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Review

Advances in liquid chromatography and related methodologies for therapeutic drug monitoring*

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Abstract: In this article the merits of current liquid chromatography (LC) columns and techniques are reviewed, to include the following topics: (1) a brief introduction to rational therapeutic drug monitoring (TDM) to justify drug measurements; (2) selected recent survey results from the College of American Pathologists (CAP) to establish the current utilization pattern of LC for TDM in the USA; (3) LC analyses of major classes of drugs — antiarrhythmics, antidepressants, antiepileptics, antimicrobials, cyclosporine, and others — with emphasis on analysis of these drugs in human serum or plasma, by focusing on the less usual, reversed-phase functional groups such as CN and phenyl, and by the use of "mini" columns, silica and polymeric columns, the emphasis being reduced on the well-established C-18 columns; (4) high-speed LC; (5) various approaches of direct sample analysis — solvent extraction, automated sample processing, column switching, micro-injections, micellar chromatography, electrochemical detection with photolytic derivation, and the internal surface reversed-phase column of Pinkerton; (6) microbore LC drug analysis; (7) clinical chiral separation; and (8) overall conclusions.

Keywords: *Therapeutic drug monitoring; direct sample analysis; microbore liquid chromatography; multimodal liquid chromatography; drug interferences; clinical direct sample analysis.*

Introduction

Therapeutic drug monitoring (TDM) has become a well-established clinical specialty [1–4]. In performing rational TDM, the clinical laboratory specialists quantify drug concentrations, and interact with other clinicians in the interpretation of plasma concentrations and other pertinent clinical data. In the determination of drug concentrations, the majority of the clinical laboratories in the USA utilizes the well-established immunoassay auto-analyzers, while various modes of chromatography are limited to complementary rôles. However, immunoassay is generally more expensive,

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and subject to potential cross-reactivity and interference by other drugs and metabolites. These disadvantages may be overcome in liquid chromatography (LC) by applying some recently introduced columns and analytical techniques. They include manual and automated direct sample analysis (DSA), chiral separation and microbore LC (MBLC). Additionally, other advantages of LC would include: low cost and possible simultaneous multi-drug and metabolite analysis.

Rationale for therapeutic drug monitoring

As proposed by Pippenger, TDM is a recently established specialty, benefitting from the contributions and advances of other specialties such as clinical pharmacology, clinical pathology, clinical chemistry, analytical chemistry, toxicology and other clinical specialties such as psychiatry, infectious disease specialists and cardiology [1]. Its primary benefit to the patient is to achieve rational drug therapy with the aid of timely measurements and interpretation of drug concentrations. Within the clinical laboratory, TDM might have been initiated by Wuth's investigation of serum bromide concentrations in differentiating psychosis as a result of bromide intoxication or organic causes [7]. With the advent of gas–liquid chromatography in the late 1960s, drug quantitation was readily achieved with acceptable reliability and precision. In the 1970s both LC and immunoassay offered even faster turn-round time and better precision. These developments were quickly followed by the immuno-autoanalyzers in the early 1980s.

Nowadays, clinical applications of TDM would possibly include: (a) checking for non-compliance; (b) adjusting dosage to coping with individual variations in drug metabolism; (c) identifying altered drug utilization as a result of disease states; (d) compensating for changing physiological states; and (e) establishing the baseline concentrations for optimal therapeutic response. The pharmacological basis for TDM is the equilibrium established between the free drugs and the receptors. After having achieved steady-state drug concentrations, the clinical response may be correlated to a therapeutic range, such as in the seizure control therapy using phenobarbital. Thus for drugs which are irreversibly bound to enzymes, such as monoamine oxidase inhibitors for the treatment of depression, TDM would not be justified [8]. At present, TDM is routinely carried out for the following drug groups: antiarrhythmics, antiasthmatics, antidepressants, antiepileptics, antimicrobials, antineoplastics, neuroleptics and immunosuppressants.

Surveys of the College of American Pathologists

In ensuring the clinical efficacy of the various methodologies and the proficiency of the personnel, clinical laboratories in the USA subscribe to survey programmes such as those organized by the College of American Pathologists (CAP), the American Association for Clinical Chemistry and other organizations. Through these programmes, each laboratory periodically (most likely monthly) perform drug measurements of samples supplied by these organizations. The results are then compared with the target values, and with those of other participating laboratories. Table 1 illustrates the participant summary of the 1987 December CAP survey for theophylline [9]. Theophylline, an antiasthmatic, was quantified exclusively by immunoassay by the 3715 participating laboratories — 54% using fluorescence polarization immunoassay (FPIA), and 12% using turbidometric immunoassay. Due to lower efficiency compared with the immunoassays, LC was not used by any clinical laboratory. In a survey on phenobarbital, out of a total of 3352 laboratories, 56% utilized FPIA, followed by 34% using enzyme immunoassay (Dupont

Table 1
Selected participant summaries of the December 1987 CAP survey on theophylline

Drug/Method	No. laboratories	Mean	SD	RSD	Median	Low value	High value
Theophylline ($\mu g\ ml^{-1}$)							
Specimen Z-14	Target value	21.0					
Enzyme immunoassay							
American dade	156	18.20	1.20	6.6	18.2	15.4	21.8
Dupont	353	19.27	0.92	4.8	19.3	16.4	22.5
Syva Company	283	20.58	1.49	7.2	20.5	16.0	25.5
Enzyme inhibition							
Kodak	138	23.34	1.30	5.6	23.3	19.7	27.0
Fluorescent immunoassay							
Ames	47	20.95	1.13	5.4	20.7	18.4	24.6
Fluorescent polarization immunoassay							
Abbot laboratories	2020	20.07	0.93	4.6	20.1	17.0	23.1
Colony laboratories	29	19.42	1.32	6.8	19.4	16.0	23.0
Innotron of Oregon	8	19.58	0.97	4.9	19.7	17.6	20.8
Immunonephelometry/Immunoturbidimetry							
Beckman Instruments	26	20.02	2.23	11.1	19.3	14.2	24.4
Latex agglutination inhibition							
Technicon	32	19.78	1.28	6.5	19.8	16.5	23.1
Photometry							
Fluorometry	9	20.14	1.28	6.3	20.6	17.9	21.9
Radioimmunoassay							
Clinical assays	19	18.52	1.84	10.0	18.4	15.0	22.0
Turbidometric immunoassay							
Dupont	464	19.23	0.88	4.6	19.2	16.4	22.0
All methods/all results	3715	19.92	1.30	6.5	19.9	15.6	24.3

and Syva), and <1% utilized LC for quantification. In this instance, LC was probably used because of possible simultaneous measurement with other antiepileptics such as carbamazepine. The measurement of antidepressants such as amitriptyline involved only 128 laboratories out of a possible maximum 3715 (3%), and of these about 47% of the laboratories utilized LC.

Judging from these survey results, LC is seldom used in US clinical laboratories, except for the measurement of antidepressants, cyclosporine, chloramphenicol and a few other drugs. It may be that the presently available LC columns and technologies do not satisfy the practical and economic needs of clinical TDM measurements in the USA. However, LC may remain as a practical alternative for TDM in other countries. With the advent of new columns, new technologies, and the recent emphasis on drug confirmation for substance abuse testing, together these factors might revitalize the applications of liquid, gas–liquid and other forms of chromatography in US clinical laboratories [5].

Liquid Chromatographic Analysis of Major Classes of Drugs

In re-assessing the rôle of LC for TDM, the following review emphasizes the "less popular" reversed-phase (RP) packings, silica and polymeric columns. Since C-18 columns, the widely used and well-established RP columns, have been used in the majority of LC RP drug assays, a few selected examples are included to demonstrate individual separations. Furthermore, this review focuses on studies performed after 1985, with human serum, plasma or whole blood. Readers are referred to other sources for publications prior to 1985 [4]. Just as important, advances in solid-phase extraction technologies have greatly enhanced drug monitoring. McDowall *et al.* [10] extensively surveyed both manual and automated methods and their experience [10]. They suggested two possible future trends: (1) more specific phases; and (2) totally integrated automation in combination with the chromatographs. When compared with the traditional liquid–liquid extraction, Ruane and Wilson noted the possibility to exploit unique, subtle "secondary" interactions of solid-phase extraction packings [11].

Antiarrhythmics

Flanagan *et al.* reviewed the LC analyses of new antiarrhythmics using silica columns and methanolic mobile phase containing ionic modifier [12]. Retention and peak shapes depend on pH, ionic strength, solvent composition, and nature of the stationary phase. Sample preparation and detection modes, including electrochemical (EC), were discussed. Chosen examples included verapamil, amiodarone, propafenone, lignocaine, atenolol and selected metabolites.

Use of silica column in a "RP" mode with an aqueous mobile phase and acetonitrile (ACN) for the analysis of disopyramide and mono-*N*-dealkyldisopyramide was proposed by Wang *et al.* [13]. In order to avoid dissolution of the silica, and thus the possible voiding of the analytical column, a guard column was used to saturate the mobile phase, 0.015 M ammonium phosphate—ACN (3:7, v/v), with silica. Measurements by this procedure were well-correlated to those of FPIA. However, the authors did not establish column life and advantages as compared to RP methods.

Recent studies showed increasing application of cyano (CN)-columns. Proelss and Townsend analysed five antiarrhythmics — lidocaine, tocainide, procainamide, quinidine and disopyramide — and their major active metabolites by using a CN-column at 40°C with ACN–MeOH–(phosphate + triethylamine) (60:7:33, v/v/v) as the mobile

phase [14]. At therapeutic concentrations, hydroxyzine, verapamil and other local anaesthetics interfere. This procedure, using an isocratic mobile phase, avoided the lengthy equilibration associated with gradient elution, and may be completed within 40–45 min. Using a 4-μm CN-column, and ACN–acetate, MacKichan and Shields developed a sensitive and specific RP quinidine analysis [15]. A natural contaminant, dihydroquinidine and an unidentified interference were resolved from quinidine. Johnson and Khalil determined verapamil and norverapamil by using a CN-column at 40°C, with ACN–acetate (65:35, v/v) as the mobile phase, and fluorescence detection [16]. Using a CN-column and acetate–ACN (55:45, v/v) as the mobile phase, these same authors showed the analysis of diltiazem and desacetyl-diltiazem — a calcium channel blocking agent for treating vasospastic angina and chronic stable angina [17]. Prior to analysis, plasma was extracted with *n*-hexane–isopropanol (99:1, v/v). No interference was detected by some commonly used drugs. Lesne and Pellegrin described another approach of analysing amiodarone and desethylamiadrone using a CN-column and hexane–isopropanol–sulphuric acid (45:55:0.06, v/v/v) as the mobile phase [18]. Peaks of interest were well resolved within a total analysis time of about 15 min.

Straka *et al.* analysed the recently introduced flecainide using a phenyl column with ACN–dil. phosphoric acid (35:65, v/v) as the mobile phase, and fluorescence detection [19]. Measurements of this method and FPIA were well-correlated ($r = 0.98$). But the method was lengthy and required a large sample volume of 1 ml, as compared with 50 μl required by FPIA. Jamali *et al.* developed a very sensitive RP procainamide assay using a 5 μm cartridge with water–MeOH–acetic acid–TEA (75:25:11:0.03, v/v/v/v) as the mobile phase [20]. A longer elimination half-life of procainamide, 8.52 h, was estimated from the 16- and 24-h data points.

Antiasthmatics

In studying the selectivity of the phenyl column, the Author compared its capability for the analysis of theophylline, caffeine and other xanthines to that by the C-18 column [21]. The mobile phase was phosphate–ACN, and detection was at 280 nm. Using either isocratic or gradient mode, theophylline was resolved from 1,7-dimethylxanthine, a caffeine metabolite. Column life was limited to about 800 injections.

Antidepressants

Fazio *et al.* [22] and the Author [4, 23] recently reviewed antidepressant measurements by LC. RP and normal phase analyses of first and second generation drugs, atypical antidepressants and selected metabolites were surveyed, with emphasis on the use of high carbon loading columns (15–20%) to resolve structurally similar hydroxylated metabolites such as 2-hydroxy-desipramine and -imipramine, and 7- and 8-hydroxy-amoxapines. Based on the Author's experience, the RP chromatographic parameters have been described [4]. As explained below, the phenyl column, used in the RP mode in combination with the *n*-nonylamine mobile phase, offers unique selectivity, useful for resolving drug interference by neuroleptics and benzodiazepines as shown in Table 2 [23]. In addressing this problem, an interactive approach was suggested using the following guidelines [24].

(1) Use the correct blood collection tubes. If not, repeat blood collection as soon as possible, or send the original sample to another laboratory with compatible methodology.

Table 2
List of drugs interfering with immunoassays and LC assays (ref. 24, p. 430)

Interfering drug	Method	% Cross-reactivity
(A) Immunoassays		
Diphenhydramine	EMIT-SEMI	—
Doxepin and *N*-desmethyl doxepin	EMIT	—
Chlorpromazine	EMIT	—
Trimipramine and metabolite	EMIT	—
Protriptyline	EMIT	—
Perphenazine	FPIA-SEMI	28
Cyproheptadine	FPIA-SEMI	33
Cyclobenzapine	FPIA-SEMI	35
Maprotiline	FPIA-SEMI	14
Promethazine	FPIA-SEMI	16
(B) Liquid chromatography assays		
Flexeril, chlorpromazine, thioridazine, loxapine	C-18	—
Triazolam, NOR, IMI, DES, protriptyline	C-18	—
Thioridazine	Phenyl	—
7-OH-Amoxapine, nifedipine, methylphenidate,	CN	—
chlorpromazine sulphoxide, disopyramide, quinidine		—
phnylbutazone, thiothixene		—
cyclobenzapine, maprotiline		—
verapamil and haloperidol		—

EMIT-SEMI, EMIT semi-quantitative; EMIT, quantitative; and FPIA-SEMI, FPIA semiquantitative.

(2) Review the patient history with emphasis on medication. If necessary, contact the therapist. Check the co-medications against the list of interfering drugs of the assay. Pay particular attention to the neuroleptics, benzodiazepines, and other drugs with similar tricyclic or tetracyclic structure.
(3) After the analysis, check that the ratio of parent drug/demethylated metabolite is between 0.5–3.0, and that the total concentration is $<500\ \mu g\ l^{-1}$. For patient concentrations beyond these ranges, check the patient history for potential interfering drugs, drug-to-drug interaction, and toxicity as a result of overdose.
(4) For resolving potential drug/metabolite interference, use an alternative method, such as immunoassay, alter chromatographic conditions, or use another chromatographic procedure, such as a phenyl column with different selectivity, as shown by a previous study [24] and discussed in the following paragraph.

Since the pheny ring offers added selectivity via the mechanisms of cyclic interaction, π–π interaction and other aromatic stacking interaction, the desired resolution from interference may be achieved due to the selectivity of the phenyl packing for the heterocyclic tricyclic antidepressants [24]. Indeed, Fig. 1 shows the successful resolution of interference by using a phenyl column. However, Fig. 2 shows that thioridazine co-eluted with the internal standard. Thus, the phenyl column does not resolve all interference.

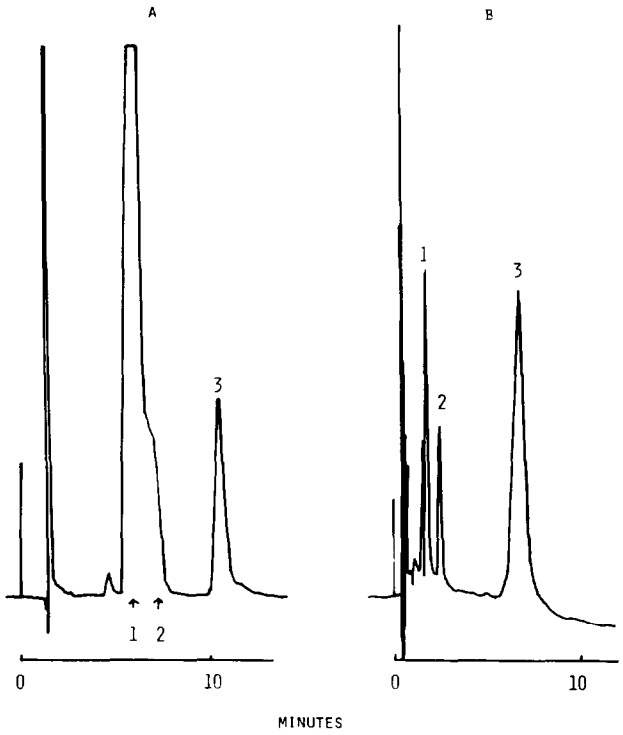

Figure 1
Chromatograms of plasma extract of a patient medicated with doxepin and other drugs, (A) C-18, and (B) phenyl column. (Note that the interference was resolved by the phenyl column.) Peak identification: 1, N-DOX, 120 $\mu g\,l^{-1}$; 2, DOX, 113 $\mu g\,l^{-1}$; and 3, IS — NOR for (A), and clomipramine for (B) adapted from ref. 24.

Lin and Frade demonstrated simultaneous measurement of eight tricyclic antidepressants using a 5-μm CN-column with phosphate–ACN–MeOH (28:58:14, v/v/v) as the mobile phase [25]. The tricyclic drugs were: amitriptyline, imipramine, doxepin, trimipramine, desipramine, nortriptyline, desmethyldoxepin and protriptyline. Systematic resolution and selectivity studies were performed on various CN-, phenyl- C-18 and silica columns with various mobile phases. Solid-phase cartridges with CN-packing was used for extraction. In choosing the CN-column, the authors suggested some precautions such as monitoring for back-pressure build-up, mobile-phase equilibration, and maintaining injection sample size of 100 μl. Some neuroleptics interfered with this assay. Lovett *et al.* described a simple method for determination of trazodone in serum, using a CN-column and ammonium phosphate–ACN (7:3, v/v) as the mobile phase [26]. Retention times of trazodone and its metabolite, 1(*m*-chloro-phenyl)-piperazine were 4.8 and 15.7 min, respectively. However, the metabolite was not detected in the serum of a volunteer administered with a 100 mg oral dose of trazodone.

Antiepileptics

Juergens recently reviewed conventional LC assays of antiepileptics, followed by the microbore analysis [27]. Wad studied the degradation of clonazepam in serum by analysis with C-8 column with water–ACN (72–62% water) as the mobile phase [28]. When

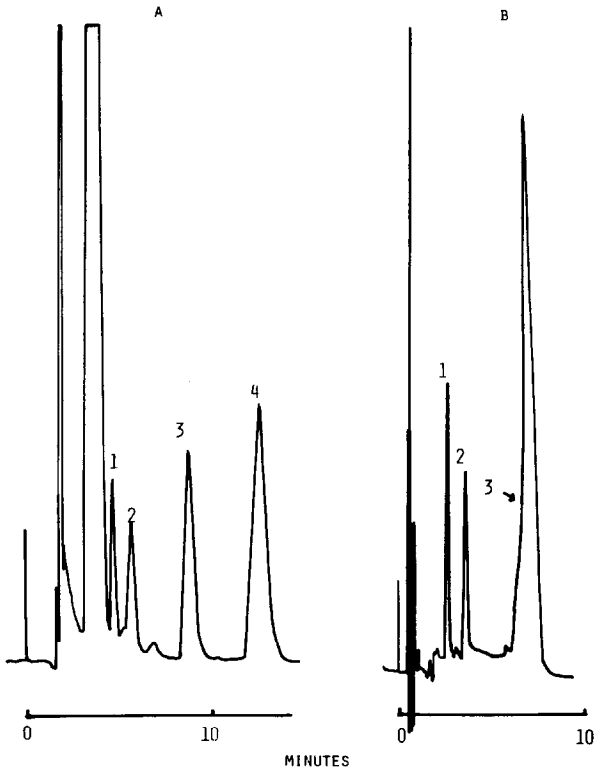

Figure 2
Chromatograms of plasma extract of a patient medicated with imipramine and thioridazine; (A) C-18, (B), phenyl column. (Note that the interference was not resolved by the phenyl column.) Peak identification: 1, DES, 121 $\mu g\,l^{-1}$; 2, IMI, 126 $\mu g\,l^{-1}$; 3, IS; and 4, thioridazine. Adapted from ref. 24.

exposed to direct sunlight, the clonazepam concentration decreased from about 290 to 0 nmol l^{-1} in about 45 min. Thus, the authors recommended dark sample tubes or tubes wrapped with aluminium foil. Lin used a CN-column with phosphate–ACN–MeOH (75:10:15, v/v/v) as mobile phase [29]. After 8 months and 500 serum samples, the column performance was satisfactory. Dusci and Hackett showed the simultaneous determination of clobazam and *N*-desmethyl clobazam using a phenyl column with phosphate–ACN (6:4, v/v) as the mobile phase [30]. Ratios of *N*-desmethylclobazam to clobazam ranged from 20:1 for patients on multi-drugs, to 2:1 for patient medicated only with clobazam, showing that *N*-demethylation of clobazam was affected by other co-administered antiepileptics.

Antimicrobials

Ristuccia extensively reviewed LC assays of antimicrobials [31]. McAteer *et al.* demonstrated an isocratic, rapid RP analysis of cephalosporins — cefixime, cefactor, cefadroxil and cephradine — by using a C-8 column with MeOH–phosphate (2:8, v/v) as the mobile phase [32]. Two standard curves were established for therapeutic and high (1–100 mg l^{-1}) concentrations. No interference was noted from a selected group of drugs. Dubourg *et al.* described analysis of chloramphenicol in plasma, milk, urine and

tissues, using a 10-μm C-18 column with acetate–ACN as the mobile phase [33]. Detection limits for plasma and milk were 20 and 10 ng l^{-1} respectively.

Cyclosporine

Critical issues in cyclosporine monitoring were recently addressed by a Task Force, jointly sponsored by the Therapeutic Drug Monitoring and Clinical Toxicology Division of the American Association for Clinical Chemistry, and the National Academy of Clinical Biochemistry [34]. In this comprehensive report, the clinical pharmacology and methodological issues were critically assessed, followed by eight recommendations regarding to the sample requirement, and specific methods such as LC and immunoassays using monoclonal antibodies.

Sangalli and Bonati described the whole blood analysis of cyclosporine using a small C-8 column (30 × 4 mm) with ACN–water–MeOH (53.5:32:14.5, v/v/v) as the mobile phase [35]. The procedure was specific, precise and reproducible. When compared to radioimmunoassay (RIA) which shows cross-reactivities with metabolites, the LC measurements were lower because of the greater specificity.

Lensmeyer *et al.* demonstrated, after extensive studies, the isocratic analysis of cyclosporine and nine metabolites — M1, M8, M17, M18, M21, M25, M26, M203–218 and MUNDF1, in whole blood using a CN-column with water–ACN–THF–acetic acid–*n*-butylamine (600:390:20:0.16:0.10, by vol) as the mobile phase at 58°C [36]. Analysis, as shown in Fig. 3, was completed in about 40 min. Sample preparation included solid-phase extraction of whole blood, after haemolysis, with uncapped CN-cartridges. The authors also evaluated other columns — silica, C-8 and semi-preparative CN. The chosen CN-column was a compromise in assuring a reasonable analysis time. Precautions for ensuring column stability included the use of a saturation column and continuous flushing at low flow-rate between assay. The proposed separation mechanism would include both RP and adsorptive interaction. In their second study, the LC method

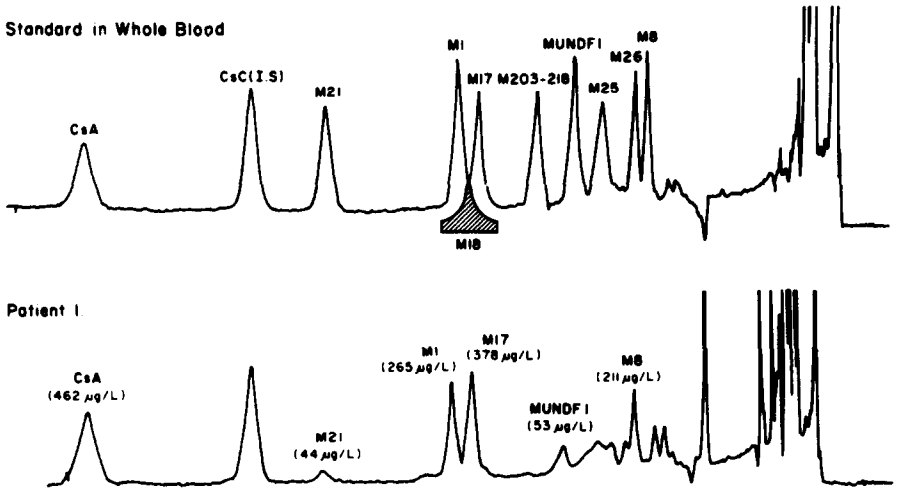

Figure 3
Typical chromatograms of extracts from whole blood supplemented with CsA and metabolites at concentrations of 400 μg l^{-1} each (M18 at 200 μg l^{-1}), and the whole-blood sample collected from a patient before the next dose of CsA. Adapted from ref. 36.

was compared with RIA, showing diverse correlations (r = 0.058–0.993). Thus the authors discouraged the use of RIA/LC ratios to estimate metabolite concentrations.

Kabra *et al.* showed automated solid-phase extraction, followed by analysis with a C-8 column and ACN–MeOH–water (7:2:11, v/v/v) as the mobile phase at 70°C [37]. Sample preparation included mixing with an internal standard mixture of MeOH and zinc sulphate to ensure complete protein precipitation. After conditioning the C-8 cartridges inside the cassette, aliquots of the supernatants were transferred into the reservoirs, followed by several washings. Then, the cassettes were inserted into an advanced automated sample processor™ (AASP) for further automated extraction and injection into the analytical column. The authors emphasized the advantages of the large-scale automation and thus the efficiency, and of the lack of interference by other drugs, steroids and late eluting peaks.

Charles *et al.* illustrated RP analysis with a C-18 column with column life of more than 1000 injections [38]. An old column, placed before the autosampler, was maintained at the same temperature, 70°C, as the analytical column. Solid-phase extraction was performed with C-18 cartridges. Between 20–60 specimens could be processed in a 24-h period.

Bowers and Singh [39] demonstrated the gradient elution of cyclosporine and its metabolites in blood and bile, using a RP column at 70°C. Metabolites **17** and **1** were quantified, and confirmed by radiolabelled products of rat liver perfusion studies. Total analysis time was 55 min.

Christians *et al.* [40] and Brossat *et al.* [41] recently described analysis of cyclosporine using gradient elution. Sample preparation was achieved by using solid-phase extraction columns. Wallemacq and Lesne utilized a CN-column with hexane–isopropanol as the mobile phase [42].

Neuroleptics

Chakraborty *et al.* compared the measurement of thioridazine by LC with two RIA methods [43]. The LC assay was developed to measure thioridazine and two major active metabolites — mesoridazine and sulphoridazine. A CN-column was used with acetate–ACN (2:8, v/v) as the mobile phase. Other metabolites — *N*-desmethylthioridazine, thioridazine-5-sulphoxide and thioridazine disulphoxide did not interfere. Correlation between the LC and the new RIA methods was acceptable (r = 0.916). The LC assay was sensitive enough for single dose pharmacokinetic study.

Furlanut *et al.* described the analysis of benperidol with a C-8 column, and acetate–ACN (6:4, v/v) as the mobile phase in combination with electrochemical detection [44]. Solid-phase extraction was carried out by C-8 cartridges.

Midha *et al.* analysed chlorpromazine and six metabolites: 7-hydroxy-chlorpromazine, *N*-monodesmethyl-chlorpromazine, 7-hydroxy-*N*-monodes-methyl-chlorpromazine, chlorpromazine-sulphoxide, chlorpromazine *N*-oxide, and *N*-monodesmethyl-chlorpromazine-sulphoxide. A CN-column was used with acetate–ACN–diethylamine (8:92:0.05, v/v/v) as the mobile phase [45]. Correlation with the RIA assay was variable. The authors concluded that the application of this assay for multianalyte analysis would be limited.

High-speed Liquid Chromatography

Dwyer and Brown recently reviewed the instrumentation and applications of high-

speed liquid chromatography (HSLC) [46]. Two reasons were suggested for the slow pace of its acceptance: firstly, HSLC may be chosen over conventional LC for only special applications, and secondly, the slow evolution of instrumentation may have hindered its wide-spread application. It is hoped that the advantages of faster analysis time, increased mass sensitivity and high efficiency would be useful for analysis of complex mixtures. Kabra and Marton demonstrated the 70-s analysis of theophylline using a 12.5-cm 5-μm C-18 column [47]. By using a shorter 3.3-cm 3-μm C-18 column, Dong and Gant analysed theophylline in 45 s [48]. Kinberger and Holmen, using a 10-cm 3-μm C-18 column, analysed carbamazepine and phenytoin within 2 min [49]. Dong and DiCesare analysed tricyclics and barbiturates in 1–3 min [50]. Jehl *et al.* compared conventional and HSLC for the analysis of 15 β-lactam drugs [51]. Following sample preparation, conventional LC analyses were performed with a 5-μm C-18 column (15 or 25 cm), while high-speed assays were performed with a 3-μm C-18 column (7.5 cm). Composition of the mobile phase was optimized according to the antimicrobial. Mean analysis times for conventional and HSLC were 2.3 and 6.6 min, respectively. The authors advocated the following advantages for the high-speed method: rapid equilibration, short retention times, lower detection limit and longer column life resulting in lower unit analysis cost.

Direct Sample Analysis

Application of LC for TDM in USA is limited as shown by CAP surveys, mainly due to the wide availability of well-established immunoassays and the perceived difficulties associated with using LC on a routine, clinical basis. The difficulties would possibly include labour-intensive sample preparation, limited column life, lack of totally integrated automated-sample preparation, analysis and data reduction. Consequently, LC may not be as precise as immunoassay. However, LC may perform simultaneous determination of drugs and/or metabolites, and may therefore become more cost-effective than immunoassays. With the advent of "direct sample analysis" (DSA), some of these perceived difficulties may be minimized or eliminated. Total automation may become more attainable, resulting in increased efficiency, precision and lowered exposure of the analyst to biohazardous samples. This is a distinct benefit. While there is no clear indication of clinical laboratory workers contracting AIDS, it is advisable, however, to perform drug measurement with maximum achievable biosafety guidelines. DSA may help fulfil the requirements of these guidelines. Furthermore, DSA would enhance the measurement of light-sensitive drugs such as the calcium channel blocker, nifedipine. The Author [24] and Shihabi [6] have independently reviewed the various approaches. Based on the literature and the Author's own experience, the following outlines the various approaches and some suggested guidelines.

Advanced automated sample processor™

Prior to introducing the samples into AASP for tricyclic antidepressant analysis, Ni *et al.* recommended mixing the plasma with the internal standard solution and phosphate [52]. These mixtures were transferred to the reservoir and then to the cartridges. After programmed back-flushing, the eluents were directed into a CN-column. As shown previously, Kabra *et al.* also recently demonstrated automated cyclosporine analysis by AASP [37].

Solvent extraction

Snyder *et al.* established the automated "Fast-LC" for drug analysis using Technicon's clinical chemistry technology for extraction [53]. Aliquots of serum were loaded into containers/cuvettes, and pipetted into the mixing and extracting coils. Extracts were directly injected onto the analytical columns. This system was developed for antiepileptics, antidepressants and other drugs.

Column switching

Numerous recent publications demonstrated the interest and potential of this important approach. Neilen *et al.* analysed etoposide by using on-line trace-enrichment with a micro pre-column, followed by eluting with mobile phase [54]. Two LC pumps were required for the pre-column and analytical column, respectively. This technique of loading serum onto the pre-column, may be used for field sampling in the doctor's office. Then, the loaded pre-column may be transported back to the laboratories for LC analysis. With instrumentation similar to the above, Nozaki *et al.* measured acetaminophen [55]. Aliquots of serum were mixed with an internal standard and injected into a bovine-ODS column, eluted with 5% MeOH in 7 mM phosphate. Aliquots were selectively injected onto the analytical column Nucleosil ODS. The procedure was precise and linear, and offered good recovery.

In separating the protein from the drug molecules — ethosuximide, primidone, phenobarbital, carbamazepine and phenytoin — and metabolite molecules — cabamazepine-10,11-epoxide and carbamazepine-10,11-dihydroxide — Matsumoto *et al.* introduced serum containing these antiepileptics into a C-18 pre-column treated with bovine serum albumin [56]. Protein molecules were eluted, while drug molecules were retained. By using column switching, the retained drugs were eluted and injected onto a column of 12 nm pore size, non-end-capped polylayer of C-18, and analysed by gradient elution. When compared to a column of 8 nm pore size with an end-capped monolayer of C-18, the above column was capable of resolving carbamazepine epoxide from phenobarbital. The sample volume was 20 μl. Washing solutions and mobile phases for the pre-column comprised mixtures of phosphate and ACN. Analytical column mobile phases were mixtures of phosphate and ACN (22–35%). Separations were carried out at room temperature or 40°C. The authors noted that over 100 samples could be analysed, but it was necessary to replace end-fittings after each 50 samples.

Daoud *et al.* evaluated a pre-column venting plug system connected to an analytical column for analysing lidocaine and its metabolites [57]. Aliquots of plasma (100 μl) were injected onto the pre-column and washed with phosphate, and subsequently directed into the RP analytical column. The pre-column should be filled with 10-μm particles, washed with phosphate buffer (pH 2), and closed with screen filters instead of frits. Under these conditions, 65 injections of 100 μl each, could be performed before changing the pre-column. Using this technique, theophylline was also quantified, as shown earlier [58]. Similar instrumentation was used by Ascalone and Dalbo to analyse diltiazem [59]. Prior to injecting onto the pre-column, plasma was mixed with internal standard solution, followed by centrifugation. Then, the clear supernatant portions were analysed. The authors recommended changing the pre-column after about 100 injections. An added advantage, not noted by the Author, is the minimized exposure of light-sensitive diltiazam.

Tamai *et al.* investigated direct injection of whole blood containing hydrophobic drugs incorporated into blood corpuscles [60] or adsorbed on blood cell membranes [61], and

whole blood containing hydrophilic drugs [62]. To demonstrate the analysis of hydrophobic drug in blood corpuscles, 50 μl of whole blood or plasma containing carbamazepine was injected onto the pre-column packed with spherical ODS 7 nm pore size particles, and was haemolysed. Then proteins, blood cytomembranes and hydrophilic components were eluted with water–phosphate using various gradients. Most of the haemoglobin was eluted in the void volume by water. By using an end-fitting with a pore size larger than 40 μm, clogging was avoided. Using phosphate–ACN (75:25, v/v), carbamazepine was eluted from the pre-column, and was injected onto the analytical column (80 × 4 mm, i.d.) (TDK G1 ODS 120T, 5 μm) for quantification. Simultaneously, the pre-column was conditioned by a succession of solvents — phosphate–ACN (1:1, v/v), a surfactant solution of 0.5% SDS, MeOH and water. Precision was about 2% RSD. In the determination of hydrophobic drugs, such as chlorpromazine, adsorbed on blood cell membranes, the chosen pre-column packing was TSK Gel HW-65 [61]. Recovery was 103.3%, and precision was 3.4% RSD. Determination of hydrophilic drug was demonstrated for procainamide and *N*-acetylprocainamide by using Butyl Toyopearl 650-M as the pre-column packing, and Nucleosil 5SA as the analytical column. Recovery was quantitative, and precision was <4% RSD. More than 100 samples may be performed by the above procedures.

Micro-injections

Direct analysis of pentobarbital in serum by using a polymeric (PRP-1) column was shown by Shihabi [63]. Figure 4 shows the analysis of pentobarbital. Serum precipitation was minimized by injecting only 2 μl, and by using a mobile phase with high pH. Column life was greater than 300 injections. Wide pore particles (300 Å) and low hydrophobicity silica columns were used for the analysis of theophylline [6]. By packing his own

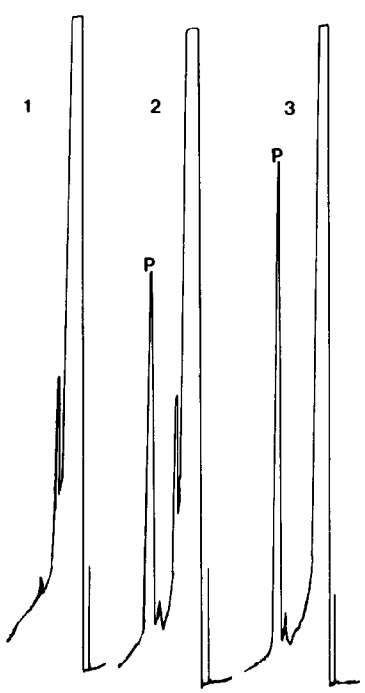

Figure 4
Representative chromatograms of: 1, patient serum free from pentobarbital; 2, patient treated with pentobarbital; and 3, aqueous standard 50 mg l^{-1}. P = pentobarbital peak; retention time, 6 min. Serum, 2 μl was injected directly onto the column, eluted as described, with detection at 254 nm. Adapted from ref. 63.

columns, the author estimated that this approach would cost $0.10 per sample, compared with $1–3.00 per sample by immunoassay.

Micellar chromatography

Love *et al.* demonstrated the use of surfactant mobile phases such as anionic SDS and non-ionic Brij-35 for direct drug analysis in serum [64]. The principle is based on the proposed reactions that the surfactant monomers, at about the critical micellar concentration, would bind to insoluble serum components, solubilizing them as a result of the charged/polar coating. Free monomers and/or micelles displace the protein-bound drugs. These serum protein micelle complexes would elute immediately after the solvent front, while the elution of the drug/micelle monomers would depend upon the SDS micelle concentration. Selective fluorescence would enhance the analysis of quinidine by minimizing the background noise, and eliminating any solvent front protein–micelle complex peaks.

Electrochemical detection with photolytic derivatization

Selavka and Krull demonstrated the use of LC electrochemical detection (EC) for the analysis of morphine, narcotic, benzodiazepine, cannabinoid, fentanyl and tricyclic antidepressants. With post-column photolytic derivatization, components with no inherent electroactivity would be rendered detectable [65]. In addition to the retention time data, identification may be confirmed by the dual parallel EC approach, and with irradiation discontinued, the disappearance of the peak for compounds amenable only to LC–photolysis–EC. The authors demonstrated analysis of β-lactam, morphine, nalorphine, pethidine, cannabinoids and metabolites, cocaine, benzoylecgonine and ecgonine, barbiturates, methylphenidate, lysergic acid diethylamide, benzodiazepines and others.

Bimodal, internal surface reversed phase

Pinkerton [66–71] established the internal surface reversed-phase (ISRP) — a unique mode of multi-modal separation. ISRP may be termed as a bimodal separation — the RP mode, with possible ionic interaction, is carried out by the hydrophobic functional group, a polypeptide, glycine-L-phenylalanine-L-phenylalanine, bound to the internal surface of the porous silica, and the size exclusion mode is carried out by the small pore size, silica packing (pore size of 52 Å). Serum protein molecules with molecular weight of >5000 undergo size exclusion separation, and elute close to the solvent front peaks. Protein adsorption is minimized by the external, hydrophilic glyceryl-propyl bonded phase. The chemistry and characteristics of this novel packing has been thoroughly characterized by Pinkerton [66–71].

Because of the small sample requirements, the Author's laboratory has begun a systematic feasibility study of using ISRP for neonatal and paediatric drug monitoring, initially for monitoring carbamazepine, and comparing these measurements with those of an established immunoassay method [72].

The ISRP column (25 cm long) was connected to a guard column, and was eluted with the mobile phase 0.1 M phosphate (pH 6.8)–isopropanol–THF (86:10:4, v/v/v). Flow rate was 1 ml min^{-1}. Detection wavelength was 254 nm at 0.005 AUFS. Aliquots (10–100 μl) of serum were centrifuged at 9500*g* for about 10 min. Then, 1-μl aliquots were injected into the column. Figure 5 shows the results of such analysis for carbamazepine in serum. Calibration was linear up to 20 mg l^{-1}. This assay was

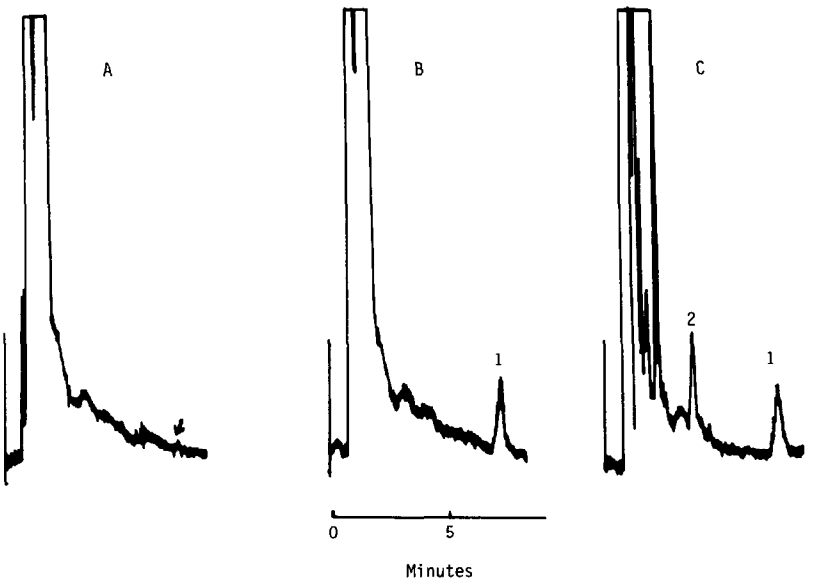

Figure 5
ISRP chromatograms of carbamazepine in serum: (A) drug-free serum; (B) 8 mg l^{-1} standard; and (C) patient serum with estimated concentration of 8 mg l^{-1}. Adapted from ref. 72.

compared with a clinical (Abbott) FPIA for carbamazepine for 20 patient specimens. Both linear regression and paired *t*-test showed that these two techniques were comparable.

The retention properties and drug recovery of this technique was investigated by Nakagawa *et al.* [73]. By studying the effect of pH, ionic strength, nature and concentration of organic modifiers, ion-pairing agent and surfactant on the capacity factor of methylparaben, p-electron interactions were said to account for the major contribution to retention mechanisms, influenced by secondary weak cation-exchange process for the retention of ionic analytes. SDS increases retention of certain drugs at low pH. For the separation of hydrophobic drugs, organic modifier hastens separation and enhances recovery, while an aqueous mobile phase, even with lower recovery, was recommended for hydrophilic drugs. Furthermore, a technical recommendation was made for the use of PTFE filter in place of stainless-steel frits.

From the Author's own experience, column life and reproducibility depend, in part, on thorough conditioning of the column before and after analysis. As an alternative to the lengthy column regeneration recommended by the supplier, Pinkerton (personal communication) advised reversing the inlet and outlet column connections, and then regenerating the column by continuous elution of the column with 5% isopropanol in phosphate. Occasional lengthy regeneration may be carried out by using ethylene glycol–water (1:1, v/v).

Silica with aqueous mobile phase

Using silica columns, Adamovics described the analysis of 16 antibiotics and antimicrobials with aqueous mobile phases to prevent protein precipitation [74]. The

author suggested that protein saturation of the column did not affect drug quantification. After 200 injections, back-pressure increased as a result of decreased column porosity.

In addition to the above eight approaches, the use of robotics in the pharmaceutical industry has become routine, but its application in clinical analysis awaits further study.

Guidelines for clinical direct sample analysis

Based on the above approaches and the Author's experience, the following guidelines are suggested for clinical direct sample analysis.

(1) Establish a column life by noting the injection volume and number.
(2) Limit the analysis of a single group of drugs to a particular column and mobile-phase composition. This would enhance equilibration, and extend column life.
(3) As a result of possible system variance such as injection volume, it is strongly suggested that duplicate injections should be made for standard, quality control and patient samples. The peak height or peak area values should be within 10% of each other. Furthermore, standards and quality controls should be placed at random positions to check on system performance.
(4) Because of possible multi-drug therapy, patient samples may contain several drugs and metabolites. In order to ascertain that the interested drug peak is not co-eluting with another drug/metabolite/endogenous substance, patient samples should be analysed twice and in random order. If possible, photodiode array UV detection should be utilized to establish peak purity.
(5) As result of DSA, automation may be easily achieved in order to minimize exposure of the analysts. This may be followed by containment design — "closed systems" such as those used in the nuclear industry.

Microbore Analysis

Horvath *et al.* [75] demonstrated the use of small-bore columns packed with pellicular particles. Two recently published volumes of the *Journal of Chromatography Library*, Vols 28 [76] and 30 [77] represent authoritative sources of microcolumn LC. The Author recently reviewed this subject [24]. The advantages of microbore (MB) LC include low solvent consumption and enhanced mass sensitivity. In Scott's estimation of the relative cost of using columns with different column internal diameters (i.d.) ranging from 0.51 mm to the standard 4.6 mm, i.d., columns [78], cost savings of 20-fold are achieved when using the 1.02-mm instead of the 4.6-mm column. According to Scott [78], enhanced mass sensitivity may be accounted for by the following equation for estimating the minimum detectable mass (m):

$$m = 2\pi r^2 1\psi(1 + k')C/\sqrt{N},$$

where

C = minimum detectable concentration
r = column radius
ψ = fraction of mobile phase-occupied volume
k' = capacity factor of the solute
N = column efficiency in theoretical plates.

Thus, m is directly dependent on the square of the radius of the column. The relative

mass sensitivity of the 4.6 and 1.0-mm, i.d., columns would be 232 and 11 ng, respectively — a 20-fold increase in sensitivity. The optimal column configuration would have 1–2 mm, i.d., with column length from 10 to 25 cm.

To explore MBLC for limited-sample-size analysis with possible application in paediatric and neonatal TDM, MBLC assays of theophylline, caffeine, procainamide, *N*-acetyl procainamide and chloramphenicol were recently published [79, 80]. Theophylline and caffeine analysis was performed by using protein precipitation with 10% trichloroacetic acid, and analysis with a 3-μm C-18 column (100 × 1 mm, i.d.) packed with Spherisorb™ particles. The mobile phase was 0.05 M, phosphate (pH 5.0)–acetonitrile (93:7, v/v), delivered at 80 μl min^{-1}; detection wavelength was 280 mm, 0.002 AUFS. Figure 6 shows the MBLC analysis of theophylline (TH) and caffeine (CA). Extractions with methylene chloride were performed for the MBLC analysis of procainamide (PA) and *N*-acetyl procainamide (NAPA). After re-constitution with the mobile phase, 0.025 M phosphate (pH 3)–acetonitrile (9:1, v/v), the mixtures were analysed by the above column. The flow rate was 100 μl min^{-1}, and the detection wavelength was 254 mm, 0.005 AUFS. Figure 7 shows the MBLC chromatograms. In designing the MBLC assay for serum from patients treated with intravenous doses of chloramphenicol succinate esters, the strategy to resolve chloramphenicol from the prodrug esters was based on increased interaction of analytes with a high carbon-loading column (20% instead of 10%). This column might offer the required enhancement of selectivity for the

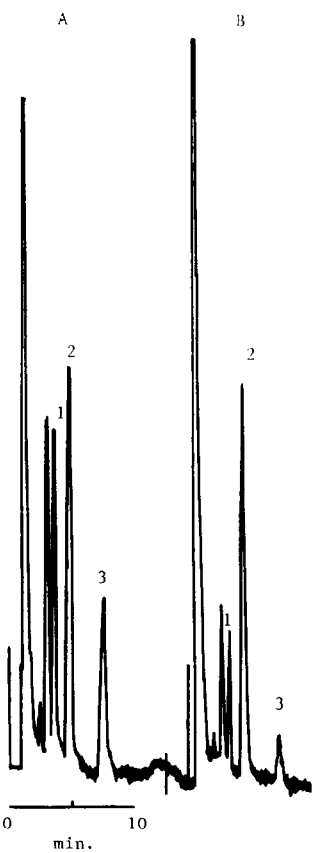

Figure 6
MBLC chromatograms of serum extracts of: (a) 10 mg l^{-1} standard, and (b) a patient. Key: TH, 4 mg l^{-1}; and CA, 3 mg l^{-1}. Peak identification; 1, TH; 2, IS; and 3, CA. Adapted from ref. 79.

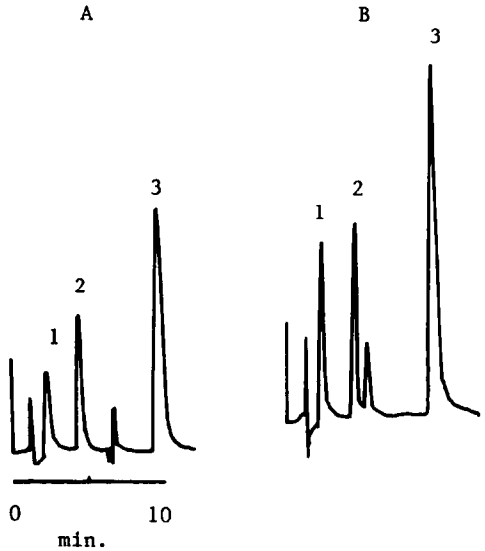

Figure 7
MBLC chromatograms of serum extracts of: (a) 5 mg l^{-1} standard; and (b) a patient (PA = 7 mg l^{-1} and NAPA = 5 mg l^{-1}). Peak identification: 1, PA; 2, NAPA; and 3, IS. Adapted from ref. 79.

separation of structurally similar analytes such as the 3- and 1-succinate esters of chloramphenicol. The separation was in fact achieved at ambient temperature as shown by Fig. 8. Prior to the analysis, protein precipitation was achieved by mixing 5 μl of serum with 20 μl of methanolic solution containing the internal standard, 5-ethyl 5-*p*-tolybarbituric acid. Based on the above studies, clinical MBLC assays would require a micro-sample size of 5–10 μl, careful sample preparation, but only minimal re-training of personnel.

Juergens reviewed antiepileptic analysis by conventional LC, and demonstrated MBLC analysis of 14 antiepileptics [27]. After extracting 0.5 ml of serum with ethyl acetate and evaporating the organic phase, 5 μl of reconstituted mixture was injected onto a C-18 5-μm column (200 × 2.1 mm, i.d.), and analysed by using gradient elution with a mobile phase, consisting of phosphate–ACN at 300 μl min^{-1}. With the use of an in-line filter, 4000 injections were achieved without loss of resolution. The author noted a 70% cost saving in solvent, and in a later study, analysed these drugs in brain tissue [81]. Shipe *et al.* demonstrated analysis of bethanide using a column similar to the above with ion-pairing agent in acetate–ACN (9:1, v/v) as the mobile phase, at a flow rate of 250 μl min^{-1} [82]. The column was replaced after 500 plasma sample analyses. Annesley demonstrated the advantages of MBLC for the analysis of cyclosporine-reduced sample size (0.2–0.5 ml), reduced solvent cost and increased resolution and sensitivity [83]. Recently, MBLC assay of flecainide was developed in that laboratory [84]. Hyldburg evaluated the feasibility of a radially compressed MB column [85]. Koenigbauer *et al.* utilized pre-column enrichment, followed by column switching into a MB column for analysing diazepam in serum. The column was a C-18 5-μm (250 × 1 mm, i.d.), and the mobile phase was MeOH–water (65:35, v/v), delivered at 60 μl min^{-1}. Lien *et al.* determined tamoxifen and four metabolites by using a C-18 5-μm (100 × 2.1 mm, i.d.) column at a flow rate of 300 μl min^{-1}. Deproteinized samples were loaded onto a pre-

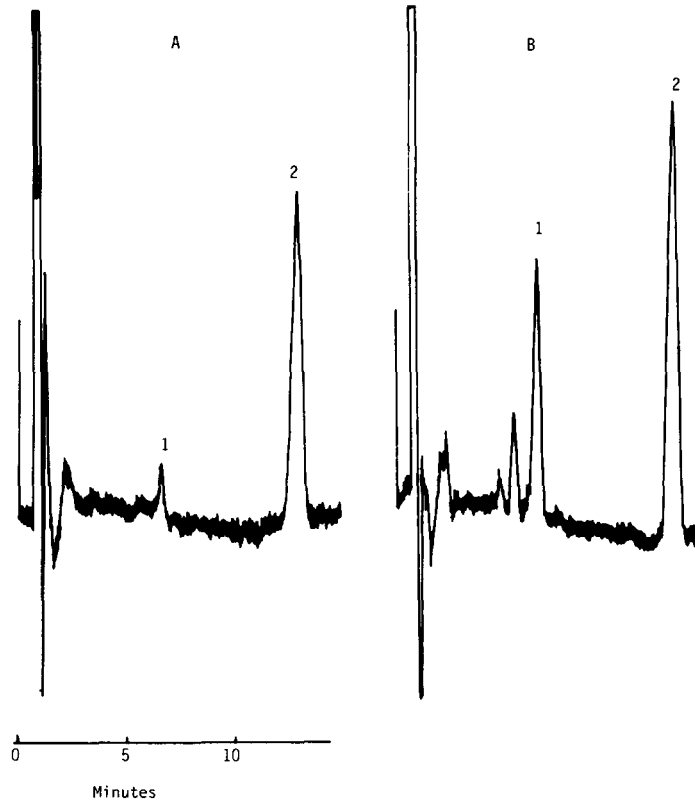

Figure 8
Microbore liquid chromatograms of 0.5-μl aliquots of supernatant from procedure B. Chromatogram A shows 6 mg l^{-1} of chloramphenicol following oral administration of chloramphenicol palmitate, and chromatogram B shows 21 mg l^{-1} of chloramphenicol following IV administration of chloramphenicol succinate. Peak identification: 1, chloramphenicol; and 2, IS. Adapted from ref. 80.

column, and pre-concentrated by eluting with a mixture of water–ACN. Detection was achieved by fluorescence activation by UV light. Automated sample extraction by the pre-column was an additional advantage.

Based on the above examples and the Author's own experience, MBLC, by using 1 or 2 mm (i.d.) columns, may be used clinically for drug analysis with established advantages. For clinical MBLC analysis, conventional LC may be adapted for coupling to microcolumns. Just as in the case of HSLC, it seems to be limited to special applications. Because of the small sample size, MBLC should be well suited to paediatric and neonatal drug monitoring.

Clinical Chiral Separation

With the advent of new packings for chiral separation, its clinical application is being recognized as shown by a recent review by Drayer [88]. Furthermore, the reader is referred to other authoritative reviews by Wainer and Armstrong, and in a special issue

of the *Journal of Liquid Chromatography*, entitled "Optical Resolution by Liquid Chromatography" [89]. Wainer *et al.* demonstrated the simultaneous analysis of (*R*)- and (*S*)-glutethimide, and their 4-hydroxy metabolites by using a Pirkle-type column and gradient elution [90]. Tan and Soldin studied stereoselective disposition kinetics of salbutamol by analysing urine with a chiral alpha$_1$-acid glycoprotein column (Enantio-Pac) with an aqueous mobile phase and amperometric detection [91]. McErlane *et al.* carried out a similar study of mexiletine by analysing the 2-naphthalyl derivative on a Pirkle column [92]. Bertucci *et al.* described the performance of SiSQuinmei I, one of the SiSQuin new chiral phase, formed by bonding with quinine [93]. Resolution of lorazepam enantiomers was achieved by using hexane–isopropanol–methylene chloride (100:28:24, v/v/v) using both UV and circular dichroic detectors. Other drugs included etozoline, oxazepam, mesantoin and ibuprofen. Most recently, the new chiral-AGP column (Chrom Tech AB, Sweden) has been shown to have considerable potential for chiral studies in clinical chemistry.

Conclusion

While LC was instrumental in advancing TDM in the 1970s, immunoassays now dominate the routine clinical measurement in the clinical laboratory in the USA. This article reviews advances in LC columns and related methodologies which may be applicable to clinical laboratories. While much has been published about the advantages of LC, the reality is that thus far, it is perceived to be a relatively more difficult but versatile technique. The advances noted certainly would be of interest to clinical chemists.

In the area of column packings, CN-columns have definitely been established as a viable alternative to C-18, with the possibility of being applied in both RP and normal-phase modes. Latest applications would include the analysis of cyclosporine. Another packing, phenyl, should be of increasing interest for drug analysts, since the phenyl ring would offer added selectivity via mechanisms of cyclic interaction, π–π interaction and other aromatic stacking interaction. Recently, Apfel *et al.* demonstrated the synthesis of a theophylline bonded phase, with the separation characteristics of a RP packing [94]. This signals the beginning of the use of drugs as functional groups, which may offer unique selectivity, especially important for resolving drug interference problems. This new class of column may be appropriately termed a "drug bonded phase column". Because of the interference problems encountered in antidepressant analysis, the selectivity of a "tricyclic bonded phase column" would be of interest. As an indication, the phenyl column was useful in resolving interference from certain neuroleptics. At the other extreme, silica columns have received renewed interest, such as in combination with the use of aqueous mobile phases, offering unique advantage. MBLC, because of its small sample size requirement should be useful in the areas of paediatric and neonatal drug monitoring.

DSA may represent a realistic alternative to the popular immunoassay for TDM, either through manipulation prior to analysis, such as solvent extraction, solid-phase extraction, robotics and column switching, or by utilizing the selectivity of the packing such as ISRP. The ease of use would certainly enhance its application in the clinical laboratories. The key would be total automation of sample preparation, analysis and data reduction. It would be important to develop guidelines to ensure method validation, peak purity, and acceptable precision.

Other areas of potential interest would include chiral separation and automated sample preparation to minimize exposure to personnel. Supercritical fluid chromatography and extraction are now becoming well-established, and their routine clinical applications awaits further studies.

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