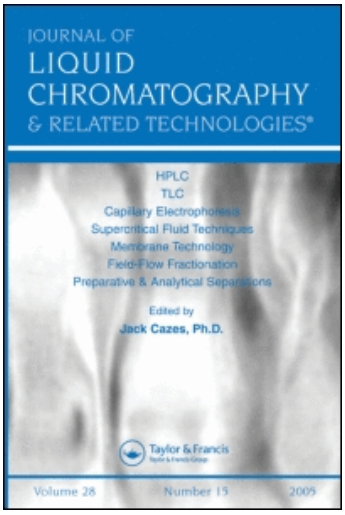

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Microbore Liquid Chromatography for Therapeutic Drug Monitoring and Toxicology: Clinical Analyses of Theophylline, Caffeine, Procainamide, and N-Acetyl Procainamide

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MICROBORE LIQUID CHROMATOGRAPHY FOR THERAPEUTIC DRUG MONITORING AND TOXICOLOGY: CLINICAL ANALYSES OF THEOPHYLLINE, CAFFEINE, PROCAINAMIDE, AND N-ACETYL PROCAINAMIDE

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ABSTRACT

With the recent technical advances in microbore liquid chromatography, routine applications, such as those in a clinical laboratory setting, may be possible. The present studies utilized readily available, commercial equipments and columns for clinical drug analyses for Therapeutic Drug Monitoring and Toxicology. Drugs would include an antiashmatic - theophylline, an antiarrhythmic - procainamide and its metabolite, N-acetyl procainamide, and caffeine - used for the treatment of neonatal apnea. Extraction procedures were modified from published methods, followed by optimized microbore liquid chromatographic analysis.

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Non-standard abbreviations - TH, theophylline; CA, caffeine; PA, procainamide; and NAPA, N-acetyl procainamide.

The microbore liquid chromatograph consisted of a syringe pump, an injector, a 3 um C-18, 1 mm i.d. column and a variable wavelength detector with 1 uL cell volume. Flow rates and analysis times ranged from 80 to 100 uL/min. and 8 to 12 minutes respectively. Precision studies showed acceptable within-run and day-to-day coefficients of variation. For the measurement of patients' drugs concentration, the results of these procedures correlated with those of conventional liquid chromatographic measurements and fluorescence polarization immunoassay. The microbore liquid chromatographic assays were simple, reliable, and utilized small amount of mobile phases. They may be readily adapted by clinical laboratory with minimal personnel retraining.

INTRODUCTION

With the increasing use of therapeutic agents, and the epidemic abuse of illicit drugs in our society, the clinical laboratories are expanding and improving in the detection/quantitation of drugs and metabolites (1-3). The specialties of Toxicology, and the more recent Therapeutic Drug Monitoring have thus been revitalized by such needs, and have responded to the challenge by using advanced technologies such as immunoassays and chromatography. Due to the potential medicolegal implication of the test results, it would be important to be able to accurately identify/quantitate the drug of interest, and to be able to confirm the results whenever possible. With the prospective payment such as diagnostic related group(DRG) for health care cost containment, the need to minimize hospital cost could affect the operating budget of laboratories performing drug analyses. For the above reasons, it would be pertinent to consider alternative methods.

In considering instrumentation for clinical drug analyses, the majority of the laboratory utilizes immunoassays, supplemented

by chromatography. With the technology such as microbore liquid chromatography(MBLC), clinical application may be performed with readily available commercial equipments and columns. The author recently reviewed the status of MBLC (4), including selected theoretical and technical considerations, and some preliminary studies of MBLC drug assays. The advantages of MBLC would include enhanced mass sensitivity, reduced solvent consumption and others. Shipe et.al. described a MBLC assay bethanidine in plasma (5), while Annesley et. al. analyzed cyclosporine using MBLC with small size sample(6). Both studies utilized either conventional or modified LC equipments, with 2 mm i.d. columns, flow rate of 250 uL/min. and injection volumes of 20 or 50 uL. Dezaro et.al. demonstrated a high speed analysis of TH using kinetically optimized MBLC (7).

In this article, clinical, MBLC assays of theophylline (TH), caffeine (CA), procainamide (PA) and N-acetyl procainamide (NAPA), are summarized, with comparison to either established, conventional LC assays (8,9), and/or to fluorescence polarization immunoassays (10). The emphasis was on using commercially available equipment so that the described applications may be readily transferred to another clinical laboratory. Particular attention would be given to the technical feasibility in a clinical laboratory setting.

MATERIALS AND METHODS

Reagents

Acetonitrile, methanol, methylene chloride, distilled in glass, ultra-violet grade, were obtained from Burdick and Jackson

(Muskegon, MI 49442). Theophylline, beta-hydroxyethyl theophylline, caffeine, procainamide HCL, N-acetyl procainamide, N-propionyl procainamide were purchased from Sigma chemical Co. (St. Louis, MO 63178).

Standards

Drug standards and quality controls of TH and CA were prepared as previously described(8). PA and NAPA assay was modified from our clinical assay, and that of Kabra(9). To prepare the primary stock solutions, 100 mg/L, three separate 100 ml volumetric flasks were added 11.6 mg of PA HCL, and 10 mg each of NAPA and N-propionyl procainamide(I.S), followed by adding methanol to the mark. Then, aliquots of these primary stocks were added to aliquots of serum for the preparation of 2.5, 5,10 and 30 mg/L standards. Concentration of the internal standard was 10 mg/L.

Mobile Phase

For the analysis of TH and CA, acetate, 0.05 M, pH = 5.0 was prepared, mixed with acetonitrile (93:7), and degassed prior to analysis. For the analysis of PA and NAPA, 0.025 M phosphate, pH= 3 was prepared, mixed with acetonitrile(9:1), and degassed prior to analysis.

Instrumentation

For the microbore analyses, the chromatograph consisted of a syringe pump, microMetric^{TM}(LDC/Milton Roy, Riviera Beach, Fl), 5

mL capacity, 1 or 0.5 uL injectors; Models, 7140 and 7520 (Rheodyne, Calif), a 3 um, C-18 column, (1 mm i.d. and 10 cm length) packed with Spherisorb^{TM} particles, and a variable wavelength detector,spectroMonitor D (LDC/Milton Roy)set at either 254 or 280 nm, equipped with either a microbore flow cell, microCell^{TM}(3 uL, 1 mm flow-path and total sample swept volume = 3 uL) or conventional flow cell maxN^{TM} (1 uL,3 mm flow-path and total sample swept volume= 9 uL).

For the conventional analyses, the chromatograph consisted of a model 9533 ternary gradient liquid chromatograph, a 7125 Rheodyne injector, a model 9523 variable wavelength detector. (IBM Instrument, Danbury, Conn),

The chromatograms were recorded on an Omniscribe recorder.

Sample preparation

For TH and CA analyses, the procedure was identical to a recently published study(8), using trichloroacetic acid(10%) for protein precipitation. For PA and NAPA analysis, 250 uL each of standards, control and patient's sera in a series of polypropylene conical tubes, were vortex-mixed with 250 uL aliquots of internal standard. Then, 50 uL of 5 N NaOH was added and vortexed. The alkalinized mixtures were extracted by 1 mL aliquots of methylene chloride by rotation for 15 minutes. Then, these tubes were centrifuged at 9500 x g for 15 minutes. After discarding the upper layer, the lower, clear layer was carefully transferred to another set of conical polypropylene tubes. The extraction solvent was

vaporized by passing nitrogen. The extracts were reconstituted with 125 uL of mobile phase, followed by centrifugation for 5 minutes at 9500 x g to yield a clear, particle-free solution. Then, aliquots were injected for analysis.

Chromatographic parameters

For TH and CA analyses with the conventional column, the flow rate was 2 mL/min., wavelength ▫ 280 nm, 0.02 AUFS. MBLC analysis utilized a flow rate of 80 uL/min., injection volume =1 uL, and wavelength ▫ 280 nm, 0.002 AUFS. PA and NAPA analysis with the uBondapak C-18 was carried out with a flow-rate of 2 mL/min., and 254 nm, 0.02 AUFS, while the MB analysis utilized 100 uL/min., injection volume of 0.5 uL and detection at 254 nm, 0.005 AUFS.

Quantitation and Statistical analysis

Calibration curves of peak height ratios plotting against drug concentrations were performed by the linear regression program of the Advanced Statistical Analysis(Radio shack, Fort Worth, TX). From these plots, drug concentrations of quality control and patients were estimated. Correlation of these values with those of other methods were also carried out by the same program.

RESULTS

Figure 1 shows the chromatograms of MBLC analyses of TH and CA in the serum extracts of a standard and a patient. Retention volumes of TH, IS and CA are 256, 352 and 576 uL respectively. Analysis time was about 8 minutes, with total elution volume of

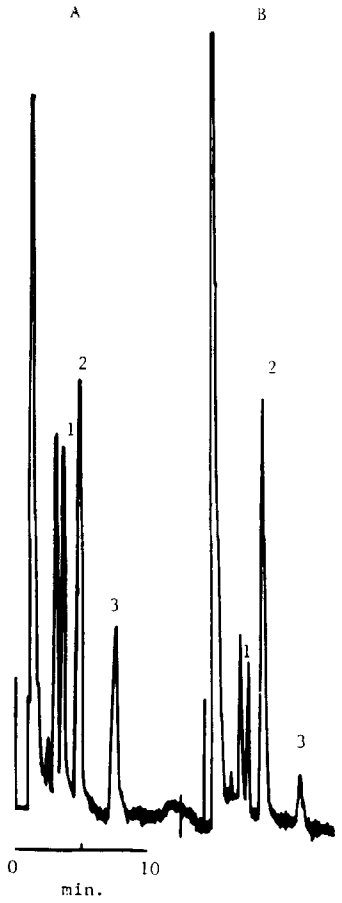

Figure 1. MBLC chromatograms of serum extracts of : (a) 10 mg/L standard, and (b) a patient. (TH = 4 mg/L and CA = 3 mg/L). Peaks identification = 1. TH., 2. IS., and 3. CA.

640 uL. Regression analyses of calibration curves from 2 to 20 mg/L are shown in table I. And the sensitivity for TH and CA, defined as S/N = 3, are estimated to be 1.0 and 1.5 mg/L respectively. Precision data are outlined in table II. Results of correlation studies with conventional LC and FPIA are included for table III.

MBLC chromatograms for PA and NAPA are included in figure 2. Retention volumes of PA, NAPA and IS are 220, 450 and 950 uL respectively. Regression analyses of calibration curves (5 to 30 mg/L) and precision studies are shown in table II and I respectively. Sensitivity (S/N=3) for PA and NAPA were about 0.3 and 0.2 mg/L respectively. Results of correlation studies with other methods are outlined in table III.

The following drugs were analyzed for possible interference by both methods:amitriptyline, acetaminophen, amoxapine, cimetidine, chlorpromazine, codeine, chloramphenicol, chlordiazepoxide, clomipramine, desipramine, diazepam, doxepin, demoxepam,flurazepam, imipramine, lorazepam, maprotiline, meperidine, nortriptyline, prochlorperazine, propoxyphene, perphenazine, phenytoin, phenobarbital, pentobarbital, thioridazine, trazodone and secobarbital. In addition, 1-methyluric acid , 1,7-dimethyluric acid, 3-methylxanthine, 1,7-dimethylxanthine and 1-methylxanthine were checked with the TH and CA method. The following interferences were observed: TH by acetaminophen and pentobarbital; CA by cimetidine and codeine; and PA by cimetidine.

Table I. Regression Analyses of Calibration Curves

Drug	r	Slope	Intercept
TH	0.999	0.069	0.032
CA	0.993	0.040	0.056
PA	0.997	0.109	-0.013
NAPA	0.993	0.155	0.036

Table II : Precision Data

	With-run		Day-To-Day	
Drugs	CV	n	CV	n
TH	1.5 %	5	6.4 %	30
CA	3.5 %	5	7.4 %	30
PA	3.8 %	5	6.5 %	25
NAPA	4.3 %	5	7.1 %	25

Table III. Linear Regression Analyses of Selected Methods Compared to MBLC

Method	r	Slope	Int.	n
TH-Con	0.987	0.939	0.350	22
TH-FPIA	0.992	1.022	0.946	14
PA-CON	0.985	0.944	-0.054	10
PA-CON	0.990	1.056	-0.314	52
NAPA-CON	0.983	1.087	-0.516	52

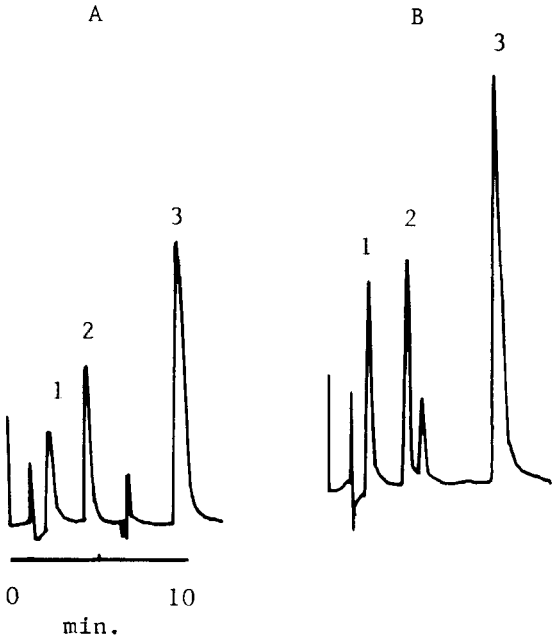

Figure 2. MBLC chromatograms of serum extracts of: (a) 5 mg/L standard, and (b) a patient (PA = 7 mg/L and NAPA = 5 mg/L). Peaks identification: 1. PA., 2. NAPA., and 3. IS.

DISCUSSION

Two major advantages of microbore LC, namely enhanced mass sensitivity and reduced solvent consumption(4,10-14), would be of interest to clinical chemists engaged in Therapeutic Drug Monitoring and Toxicology. In order to capitalize on the first advantage, stringent requirements on instrument would have to be fulfilled in order to minimize band spreading(15). While it is not within the usual capability of a clinical laboratory to perfect modification of existing LC for this purpose, the presently available commercial instrument would be useful for MBLC drug assay. This study attempts to perform selected MBLC drug assays as a model for further investigation with the possible applications in the areas of neonatal and pediatric drug monitoring, micro-drug analysis such as pharmacokinetics studies employing small animals, amino acid/peptide analyses and drug screening. The second advantage, reduced solvent consumption, would be of interest in reducing operation budget. However, on a relative basis, the percent of the operating budget for solvent is not significant(< 10 %) to the extent that choosing MBLC would realize a substantial saving. Thus, this study would document, but not emphasis on the reduced solvent consumption in a clinical laboratory setting. The readers would be advised to draw their own conclusion as to the relative importance of this advantage of MBLC for routine clinical drug assay.

As pointed out in a previous review(4), clinical application of MBLC is relatively new and few. Both studies by Shipe (5) and

Annesly (6) utilized 2 mm i.d. columns and large injection volumes (20 to 50 uL), which are not considered to optimal for MBLC analyses. Information on flow-cell dimension was not available. As pointed out by Scott(11) and Tehrani(14), column diameter, injector and detector flow-cell volume should be minimized to avoid band spreading. The present study utilized dedicated MBLC instrument - a syringe pump, small volume injector (1 or 0.5 uL), and small flow-cell (1.0 uL). As shown by the results, these parameters would be important in optimizing MBLC drug assays.

Column stability

For both studies, a 3 um, C-18 column with 1 mm i.d. and 100 mm length was used. The operating back-pressure of these analyses would be dependent on the 'age', mobile phase and column temperature, and would fluctuate between 4000 to 6000 psi at flow-rates between 80 to 100 uL per minute. With the exception of the first colum, the number of injections would range from 500 to 600, before drifting baseline and/or skewed peak shape rendering the chromatograms unusable as a result of inaccurate peak height ratio calculations. (The lower number of injection for the first column, about 350, was atypical, probably due to the "break-in" process!). From the author's experience, these compared favorably with that of the conventional column. Due to complexity of the extract from patient's sample and the lack of a guard column, the column life is acceptable . During these studies, the retention time, and thus elution volumes remained stable, i.e. the capacity

factors differed by less than 10%. In the event of a high back-pressure, the column was reversed and regenerated at an initial lower flow-rate of about 30 to 40 uL per minute. This was attempted rather infrequently, and as a last attempt before discarding the column. No attempt was made to replace end frits or the packing at the inlet of the column due to possible voiding of a rather small column volume of about 80 uL.

Other chromatographic parameters

In the preliminary studies, the MBLC consisted of a 1 uL injector and a 3 uL microbore flow-cell. Subsequently, a smaller injector volume of 0.5 uL was used, since the injected amount of extracted drug would theoretically be detectable with the enhanced mass sensitivity of a MBLC. For the TH and CA MBLC assay, detector was set at the most sensitive attenuation of 0.002 AUFS, and the chromatogram would show a constant drifting baseline (0.0004 AUFS/min.), resulting in frequent adjustment between analyses. This was not improved by column insulation to minimize possible baseline drift due to column temperature fluctuation. Finally, the problem was solved by using another flow-cell (1 uL cell volume and 3 mm pathlength).

Using this instrumentation and the described mobile phases with adequate column "washing" (2:8 to 4:6 = ACN/H_2O) after each run, equilibration was achieved quickly (about 30 minutes) and dependably - a very important consideration for clinical methodology. However, despite precaution described above and in

the following paragraph, inadvertent back-pressure would increase, exceeding 7000 psi limit of the MBLC, and rendering the column unsuitable for clinical application. A guard column (not yet commercally available) might have minimized such problem.

Sample preparation

Due to the typically small i.d.tubings, the possibility of a "plugged" LC and/or columns is increased. Thus careful sample preparation would be needed to avoid introduction of particles during injection. Inherently, a simple procedure with the minimum number of extraction steps would certainly enhance such requirement. Thus, for the analysis of TH and CA, instead of extraction, a single, protein precipitation step with trichloroacetic acid was adopted, and by using conical polypropylene tubes, the final centrifugation at 9500 x g would ensure a clear, particle-free liquid phase with a solid protein pellet at the bottom. These precautions were successfully applied to last step of the PA and NAPA assay.

Assays characteristics

Both precision and recovery studies showed acceptable results. And a systematic check showed some interference. The sensitivity was acceptable (0.2 to 1.5 mg/L range). It is important to note that using a MBLC of different configuration with even smaller instrument dead volume, theoretically, a higher sensitivity limit may be achieved. However, for these clinical drug assays, the present limits would be adequate.

Correlation of patient's data with either an established LC method and/or FPIA shows acceptable result. Thus, this may be readily used for clinical laboratory TDM and Toxicology measurements. And our experience shows that minimal personnel retraining is needed for performing MBLC. By using this technique, reduction in sample size would be achievable, making it a potentially useful tool for drug measurement of neonatal and pediatric patients, sample size limited applications such as amino acid/peptides analyses, and pharmacokinetics study using small animal model such as mice and rats, and drug screening procedures for confirmation and/or initial screening purposes.

REFERENCES

1. Wong, S.H.Y., "Introduction" and " Liquid Chromatography and Other Methodologies for TDM and Toxicology", in " Therapeutic Drug Monitoring and Toxicology by Liquid Chromatography" (S.H.Y. Wong, ed.) , Marcel Dekker, New York, 1985, p.1 and p.39.

2. Poklis, A., Drugs of Abuse: An Integrated Approach to Urine Screening, Laboratory Management, 23,41, 1986.

3. Bennet, D.D., More Labs Branching Into Corporate Drug Screening, Clin. Chem. News, 12,1, 1986.

4. Wong, S.H.Y., Microbore HPLC in Drug Monitoring and Toxicology: An Overview, Lab. Management., 23,59,1985

5. Shipe, J.R., Arlinghaus, A.F., Savory, J., Wills, M.R., and DiMarco, J.P., Determination of Bethanidine in Plasma by Liquid-Chromatography with a Microbore Reversed-Phase Column, Clin. Chem., 10,1793, 1983.

6. Annesley, T., Matz, K., Balogh, L., Clayton, L., and Giacherio, D., Liquid-Chromatographic Analysis for Cyclosporine with Use of a Microbore Column and Small Sample Volume, Clin. Chem., 32,1407,1986.

7. Dezaro, D., Horn, D. and Hartwick, R.A., Kinetically Optimized Microbore HPLC in the High Speed Monitoring of Theophylline, Clin. Chem.,29, 1158, 1983.

8. Wong, S.H.Y., Marzouk, N., McHugh, S.L., Cazes, E., Simultaneous Determination of Theophylline and Caffeine by Reversed Phase Liquid Chromatography using Phenyl Column, J. of Liq. Chromatogr. , 8, 1797, 1985

9. Kabra, P.M., Chen, S., Marton, L.J., Liquid-Chromatographic Determination of Antidysrhythmic Drugs: Procainamide, Lidocaine, Quinidine, Disopyramide, and Propranolol, Ther. Drug Monit., 3,91, 1981.

10. Wong, S.H.Y., The Fluorescence Polarization Assay in TDM: Recent Development, Lab. Management, 22,36,1984.

11. Scott, R.P.W., Small-bore Column in Liquid Chromatography, in Adv. in Chromatogr. J. Giddings, et al. (eds.), 22,247,1983.

12. Kucera, P. (ed.), Microcolumn High-performance Liquid Chromatography, J. Chromatogr. Lib. V. 28,Marcel Dekker, New York, 1984.

13. Novotny, M.V. and Ishii, D. (eds.), Microcolumn Separations, J. Chromatogr. Lib. V.30, Marcel Dekker, New York, 1985.

14. Tehrani, A.Y., Enhanced Mass Sensitivity and Resolution in Microbore Liquid Chromatography by Improvements in Detection and Liquid Flow Path, LC, 3,42, 1985.

15. Sternberg, J,C., Extracolumn Contributions to Chromatographic Band Broadening, Adv. in Chromatogr, V.2,205, 1966.