggested that the separation was based on both reversed-phase and adsorptive interactions. Measurement of clinical samples showed significant concentrations of CsA, M1, M8 or M17. Kabra *et al.* [22] showed the utility of the automated sample preparation system (AASP) of Varian in the clinical analysis of cyclosporine. After mixing the whole blood sample with the internal standard, the supernatant was transferred to the rinsed C_{18} cartridges. The washed cartridges were loaded into the sample preparation system and eluted with acetonitrile–methanol–water. The authors concluded that this was a fast, cost-effective and simple procedure.

3'-Azido-3'-deoxythymidine (AZT)

Although the rationale for the TDM of AZT in AIDS patients has yet to be established, its LC has been demonstrated in volunteer studies [23, 24]. Two normal volunteers were given 200 mg oral doses of AZT and their serum and urine AZT concentrations were established by LC. After addition of two internal standards, β-hydroxyethyl theophylline and β-hydroxypropyl theophylline, 1-ml aliquots of plasma or diluted urine were extracted with a mixture of chloroform–isopropyl alcohol (95:5, v/v). The organic phase was separated and evaporated at 60°C. Extracts were reconstituted with mobile phase, and 40 μl aliquots were injected into the LC apparatus. The column was a Supelcosil LC-18 (5 μm, 4.6 mm × 15 cm). The mobile phase was acetonitrile–phosphate (9:91, v/v), maintained at 30°C. The flow rate was 1.5 ml min^{-1} and detection was at 266 nm. The retention times were: BHET, 4.8 min; BHPT, 8.4 min, and AZT, 10.1 min. With the use of two internal standards, two linear ranges were established: 0.015–3 mg l^{-1} for plasma, and 0.3–30 mg l^{-1} for urine. The two subjects showed peak plasma concentrations of 0.53 and 0.72 mg l^{-1} at about 40 min. Due to the restrictions in sample handling, analyses of

samples from AIDS patients were not attempted.

More recently, Schmid and Kupferschmidt [24] reported a clinical assay for AIDS patients' samples using a novel, specific two-step solid-phase extraction and LC assay with a quantitative recovery of 95%. By using the Varian AASP, minimum sample handling was needed, reducing possible personnel exposure to the HIV virus. Heparinized or clotted blood samples were collected from AIDS patients. To reduce the risk of infection, these samples were incubated for at least 45 min at 56°C to inactivate the virus in blood samples, as recommended by Burroughs Wellcome. Extraction was carried out in two steps: concentration into Bond-Elut columns, and elution using the AASP. After mixing with the internal standard, BW A2U, 1–3 ml aliquots were transferred to the rinsed Bond-Elut (C_{18}) columns. After washing with water and drying, chloroform was used to elute the AZT into reservoirs of the AASP (Si) cartridges. These cartridges were dried and then mounted into the AASP module for elution and subsequent injection into the LC. Prior to injection, the cartridges were purged with 125 μl of water, followed by injection with mobile phase. The LC packing material was Partisil 5 ODS-3 (4 mm × 15 cm). The mobile phase was acetonitrile–phosphate buffer (13:87, v/v) at 1 ml min^{-1}. Detection was at 267 nm. Figure 3 shows the chromatograms of extracts of patient, blank and standard. For patients receiving 400 mg AZT, peak plasma concentrations of 1–3.5 mg l^{-1} were observed 40–120 min after dosing.

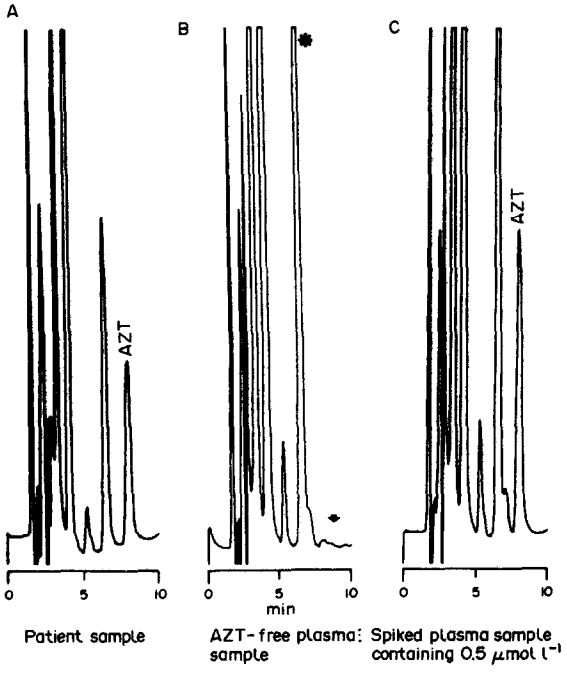

Figure 3
Chromatograms of (A) blood sample of patient on chronic AZT therapy after two-step solid-phase extraction of 1 ml plasma (C) control plasma spiked with 0.5 μmol l^{-1} AZT. (Adapted from ref. 24.)

Direct Analysis of Body Fluids by Liquid Chromatography

Traditional LC assays for TDM and toxicology inevitably include sample-preparation steps so that the final extract is compatible with the instrument. This compatibility requirement usually dictates an extract free of proteins and, sometimes, pre-concentration of the analyte above the detection limits of the method.

Direct-sample analysis (DSA) [25, 26] may be used to enhance precision because it minimizes the sample preparation steps. Other potential advantages of DSA include reduced cost of analysis, increased analyte recovery, reduced sample-size requirements (a particular advantage in the analysis of neonatal samples) and reduced exposure to toxic samples (e.g. HIV virus). For the analysis of light-sensitive drugs such as nifedipine, DSA may reduce the chance of degradation during sample preparation.

In updating the previous survey of DSA approaches [25, 26], there are currently a number of approaches available: commercial automated sample preparation systems (e.g. AASP) [22, 24], solvent extraction with "FAST-LC" [27], column switching [28], direct micro-injections into packings of silica and polymers [29, 30], an internal surface reversed-phase material [16] or a shielded hydrophobic phase [31, 32], micellar chromatography [33], the LC–photolysis–EC approach of Selavka and Krull [34], robotics, and various multi-functional–multimodal systems such as LC–LC, LC–SFC, etc. [35].

Although DSA for drug analysis has been well illustrated in the literature and practised in pharmaceutical industries using column switching, this approach was not demonstrated to be of routine and practical application until the advent of the micellar chromatography and, more prominently, Pinkerton's novel concept of internal surface reversed-phase (ISRP) [16]. The primary reason for the lack of acceptability may be the need to purchase new

and often sizable pieces of instrumentation. Bimodal separations using ISRP columns depend on the size exclusion of proteinous molecules with molecular weights >5000 by the silica packing with small pore size of about 52 Å, and the reversed-phase mode with ionic characteristics of the drug/metabolites afforded by the glycine-L-phenylalanine-L-phenylalanine chemically bonded inside the pores.

Niwa *et al.* have applied ISRP to study the accumulation of indoxyl sulphate [36], an inhibitor of drug-binding in serum of uraemic patients. Indoxyl sulphate is formed as a result of the intestinal bacteria metabolism of L-tryptophan to indole, which is further metabolized to indoxyl sulphate by the liver. Accumulation of indoxyl sulphate in uraemic patients is due to the decreased or lack of excretion by the kidney. The accumulation leads to inhibition of drug–protein binding, such as salicylate to albumin. Prior to LC analysis, the serum was filtered (0.20 μm), and 10-μl aliquots were injected. The ISRP column was 15 cm × 4.6 mm, protected by a 1 cm × 3 mm guard column cartridge. The mobile phase was acetic acid–isopropanol–tetrahydrofuran (84:10:6, v/v/v). The flow rate was 1 ml min^{-1}, and detection was at 270 nm. Figure 4 shows marked elevation of indoxyl sulphate in serum of a uraemic patient compared with normal serum.

Recently, another DSA column packing, the shielded hydrophobic phase (Hisep) was introduced by Gisch *et al.* [31]. Direct injection of body fluids into shielded hydrophobic phases relies on the exclusion of macromolecules such as protein by a polymeric hydrophilic network of bonded polyethylene oxide, while small molecules such as drugs/metabolites can penetrate the network to interact with a phenyl moiety bonded to a support. The author showed a preliminary study in the clinical analysis of phenobarbital [32]. The column was 4.6 mm × 15 cm, protected by a guard column cartridge, 4.6 mm × 2 cm. In order to prevent injection of particles, the serum was centrifuged at 9500*g* for about 20 min. Aliquots (10 μl) were injected for analysis. The mobile phase was acetonitrile–phosphate (pH 7.4; 1:9, v/v), pumped at 2 ml min^{-1}. The low concentration of acetonitrile (10%) was needed to prevent precipitation of proteins. Figure 5 shows the analyses of drug-free serum, standard and patient. Phenobarbital eluted at about 4 min. When compared with the well-established fluorescence polarization immunoassay, a correlation coefficient of 0.982 was obtained (n = 45).

In applying DSA for clinical drug analysis, guidelines have been proposed previously [25] and have been updated as follows:

1. Establish the column life by noting the injection volume and number (e.g. 1 μl × 1000 injections).
2. Dedicate a particular column and mobile phase composition to a single group of drugs. This maintains the column equilibration and extends its life.
3. Because of possible system variance such as injection volume, it is strongly

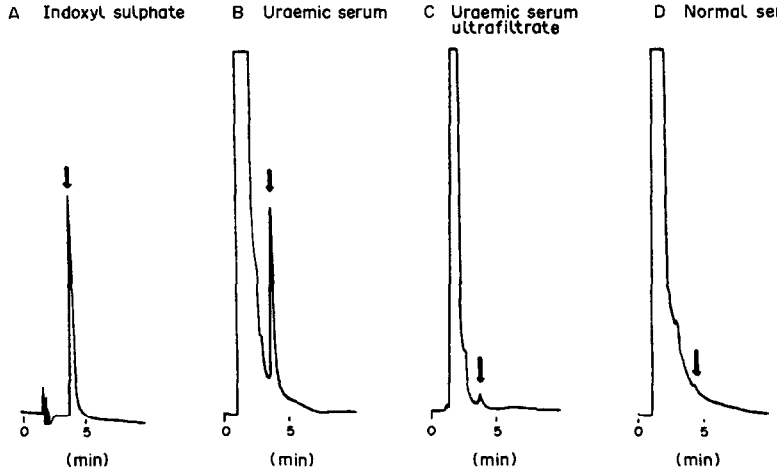

Figure 4
Chromatograms of (A) authentic indoxyl sulphate potassium salt; (B) protein-bound metabolites in uraemic serum; (C) free metabolite in uraemic serum; and (D) protein-bound metabolites in normal serum. (Adapted from ref. 36.)

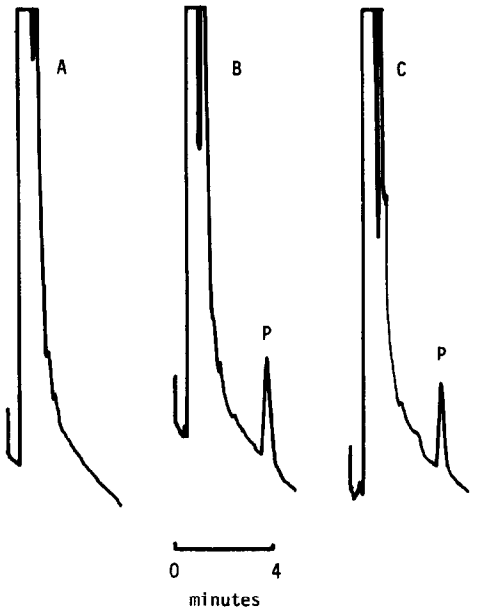

Figure 5
Chromatograms of 10-μl aliquots of serum analysed on a Shielded Hydrophobic Phase Column. A, drug-free serum; B, 20 mg l^{-1} phenobarbital calibration standard; and C, patient serum sample with an estimated concentration of 17 mg l^{-1}. By fluorescence polarization immunoassay, the drug concentrations (in mg l^{-1}) in this patient sample were: phenobarbital, 18; phenytoin, 20; primidone, 6; and carbamazepine, 3. P = phenobarbital. (Adapted from ref. 32.)

suggested that duplicate injections be made for standard, quality control and patient samples. The peak height or peak area should be within 10%. Standards and quality controls should be injected at random positions to check system performance.

4. Patient samples may contain several drugs and their metabolites, and to check for interferences from previous injections, patient samples should be analysed twice, in random order. If possible, photodiode array UV detection should be utilized to further establish peak purity.

Microbore Liquid Chromatography

The principal advantages of microbore liquid chromatography (MBLC) include enhanced mass sensitivity and reduced solvent consumption [25, 26, 37]. Since its introduction, the application of MBLC for clinical drug analysis has been limited. The reasons for this may include the generally satisfactory performance of conventional 4.6 mm, i.d., columns for clinical drug analysis, and the lack of incentive to purchase a dedicated MBLC chromatograph. It should be noted, however, that 1 and 2 mm, i.d., columns can be used with conventional LC systems. MBLC may indeed have advantages over conventional LC systems in the area of biotechnology. This author has recently reviewed the theory, instrumentation and clinical applications of MBLC [25] and noted the benefit of reduced solvent consumption which gives rise to reduced chemical waste. Within biomedical research and clinical laboratory environments, there is increasing regulation of waste disposal. While this may not be a major consideration for using MBLC, this advantage should not be overlooked.

As indicated in a recent review [37], MBLC may have advantages over conventional systems in applications of neonatal and paediatric drug monitoring where only small (20–50 μl) samples are available (Table 1). As previously demonstrated, the advent of DSA may require re-evaluation of the rôle of MBLC in clinical drug analysis.

Multidimensional–Multifunctional–Multimodal Analysis

By capitalizing on the advances in column technology and in instrumentation, this approach has been shown to offer unique applications in the field of drug analysis. Issaq *et al.* [38] recently evaluated mixed packings for the analysis of anticonvulsants and antidepressants. In their study, three different columns were used: a mixed ligand support including C_8 and cation exchange (8% carbon), a mixed packing consisting of C_8 and cation exchange (1.14:1, 11.2% carbon) and serially connected columns. The mobile phases consisted of various mixtures of methanol, acetonitrile and phosphate. The first column was shown to offer better resolution and peak shape than the other two.

Lloyd *et al.* [39] adapted a strategy of using a mixed-mode column of C_4 alkyl and phenylsulphonate functionalities for ionic exchange. Five compounds in a common cold medication were analysed: phenacetin, guaifenesine phenylephrine, norephedrine and ephedrine. The mobile phase was methanol–ammonium trifluoroacetate (25:75, v/v) pumped at 0.4 ml min^{-1}. The volatile salt was compatible with the thermospray interface of a mass spectrometer and the mixed mode column provided a viable method for the analysis of ionic and

Table 1
Comparison of MBLC 1- and 2-mm (i.d.) columns for clinical drug analysis (from ref. 37)

Drugs	Volume (μl)	Volume (μl)	Flow rate (μl min^{-1})	Detection limit (mg l^{-1})	Mobile phase* volume (ml)
1 mm (i.d.) column analyses					
Theophylline	20	0.5	80	1.0	0.8
caffeine				1.5	
Procainamide,	250	0.5	100	0.3	1.0
n-Acetyl procainamide				0.2	
Chloramphenicol	5	0.5	60	3	0.9
2 mm (i.d) Column analyses					
Cyclosporine	200–500	20	250	0.003	3.0
Flecainide	100	20	500	0.02	5.0
Carbamazepine	200–1000	10	500	0.025	5.0
10,11-epoxide				0.010	
10-11-*trans*diol					
Bethanidine	2000	50	250	0.02	7.3
Antiepileptics	500	5	300	—	3.0

*Volume of mobile phase per analysis.
—Not available.

neutral compounds that could not be performed by reversed-phase.

Another novel application was demonstrated by Lurie for the analysis of an impurity in cocaine [35] in which LC was combined with capillary SFC. Size exclusion chromatography was carried out by using two analytical polymeric columns and methylene chloride as the mobile phase. Methylene chloride was also chosen for its compatibility with the subsequent supercritical fluid chromatography analysis. The cocaine impurity eluted at about 18 min. By using a heart-cutting technique, reproducible injections were made into the SFC. The column for the SFC analysis was a SB-biphenyl-30 open tubular capillary column, and the mobile phase was carbon dioxide. Separation was carried out at 140°C with density programming from 0.25–0.65 g ml^{-1}. Detection was by FID.

Conclusions

Novel strategies for the resolution of problems in drug analysis by LC may be designed around key column parameters such as carbon load and unique functionalities for added selectivity. Despite the widespread acceptability of immunoassays for analysis, LC is sometimes the only approach available for newly introduced drugs. More recently established techniques such as microbore and chiral liquid chromatography, solid-phase extraction and the emerging modes of DSA, and multidimensional–multimodal separation have certainly enhanced clinical drug analysis. The extent of these applications may depend on the resourcefulness and perhaps the imagination of the analyst.

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