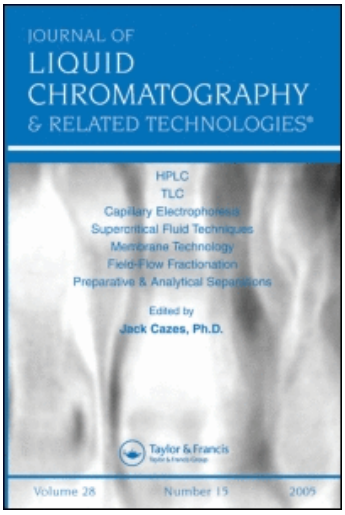

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Shielded Hydrophobic Phase for Direct Sample Analysis: Preliminary Study for the Analysis of Phenobarbital for Potential Neonatal and Pediatric Drug Monitoring

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SHIELDED HYDROPHOBIC PHASE FOR DIRECT SAMPLE ANALYSIS: PRELIMINARY STUDY FOR THE ANALYSIS OF PHENOBARBITAL FOR POTENTIAL NEONATAL AND PEDIATRIC DRUG MONITORING

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ABSTRACT

Shielded Hydrophobic Phase(SHP) for Direct Sample Analysis of phenobarbital in serum was investigated for Therapeutic Drug Monitoring by studying the column stability and utilization parameters, and by comparison to the established fluorescence polarization immunoassays. This novel packing consisted of 5 um spherical silica particles with 100 Å pores, bonded with polar and non-polar functionalities to the inside and outside of the particles. Proteins may be eluted, unretained with solvent front peaks while the drug/metabolites would undergo hydrophobic interaction, eluting later. For Direct Sample Analysis of phenobarbital in serum, 10 uL aliquots, after centrifuged at 9,500 x g for 20 minutes, were injected into the SHP guard column and column, and eluted with phosphate/ACN(9:1). Phenobarbital eluted with k' of 3.4 at

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3.8 minutes. Calibration was linear between 5 to 80 mg/L. Precision studies showed acceptable within-run and day-to-day coefficients of variation. Comparison with FPIA showed acceptable correlation. This preliminary study showed that SHP may be used for Direct Sample Analysis of phenobarbital. The simplicity of the procedure, and the small sample size may be advantageously applied for neonatal and pediatric drug monitoring. Further study will be needed to evaluate its long term stability, and applications to the monitoring of other drug groups.

Introduction

Direct Sample Analysis(DSA) by Liquid Chromatography(LC) has recently been explored for Therapeutic Drug Monitoring(TDM) and Toxicology in the clinical laboratory. This approach of drug/metabolite analysis with different configurations of LC, and with different columns were reviewed by the author (1,2), and Shihabi(3). Advantages would include, as a result of lack of sample preparation, enhanced efficiency, precision and possible automation, and possible lower cost per analysis. For the clinical laboratories engaging in TDM and toxicological drug analysis, additional advantages would possibly include micro-sample analysis, analysis of labile - light or heat sensitive - drug/metabolites, and minimized personnel exposure to bioharzardous samples. Thus, DSA may offer a practical alternative to the now predominant immunoassay.

According to the reviews(1-3), the following eight approaches have been illustrated: 1. Advanced Automated Sample Processor^{TM}(4,5), 2. Solvent extraction (6), 3. Column switching (7,8), 4. Micro-injections (9), 5. Micellar chromatography (10), 6. Electrochemical detection with photolytic derivatization (11), 7. Bimodal, internal surface reversed-phase (12-19), and 8. Silica column with aqueous mobile phase (20). Recently, a new packing, the Shielded Hydrophobic Phase(SHP), was developed by Gisch, Hunter and Feibush (21) for direct serum analysis of

drugs/metabolites. The present preliminary study summarized our experience in the clinical analysis of phenobarbital, and compared these measurements to those by a clinically established method of fluorescence polarization immunoassay(FPIA).

MATERIALS AND METHODS

Reagents

Acetonitrile(ACN), HPLC grade, and potassium dihydrogen phosphate, Baker-Analyzed, were obtained from Baker(Phillisburg, NJ).

Mobile phase

Phosphate, 0.05 M, pH = 7.0, was prepared by dissolving 6.8 gm of KH_2PO_4 in 1 liter of water, following by adjusting the pH to 7.0 by adding KOH. The solution was filtered and refrigerated until analysis. Immediately prior to assay, it was mixed with ACN(10%) and degassed.

Standards

Calibrators for the FPIA of phenobarbital from Abbott Laboratories (North Chicago, Ill) were used. These drug calibrator standards in serum were prepared with the following concentrations: 0, 5, 10, 20, 40 and 80 mg/L. Quality control samples, Antiepileptic Level I and II, were obtained from Gilford(Irvine, Ca).

Instrumentations

The LC consisted of a Series 100 pump, Model 7125 Rheodyne Injector with a 10 uL loop, TriDet detector with a 254 nm filter, and

R50 recorder from Perkin Elmer(Norwalk, Conn). The column was HYSEP^{TM}, 4.6 X 150 mm, 5 um, protected by a guard column packed with HYSEP^{TM}, 4.6 X 20 mm. Polypropylene microtubes with screw tops were obtained from Sarstedt.

Sample preparation

Prior to injecting the serum samples into the LC, aliquots, 50 uL of calibrators, quality controls and patient samples were transferred into a series of marked microtubes. These samples tubes were centrifuged at 9,500 x g for 20 minutes in order to "precipitate" any particle to the bottom of the tube, thus ensuring adequate amount of particle-free serum samples for injection. For analysis, 15 uL aliquots were loaded into the syringe for injecting into the LC.

Chromatographic parameters

Flow rate was 2 mL/min. Injection volume was 10 uL. Detection wavelength was 254 nm at 0.01 AUFS. After each daily run, the following parameters were noted in order to assess column stability for clinical drug monitoring: retention time, number of injections, total volume of injection, quantity of "washing" mobile phase, and a log of technical problem. Operating back-pressure was not recorded due to the lack of a pressure monitor.

Statistical analysis

Calibration curves and correlation study with the FPIA were analyzed by the Advanced Statistical Analysis from Radio Shack(Fort Worth, Tx).

Result

Figure 1 shows the chromatograms of 10 uL aliquots of a zero calibrator, a 20 mg/L standard, and a patient serum(17 mg/L). Retention time of phenobarbital, during the period of this preliminary study of 7 weeks, and after 180 injections, remained constant at 3.8 minutes, corresponding to a retention volume of 7.6 mL, and a k' of 3.4

Calibration studies showed that the peak heights were linearly correlated to concentrations from 5 to 80 mg/L(r = 1.000, slope = 1.074 and intercept = -0.671). Table I shows the result of the precision studies. Then, the procedure was used to quantitate patient serum samples containing therapeutic concentrations of phenobarbital as the only drug, or in combination with other antiepileptic and therapeutic agents. Comparison of patient phenobarbital concentrations estimated from a clinically established method, FPIA, and by the present procedure showed r = 0.982, slope = 0.982 and intercept = 0.409 for n = 45, indicating that both procedures yielded comparable concentrations. Table II shows the column stability and other utilization parameters. The following drugs, analyzed by the present procedure, were shown not to interfere with phenobarbital: primidone, theophylline, phenytoin, carbamazepine, salicylate, and methsuximide.

Discussion

Drug measurement by clinical laboratories in the U.S. is primarily performed by immunoassays due to the ease of operation as a result of automation, and good precision. LC is limited to the measurement of "low" concentration drugs such as tricyclic and cyclosporine. If applied,

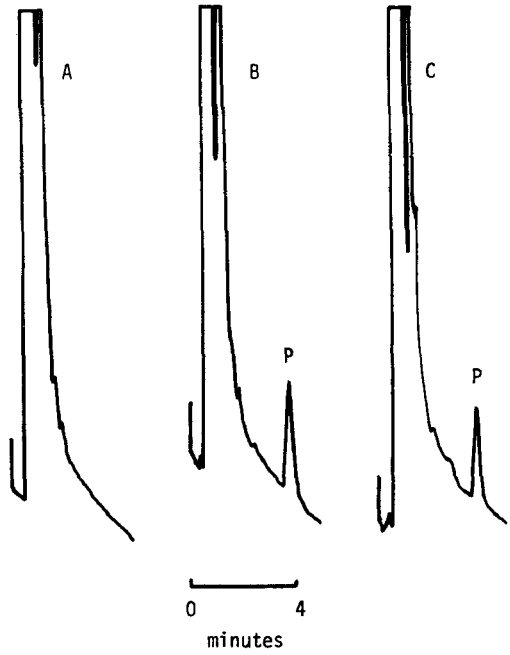

Figure 1. Chromatograms of 10 uL aliquots of serum analyzed by the Shielded Hydrophobic Phase column - A. Drug free serum, B. a 20 mg/L phenobarbital calibration standard, and C. a patient serum with estimated concentration of 17 mg/L. (From the FPIA, this patient drug concentrations, in mg/L, were : phenobarbital, 18; phenytoin, 20; primidone, 6 and carbamazepine, 3., and peak identification: P, phenobarbital).

Table I: Precision Studies of Shielded Hydrophobic Phase column for Clinical Direct Sample Analysis of phenobarbital in serum.

	Guilford Level I			Guilford Level II		
	Mean,mg/L	CV, %	n	Mean,mg/L	CV, %	n
Within-run	15.6	4.1	5	44.8	3.9	5
Day-to-day	15.8	6.1	9	41.6	5.4	8

Table II: Stability and Utilization parameters of a SHP column.

Utilization period = 3-15-88 to 5-5-88

= 7 weeks.

Retention volume of Phenobarbital (3-15-88) = 7.6 mL

(5-5-88) = 7.6 mL.

Number of injections = 180.

Guard column change after 6.5 weeks, 140 injections.

Total volume of injected serum = 1800 uL.

Total volume of mobile phase used = 4 L.

Total volume of washing mobile phase used = 1 L.

LC offers cost effectiveness, possible simultaneous measurement of drugs/metabolites, and the needed selectivity to resolve interference from other metabolites and drugs. However, it is indeed a more labor intensive technique, involving sample preparation, operation of a LC, and data reduction. Recently, solid phase extraction has greatly enhanced sample preparation (22). In order for LC to match the ease of immunoassays performed by autoanalyzer, various approaches of DSA, as outlined in the introduction and the recent reviews(1-3), may offer viable alternatives. The present study outlined our preliminary experience with a new column - Shielded Hydrophobic Phase(SHP).

According to Gisch, Hunter and Feibush, SHP is a 5 um spherical silica based packing with 100 Å pore size. The stationary phase, bonded to both the outside and inside of the particles, is consisted of both polar and nonpolar groups. Different from the bimodal, internal surface reversed-phase(12-19), protein molecules are not necessarily excluded from penetrating into the pores. The proposed separation mechanisms, according to the above authors, may be due to the lack of interaction of the proteins with the stationary phase, thus eluting close to the solvent front, while small molecules, such as drugs and metabolites, may penetrate into the stationary phase and undergo hydrophobic interaction. Consequently, they are retained and would elute after the proteins.

From the chromatograms as shown by Figure 1, phenobarbital eluted with the retention volume of 7.6 mL, and a k' of 3.4 well resolved from the solvent front peaks. Drug-free serum did not show any interference. In choosing the mobile phase, the percent of organic modifier was selected as not to result in possible protein denaturation

and thus precipitation, and yet high enough as to elute phenobarbital with a k' of 3.4 in about 4 minutes. Total analysis time, including flushing the injector, may be maintained at about 6 minutes.

In order to evaluate the clinical efficacy of SHP, Table II shows the parameters during a limited seven weeks preliminary study. Thus far, the following characteristics have been established:the total number of injection at 180, volume of serum injected at 1800 uL. These were achieved without any change of retention volume and column. However, it was necessary to replace the guard column after 6.5 weeks, about 140 injections as a result of the peak height of some of the calibration standards were more than 10% lower than those at the beginning of the study. Thus, before adopting SHP for routine TDM, long term study may be required to establish the guidelines, and to understand the various factors on column life.

Based on our experience, the following guidelines have been proposed in the recent review for **Clinical Direct Sample Analysis**(1):

1. Establish the column life by noting the injection volume and number e.g. 1 uL for 1000 times.

2. Limit the analysis of a single group of drug to a 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 should be within 10%. Further, standards and quality controls should be placed at random position to check on system performance.

4. Because of possible multi-drugs therapy, patient samples may contain multi-drugs and metabolites. In order to ascertain the interested drug peak is not co-eluting with another drug/metabolite/endogenous substance, patient samples should be analyzed twice and in random order. If possible, photodiode array UV detection should be utilized to establish peak purity.

5. As a result of DSA, automation may be easily achieved in order to minimize exposure of the analysts. This may be followed by containment design - "closed system" such as those used in nuclear industries.

This preliminary study established the potential of SHP for clinical monitoring of phenobarbital. Due to its ease and small sample size, it may be desirable to explore the application of SHP for drug monitoring of neonatal and pediatric patients. However, long term study may be needed to establish its use for other drugs, and to delineate the possible separation mechanisms. Also, it would be important to study the effect of drug protein binding on separation.

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