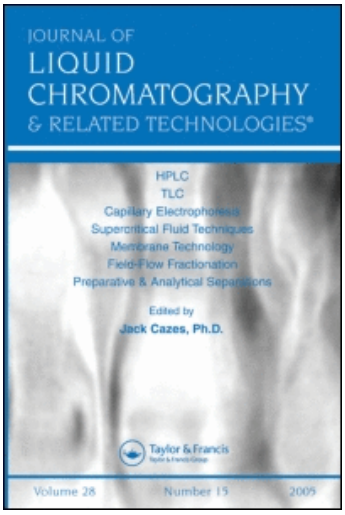

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MICROBORE LIQUID CHROMATOGRAPHY FOR PEDIATRIC AND NEONATAL THERAPEUTIC DRUG MONITORING AND TOXICOLOGY: CLINICAL ANALYSIS OF CHLORAMPHENICOL

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ABSTRACT

Microbore liquid chromatographic assay of chloramphenicol was developed, using 5 uL samples and reversed-phase liquid chromatographic analysis with a C-18, 20% carbon load column. For protein precipitation, serum samples were mixed with 20 uL of methanolic internal standard solutions. After centrifugation, 0.5 uL aliquots were injected for analysis. Two procedures were evaluated: procedure A - column = C-18, 3 um, carbon load of 10%, mobile phase = acetate/acetonitrile /tetrahydrofuran (85:15:1.5), flow rate = 80 uL/min, temperature = 50°C; procedure B - column = C-18, 5 um, carbon load of 20%, mobile phase = acetate/ acetonitrile(8:2), and flow rate = 60 uL/min. Procedure B was chosen for clinical efficacy study. The retention volumes of

chloramphenicol and internal standard were 360 and 840 uL respectively. Calibration curve was linear between 3 to 40 mg/L. And day-to-day coefficient of variation was 6.8%. Correlation study with an established clinical liquid chromatographic assay for 20 patient samples showed acceptable data: r=0.9949, slope=0.9760 and intercept=0.0025. Selected drugs did not interfere. This assay, with advantages of small sample size, easy preparation and MBLC analysis, may be readily adapted for pediatric and neonatal drug monitoring.

Introduction

Microbore(MB) liquid chromatography(LC) may be complimentary to other chromatographic and immunoassay techniques for drug analysis(1). Since the introduction of this new technology, its applications in clinical laboratory were demonstrated in only a few drug assays. Its major advantages include enhanced mass sensitivity, and reduced solvent consumption. In a clinical laboratory setting, enhanced mass sensitivity would be of more interest, and may be readily applied to micro-sample-size analysis for pediatric and neonatal drug monitoring. While it is desirable to perform MBLC analysis with dedicated instrumentation, clinical laboratory equipped with conventional LC might attempt MBLC analysis using 2 mm i.d. column, without the purchase of a dedicated MBLC.

Chloramphenicol may be quantified by microbiological assay(3), radioenzymatic assays, gas chromatographic assays(4), and readily by LC with high specificity(5-8). Recent novel LC procedures included Sood's study(9) using small sample size of 25 uL for a two step extraction, followed by LC analysis, and El-Yazigi's study(10) of direct-sample-analysis of 10 uL by reversed-phase(RP) LC. However, the later study did not establish column stability. The present study utilized small sample of 5 uL , protein precipitation, followed by RP MBLC analysis.

Clinical applications of MBLC have been demonstrated recently by the author for the analysis of theophylline , caffeine, procainamide and N-acetyl procainamide(1). Jurgen reviewed antiepileptics analyses(11), and utilized MBLC for antiepiletpic analysis in brain tissue(12). Shipe et al. analysed bethamide(13) , while Annesley demonstrated its use for cyclosporine with small sample volume(2), and more recently for flecainide(14). Hyldburg evaluated the feasibility of radially compressed microbore column(15). The present study evaluated two MBLC procedures for the analysis of chloramphenicol, using a commercially available, dedicated MBLC, for possible application in pediatric and neonatal Therapeutic Drug Monitoring and Toxicology.

MATERIALS AND METHODS

Reagents

Acetonitrile(ACN) and methanol, HPLC grade, ethyl acetate, Baker Resi-analyzed, and sodium acetate, Baker Analyzed, were obtained from Baker(Phillisburg, NJ). Chloramphenicol was obtained from National Bureau of Standard(Rockville, Md). The internal standard, 5-ethyl-5 -p-tolybarbituric acid was obtained from Aldrich(Milwaukee, Wis).

Standards

Chloramphenicol stock solution, 1 mg/ml was prepared by dissolving 10 mg of chloramphenicol in 10 ml of methanol. To prepare working standards, aliquots, 250, 375, 500 and 1000 uL of the above standard were transferred to four separate 25 mL volumetric flasks,

followed by evaporation of methanol under nitrogen. Then, drug-free serum was added to the mark. The concentrations of these four standards were: 10,15,20 and 40 mg/L. The internal standard stock solution was prepared by dissolving 10 mg in 50 ml of either methanol or ethyl acetate. Using two separate 100 mL volumetric flasks, the working internal standard solutions were prepared by mixing 6.25 ml of stock solution with either methanol or ethyl acetate to the mark. The resultant concentrations were 12.5 ug/L.

Mobile phase

Acetate, 0.1 N, pH = 6.0, was prepared by dissolving 40.8 g of sodium acetate in three liters of distilled water, followed by adding glacial acetic acid to adjust pH = 6.0. This solution was filtered and mixed with the appropiate amount of acetonitrile (8:2 or 75:25)and acetonitrile/tetrahydrofuran(85:15:1.5), followed by degassing.

Instrumentation

MBLC consisted of a microMetric^{TM} syringe pump from LDC/Milton Roy(Riviera Beach, Fl), with 5 ml capacity, connected to a Model 7520 injector from Rheodyne(Cotati, Calif), equipped with a 0.5 uL rotor, and a variable wavelength spectroMonitor D detector(LDC/Milton Roy) set at 254 nm. The flow-cell was a maxN^{TM} with 1 uL volume, 3 mm flow-path and swept volume of 9 uL. Two different columns were evaluated: 1. Spherisorb^{TM}, C-18, 3 um, and 10% carbon load; and 2. Nucleosil^{TM}, C-18, 5 um, and 20% carbon load. Both columns were 1 mm i.d. and 10 cm length, purchased from Keystone

Scientific(College Park, Pa). Column heating coil and monitor, CH1445, were purchased from SYS-TEC(Minneapolis, Minnesota). The column was embedded within two-halves of the cylindrical aluminium blocks for efficient heat transfer. Silicon heat sink compound-type Z9 from GC Electronics (Rockford, Illinois), was introduced into the grove of the aluminium blocks for enhancing heat transfer between the block and column wall. The assembled blocks and column were then wrapped with the heating coil connected to the monitor. This assembly was readily positioned between the injector and the detector without any additional connecting tubing which will add to the extra-band dispersion.

The chromatograph for the clinical analysis consisted of a Constantmetric Pump from LDC/Milton Roy, a Rheodyne 7125 injector, and model 440 detector with a 254 nm filter from Waters(Milford, Mass). The column was a uBondapak C-18(Waters), protected by a guard column packed with Bondapak C-18. Chromatograms were recorded on an Ommiscribe recorder.

Sample preparation

MBLC analysis was based on protein precipitation with methanol, while the clinical procedure, adapted from a published method(5), was based on ethyl acetate extraction.

MBLC analysis - Using a series of conical, polypropylene tubes, aliquots, 5 uL, of standards (0,10,15,20 and 40 mg/L), quality controls and patient's sera were mixed with 20 uL of methanolic working internal standard solution. The mixtures were vortexed thoroughly for protein precipitation. Then, these conical tubes were centrifuged at 9,500xg for

10 minutes. The proteinaceous materials were compacted to a fine pellet at the bottom of the conical tubes, while the supernatant, containing chloramphenicol and the internal standard, was clear and distinct. These tubes were carefully removed from the centrifuge so as not to disturb the content, and placed sequentially inside a transparent tube holder. With steady and smooth motion, and without moving the tubes, a 10 uL syringe was inserted into the clear supernatant. While CAREFULLY not disturbing the content, 5 uL of the clear supernatant was loaded without picking up any particle. Through the injector, 0.5 uL was introduced into the column.

Clinical assay - Aliquots, 100 uL, of standards, quality controls and patients' sera were extracted with ethyl acetate. After transfer, the organic phase containing chloramphenicol and the internal standard was evaporated, followed by re-constitution with methanol. Aliquots, 10 uL, were injected for analysis.

Chromatographic parameters

Two procedures were evaluated for their clinical efficacy. For procedure A, the analysis was performed with a Spherisorb^{TM} C-18 column and acetate/ACN/THF(85:15:1.5) as the mobile phase. Flow rate was 80 uL/min., and analysis temperature was 50^{o}C. For procedure B, the analysis was performed with a Nucleosil^{TM} C-18 column and acetate/ACN(80:20) as the mobile phase. Flow-rate was 60 uL/min. Detection wavelength for both procedures was set at 254 nm, 0.002 to 0.005 AUFS.

Calibration

Peak height ratios of chloramphenicol to the internal standards were plotted against their respective chloramphenicol concentrations. Linear regression analysis of the calibration curves were performed by using Advanced Statistical Analysis from Radio Shack(Fort Worth, TX). Concentrations of quality control and patient samples were estimated from these plots.

Results

Chloramphenicol and its esters were well resolved by both procedures A and B. Using procedure A, the analysis time per sample was about 9 minutes, as shown by Figure 1. Retention volumes of chloramphenicol and the internal standard were 320 and 640 uL respectively. With procedure B, the analysis time was longer, 15 minutes. Retention volumes of chloramphenicol and the internal standard were 360 and 840 uL respectively. Because of the binary mobile phase and ambient temperature separation, the simpler procedure B was chosen for further evaluation.

Calibration studies showed that peak height ratios were linearly correlated to concentrations between 3 to 40 mg/L. Sensitivity was estimated to be 3 mg/L at S/N = 3. Recovery, based on peak height of the 15 mg/L standard(n=6), was estimated to be about 95%.Precision studies showed that: within-run, mean = 14.6 mg/L, CV = 3.5% and n =6., and day-to-day, mean = 14.8 mg/L, CV = 6.8% and n = 15. When compared to an established clinical LC analysis for the measurement of 20 clinical samples, the correlation study showed that r = 0.9949, slope = 0.9760 and an intercept = 0.0025.

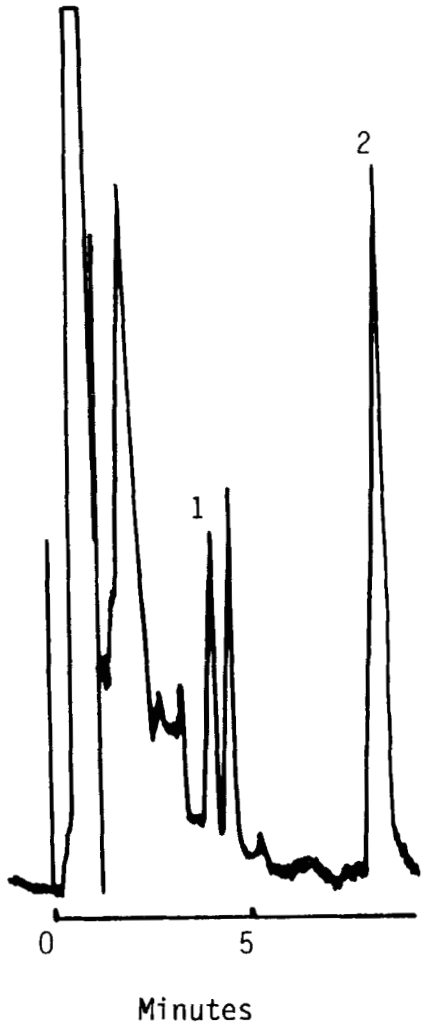

Figure 1 : Microbore liquid chromatogram of 0.5 uL aliquot of supernatant from procedure A. Chloramphenicol concentration was 21 mg/L. (Peak identification: 1 = chloramphenicol and 2 = internal standard).

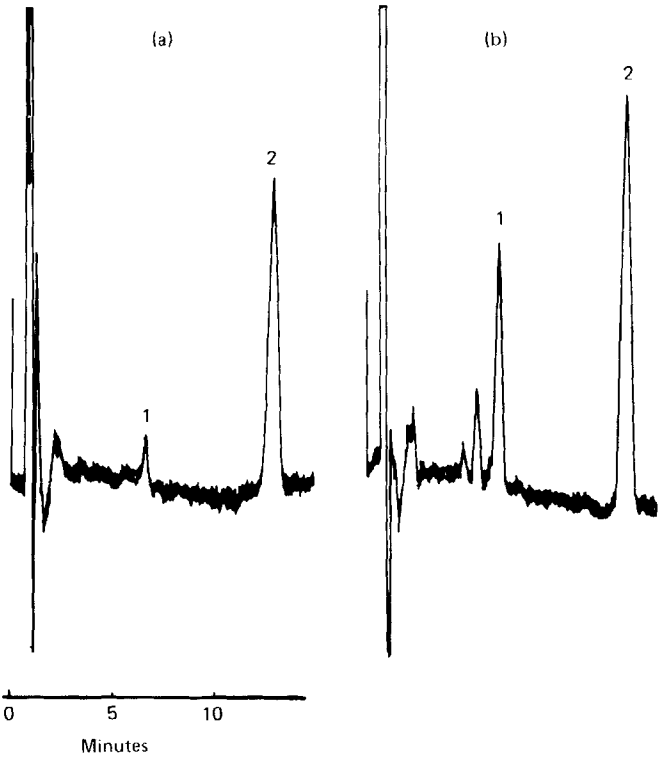

Figure 2 : Microbore liquid chromatograms of 0.5 uL aliquots of supernatant from procedure B. Chromatogram A shows 6 mg/L of chloramphenicol following oral administration of chloramphenicol palmitate, and chromatogram B shows 21 mg/L of chloramphenicol following IV administration of chloramphenicol succinate. (Peak identification: 1 = chloramphenicol and 2 = internal standard).

Interference

For checking possible chromatographic interference, the following drugs, analyzed by procedure B, did not co-elute with chloramphenicol or the internal standard: acetaminophen, cimetidine, chlorpromazine, codeine, chlordiazepoxide, desipramine, diazepam, doxepin, demoxepam, flurazepam, imipramine, meperidine, nortriptyline,

propoxyphene, phenytoin, phenobarbital, pentobarbital, thioridazine, vancomycin, cephalexin and ampicillin.

Discussion

For clinical drug analysis, enhanced mass sensitivity of MBLC may be readily capitalized for limited-sample-size application such as pediatric and neonatal drug monitoring(1,2). Previously, the author has demonstrated MBLC analysis of theophylline, caffeine, procainamide and N-acetyl procainamide. The present study was designed to explore the potential applications of MBLC for antimicrobial monitoring, and chloramphenicol was chosen in this pilot study.

For the clinical assays of chloramphenicol, the obvious choices of either the microbiological(3) or liquid chromatographic assays(4-10) would be dependent on whether the patient has been administered with other antimicrobials. If affirmative, microbiological assay would encounter potential cross-reactivity or interference from other antimicrobials, so that liquid chromatographic assay would be preferred. In order to design a clinical assay of chloramphenicol, a brief outline of its clinical pharmacology and some relevant analytic considerations are outlined as follows.

Chloramphenicol is a broad spectrum antimicrobials for the treatment of infections as a result of gram-positive and gram-negative organisms, and rickettsiae(16-23). In pediatric, it is used for the treatment of meningitis and epiglottitis caused by Hemophilus influenzae. Side effects would include: aplastic anemia, possible bone marrow suppression, and "gray" syndrome. The proposed therapeutic range is 10 to 25 mg/L.

Chloramphenicol may be administered orally or intravenously(IV) - the preferred route for pediatric and neonatal patients. Following IV administration of the prodrug, chloramphenicol succinate esters are hydrolyzed in the liver to the active chloramphenicol. Under physiological conditions, the prodrugs, 3- and 1- esters exist in a ratio of 4 to 1. For the LC analysis of serum from this patient group, both prodrug esters and chloramphenicol would be detectable as three separate peaks.

Another approach would be the oral administration of chloramphenicol palmitate. It is hydrolyzed by pancreatic lipases in the duodenum. And chromatographic analysis of the serum would show only one peak - chloramphenicol. Thus for pediatric and neonatal TDM of chloramphenicol, it would be necessary to consider both situations: analysis of chloramphenicol alone, and along with 3- and 1- succinate esters. The later has been readily achieved by the clinically established LC assay with sample size of 100 uL or more, but satisfactory analysis by MBLC was achieved in this study, only after extensive, systematic column evaluation and solvent scouting.

In addition to meeting the above requirements, the MBLC assay was designed to capitalize on enhanced mass sensitivity by using as little specimen as possible. Thus, necessary precautions would include minimized sample preparation to enhance recovery, followed by analysis by RP MBLC columns.

Sample preparation

In keeping the sample preparation as simple as possible, systematic studies were performed on the minimun sample size of 5 to 50 uL,

multi-steps extraction with various solvents, protein precipitation using various solvent such as acetonitrile, methanol and trichloroacetic acid. The finalized procedure would require 5 uL aliquots of serum samples, pippeted into a series of marked, small conical polypropylene tubes. Recent studies also showed the interest of reduced sample size. Sood utilized 25 uL of serum for a multi-step extraction(9), while El-Yazigi demonstrated direct-sample-analysis with 10 uL samples(10). However, column stability was not established for this later study.

Protein precipitation was achieved by vortex-mixing with the methanolic solution of the internal standard. Then, these samples tubes were centrifuged at high speed to yield a well-defined lower proteinaceous pellet which is firmly embedded onto the conical bottom, and a clear and well-defined supernatant, containing chloramphenicol and the internal standard. By transferring carefully these test tubes to a see-through test tubes stand, 5 uL aliquots were readily loaded into the syringe without taking up particle which would block the solvent path of the MBLC. The sample preparation, carried out in one single tube without any transfer, is thus simple and highly reproducible, and the recovery is about 95%.

MBLC analysis

The preliminary experiments, using a binary mobile phase similar to that of the clinically established assay(5), showed acceptable comparison if chloramphenicol was un-accompanied by pro-drug esters such as in the analysis of serum from patient receiving orally administered chloramphenicol palmitate. However, for the analysis of serum from patient receiving IV of prodrug succinate esters, these analyses did not

adequately resolve chloramphenicol from its pro-drug esters, resulting in over-estimated concentrations in comparison to the clinical assay. In attempting to achieve the needed resolution, solvent scouting experiments led to use of procedure A , using a ternary mobile phase: acetate/ACN/THF= 85:15:1.5 at an elevated temperature of 50°C.

Another approach, procedure B was based on the increased interaction of the analyte with a higher carbon load(20% vs 10% in the procedure A) C-18 column. This was attempted as a result of previous successful separation of polar antidepressant metabolites such as 7- and 8-hydroxy amoxapines(24). However, the analysis time was longer, about 15 minutes as compared to 9 minutes of the previous procedure. This simple analysis, carried out at ambient temperature, would be desirable for possible clinical application.

In capitalizing enhanced mass sensitivity of MBLC, and to prolong the column life, small sample size of 0.5 uL aliquots were injected for analysis. Based on our experience, without using a guard column, the number of injections was estimated to be 500 to 600, comparable to that of a previous study(1). Because of the small, injected sample size of 0.5 uL, the attenuation was set at 0.002 AUFS. The baseline was " noisy ", resulting in assay sensitivity of about 3 mg/L.

Clinical Analysis

As shown by the result, acceptable precision was established for both within-run and day-to-day studies. Selected drugs do not interfere with the analysis. The established sensitivity at 3 mg/L may be improved with better MBLC detector, but is adequate for clinical monitoring of chloramphenicol with therapeutic range of 10 to 25 mg/L. And

comparison with the clinical assay showed acceptable correlation. Because of the small sample size of 5 uL and the simple sample preparation, this procedure may be readily adapted for pediatric and neonatal TDM of chloramphenicol.

The described experience in column heating, althougth not adapted in this assay, may be readily used for elevated temperature separation for other MBLC assay. As shown by Annesley(2,14), MBLC may be performed by 2 mm i.d. column using conventional LC. This represents an "easier" approach for the clinical laboratory to attempt and to capitalize on the advantages of MBLC. In additon, there is renewed interest in direct-sample-analysis(10,25), using the following methods: micro-injection(<10 uL), column switching, micellar chromatography, internal surface reversed-phase and electrochemical detection following photolytic derivatization. And these techniques with emphasis on micro-size, in addition to MBLC, would undoubtedly enhance the analytic capability for pediatric and neonatal TDM and toxicology.

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