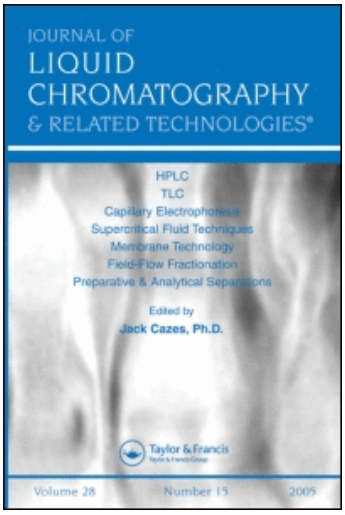

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Supercritical Fluid Chromatography for Therapeutic Drug Monitoring and Toxicology: Methodological Considerations for Open Capillary Tubular Column for the Analysis of Phenobarbital in Serum

Steven H. Y. Wong[a]; Sandy S. Dellafera[a]

[a] Drug Analysis Division Department of Laboratory Medicine, University of Connecticut School of Medicine, Farmington, Connecticut

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Original Article

SUPERCRITICAL FLUID CHROMATOGRAPHY FOR THERAPEUTIC DRUG MONITORING AND TOXICOLOGY: METHODOLOGICAL CONSIDERATIONS FOR OPEN CAPILLARY TUBULAR COLUMN FOR THE ANALYSIS OF PHENOBARBITAL IN SERUM

STEVEN H. Y. WONG
AND SANDY S. DELLAFERA
Drug Analysis Division
Department of Laboratory Medicine
University of Connecticut School of Medicine
Farmington, Connecticut 06032

Abstract

Methodological considerations are presented here for the application of SFC for clinical drug analysis using an open-tubular capillary column with polymethylsiloxane as the stationary phase. Preliminary studies of analysis of phenobarbital in serum showed that the use of liquid-liquid extractions, and recently introduced microfilters did not prevent rapid deterioration of column performance. Through systematic studies with solid-phase, C-18 extraction columns, a retention gap and a non-polar mixture of n-pentane/methylene chloride(25:75) for reconstituting the extracts, the feasibility was established. As a result of the lack of chromatographic interferences as shown by analysis of extract of drug-free serum, the procedure was used to estimate a patient's phenobarbital concentration of about 20 mg/L, comparable to a clinically established determination by fluorescence polarization immunoassay. Precision studies showed comparable mean concentrations for the measurement of quality control samples, but with marginally acceptable coefficients of variation. Preliminary extraction studies showed that other antiepileptics - phenytoin, secobarbital and pentobarbital were identifiable in SFC chromatograms.

Introduction

Supercritical Fluid Chromatography (SFC) offers a complementary analytical capability to gas liquid chromatography and high performance liquid chromatography, potentially applicable to clinical drug analysis. The major advantages of SFC would include the possibility of analysis of thermally labile drugs and metabolites, universal detection by using the flame ionization detector, possible high sensitivity by using microcolumn, differing selectivity from gas liquid chromatography and high performance liquid chromatography, and possibly faster method development. Recently, commercial instrumentations have become available for both packed and open tubular capillary columns. The feasibility of their application for clinical drug analysis may be evaluated. Thus, the purpose of this study was to assess the various methodological considerations involving in the application of one mode of SFC columns - open tubular capillary, including sample preparation and selection of chromatographic parameters. The findings may be readily extrapolated to SFC analysis by packed column with minor modifications. Having established the necessary methodological parameters, SFC assay was developed for therapeutic drug monitoring of phenobarbital, and the efficacy was established by comparison with the result from a clinically established method of fluorescence polarization immunoassay. Since barbiturate overdose is considered to be one of the major causes of poisoning, the phenobarbital protocol may also be adapted for the monitoring of other barbiturates for toxicological studies.

In the early eighties, SFC was demonstrated by Gere et al. by modifying a conventional HPLC (1). Several recent reviews (2-5), books (6,7) and two compilations of SFC chromatograms (8,9) have updated on the fundamental principles, instrumentational considerations and general applications of SFC. Since the methodology is newly established, its applications in the field of pharmaceutical and clinical drug analysis is rather limited. Later et al. (10) applied open tubular capillary column for the analysis of three classes of drugs - steroids, antibiotic and illicit drugs such as cannabinoids. Analyses were performed by using a methyl polysiloxane column, carbon dioxide as the mobile phase, and pressure programming. Without any derivatization, structurally identical epimers - dexamethasone and betamethasone were resolved at 130 ^{o}C to

minimize epimerization, whereas cortisone and hydrocortisone differing in the eleventh position by a keto functional group, were also resolved. Further, prednisolone in equine urine extract was detected, demonstrating its application in "real world" sample analysis. By using density programming, cannabinoid and six metabolites standards were resolved, and a human urine extract showed the presence of delta-cannabinoid, confirmed by an immunoassay. Elution densities were included for cocaine, phencyclidine, methaqualone, methadone, phenobarbital and propoxyphene. In applying SFC for antibiotics, a standard of oxytetracycline was analyzed without derivatization by using density programming. The authors concluded that capillary SFC offered rapid analysis, minimized sample preparation, and efficient separations such as in the analysis of isomeric, epimeric drugs. Crowther and Henion (11) demonstrated the SFC-mass spectrometric analysis of caffeine, codeine, cocaine, phenylbutazone and methocarbamol by using packed columns and a modified direct liquid-introduction interface. With a silica column, caffeine was eluted with CO_2/methanol(8/2) at about 2.3 min, as shown by a single-ion chromatogram with a m/z of 195. Codeine, eluted by using an amino column and CO_2/methanol(9/1) as the mobile phase, showed a mass spectrum(chemical ionization) with few fragments of 282 and 300 $(M+1)^+$, while cocaine, eluted similarly, showed few fragments of 182 and 304 $(M+1)^+$. Phenylbutazone and metabolites were analyzed by negative chemical ionization, while the analysis of thermally labile methocarbamol was performed with a low ion-source temperature of 140 °C. Lurie (12) demonstrated a novel combination of a size-exclusion HPLC with methylene chloride as the eluent, with a capillary SFC, and used it to identify a cocaine impurity. With the advent of supercritical fluid extraction, Nair and Huber (13) used a commercially available extractor, " SPA" from Milton-Roy (Riviera Beach, FL) to analyze caffeine, ibuprofen and an impurity in combination with HPLC. White et al. (14) used a converging-diverging restrictor for the analysis of cyclosporine, some ionophores and vitamins. In studying the elution of steroid isomers by the liquid crystalline biphenylcarboxylate esters polysiloxane stationary phase, Chang, Markides, Bradshaw and Lee (15) noted the correlation of elution order with the planarity of the molecules, with increased retention of the more-planar molecules. This finding may be applicable for the analysis of anabolic steroids abused in sports medicine. Smith and Sanagi (16) showed SFC analysis of barbiturates standards by using packed columns with polystyrene-divinylbenzene or C-18, and CO_2

with methanol as the modifier. Retention was markedly affected by the modifier content. The study did not include analysis of serum samples.

While the merits of open tubular capillary columns and packed column for clinical drug analysis remain to be established as in the applications in other areas, this study evaluated the open capillary column based on the assumption that the requirements for sample preparation and other technical considerations would be more stringent than those of packed columns. Thus, the findings would be readily transferable from the capillary columns to packed column analysis.

The first part of the study was concerned with the methodological considerations as follows: sample preparation by using the traditional liquid-liquid extraction, and by solid-phase extraction, the compatibility of solvent for reconstitution with SFC analysis, the use of microfilter to eliminate minute particles in the re-constituted extract. and systematic study of optimization of SFC parameter such as temperature, pressure or density programmings. Having established a protocol for phenobarbital, the effect of prolonged analysis of serum extracts on the performance of open-tubular capillary column was investigated. The long-term efficacy of coupling solid-phase extraction with SFC drug analysis was investigated by examining the precision, and by comparing measurement with a clinically established methodology, fluorescence polarization immunoassay, FPIA. Consequently, guidelines were suggested for enhancing SFC for clinical drug analysis of antiepileptics and others in biological samples.

Experimentals

I. Reagents - Methylene chloride, n-hexane, and n-pentane, Resi-analyzed grade, methanol, HPLC grade, and potassium phosphate, were obtained from J.T. Baker (Phillipsburg, NJ). Bond-Elut C-18 extraction column cartridges were obtained from AnalytiChem (Harbour City, CA). SF grade carbon dioxide was obtained from Scott Specialty Gases (Plumsteadville, PA). Microfilters were obtained from Rainin Inst. (Woburn, MA).

II. Drug standard solutions - To two separate 10 mL volumetric flasks, 10 mg of phenobarbital, or 40 mg of barbital, obtained from Sigma (St. Louis, MO), were added individually, followed by 10 mL of methanol. The resultant concentrations for phenobarbital and barbital were 1 and 4 ug/uL respectively.

III. Instrumentation - The SFC was consisted of a Model 500 SFC syringe pump from Lee Scientific (Salt Lake City, Utah), equipped with a pneumatic actuated Valvo-injector with a 200 nL loop. The pump was monitored by a Series 600 Controller, capable of both density and pressure programmings. SF CO_2 was filtered through a manifold equipped with a high pressure release valve. A model 5880 gas chromatograph from Hewlett Packard (Avondale, PA) was modified to provide the column compartment. The injector was affixed on top of the column compartment, with a column adaptor. The column or retention gap was connected to the injector through this adapter, with about 3 cm clearance from the top of the adaptor. The retention gap was connected to the column via a butt connector. The outlet of the column was also connected via a butt connector to a frit of 50 um i.d. The end of the frit was inserted into a capillary column adaptor into the flame ionization chamber. The column temperature, the detector and recording of the signal were controlled by the Hewlett-Packard monitor.

IV. An open tubular capillary column, SB-Methyl-100, 10 m in length and 50 um internal diameter, and a 5 m retention gap, consisting of deactivated 50 um i.d. fused silica column, were obtained from Lee Scientific.

V. Sample preparation - Two different approaches were attempted - liquid-liquid and solid-phase extractions. The first procedure was adapted from a clinical procedure previously developed for barbiturate monitoring by gas-liquid chromatography. Briefly, 1 ml aliquots of serum standards, quality control samples and patient sample serum were transferred into a series of marked tube, followed by adding 50 uL of internal standard solution of barbital. Then, potassium phosphate was added, followed by extraction with 2 mL aliquots of methylene chloride through rotation. After centrifugation, the organic layer was transferred for evaporation. The extract was re-constituted with 10 uL of methylene chloride for injection into the SFC for analysis.

The solid phase extraction protocol was modified from AnalytiChem. To 1 mL aliquots of drug-free serum were added 0, 20, 40, 80 uLs of the phenobarbital stock solution. The resultant concentrations were 0, 20, 40, and 80 ug/mL of serum. C-18 Bond-Elut cartridges were activated by passing 2 mL

aliquots of methanol and 0.1M potassium phosphate, pH=6.0. Then, 1 mL aliquots of standard, quality control, patient serum were mixed with 50 uL of barbital (4 mg/L), and 1 mL of phosphate. These mixtures were transferred into the cartridges, and eluted slowly by vacuum for at least 2 minutes. Washing was achieved by applying 1 mL aliquots of phosphate-methanol (8:2), and 1M acetic acid. After drying, the columns were rinsed by using n-hexane. Elution of phenobarbital and the internal standard was achieved by 2 mL aliquots of methylene chloride. For some procedure, after 1 ml of the organic phase was evaporated under nitrogen at 40 ^{o}C, it was transferred to microfilter reservoir. With centrifugation at 9,500 x g for 5 minutes, the filtered organic phase was collected in the lower tube. Further evaporation ensured. For the final protocol, methylene chloride from the above elution without filtration was evaporated. The residue was re-constituted in 10 to 15 uL of methylene chloride/n-pentane (75:25). About 5 to 8 uL was loaded into the syringe for SFC analysis.

VI. Chromatographic conditions - Separation temperature was isothermally maintained at 100 to 140 ^{o}C. Analyses were carried out by either density programming from 0.25 to 0.7 gm/mL at 0.02 to 0.05 gm/mL/min., or pressure programming from 100 to 300 atmos at atmos/min. Regeneration to the original density or pressure was usually achieved within 2 to 3 minutes. Injection loop was 200 nL, and the injection ratio of the split injector was about 20 to 1, indicating that the actual injected volume to be approximately 10 nL. Analysis time ranged from 10 to 13 minutes

Results and discussions

Using a traditional liquid-liquid extraction, SFC analysis of the extracts, as shown by Fig. 1A and B show that the internal standard eluted at about 6.3 min, and phenobarbital co-eluting with an interference peak at 7.6 min. In order to assess column performance, a " test-mix " of barbital and phenobarbital was injected before and after a given run. Fig. 2A shows that the SFC chromatogram at the beginning, with well defined and highly efficient separation. However, after analysis of only 6 extracts , column deactivation would result in an " unrecognizable " chromatogram. Such deactivation may be due to the injection of minute particles in the extract, and/or incompatibility of solvent

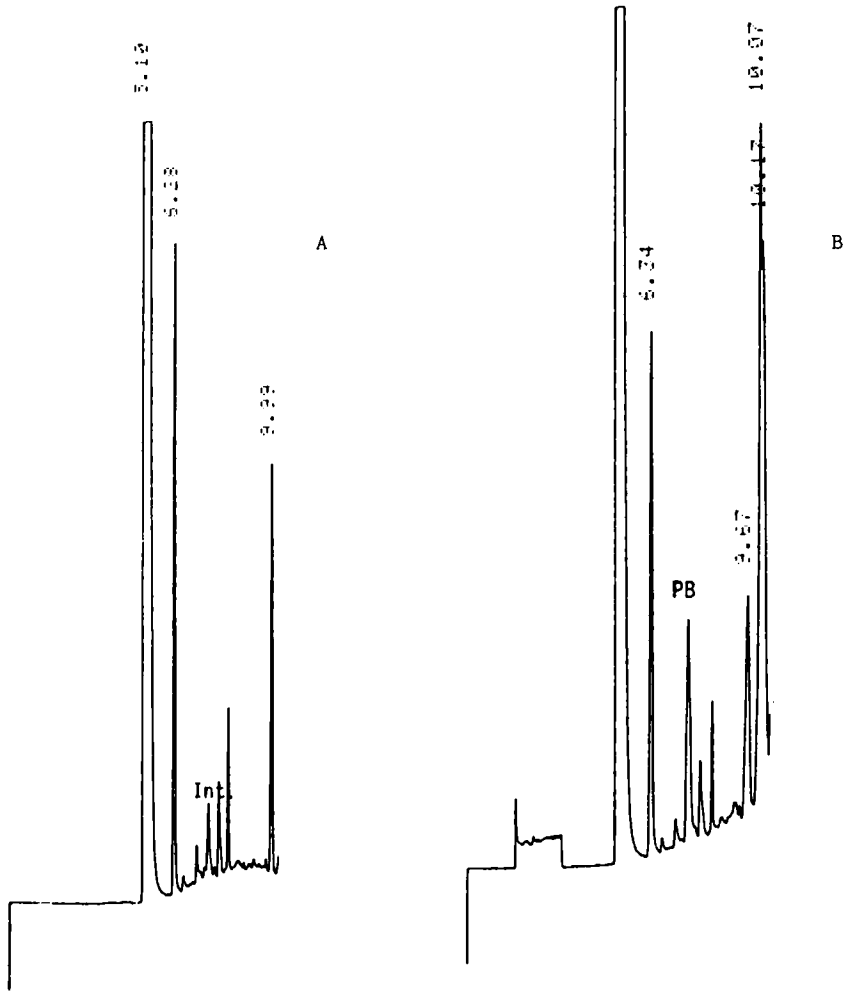

Fig. 1. SFC Chromatograms of extracts of: (A). a drug-free serum with IS added., and (B). a serum standard containing 20 mg/L of phenobarbital. SFC conditions - MP-CO_2, Column - SB-Methyl-100, Temperature - 120 ^{o}C. Pressure gradient - 100 to 300 atom. at 20 atom./min. Peak identification : IS - barbital, 6.3 min, and PB, phenobarbital, 7.3 min, co-eluting with an interference peak.

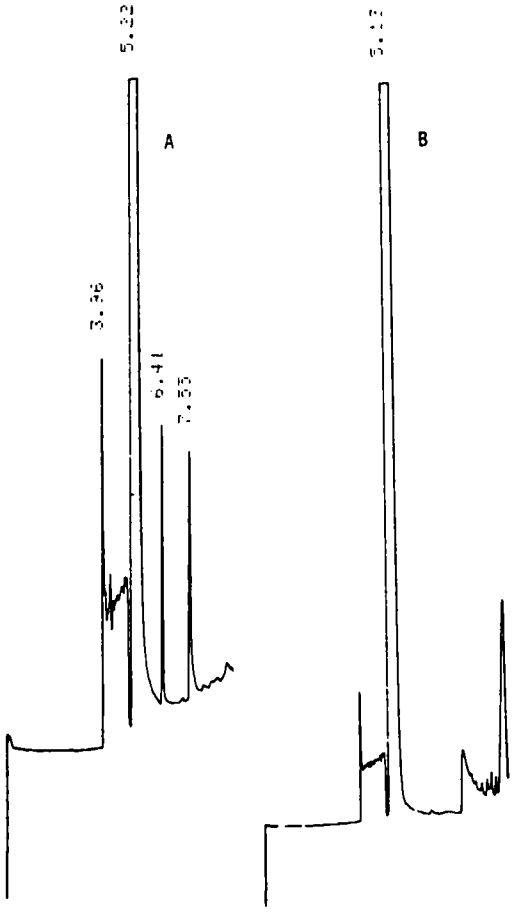

Fig. 2. SFC chromatograms of a " test-mix " of barbital and phenobarbital standards: (A). at the beginning, and (B). at the end of a run with about 6 serum extracts. Deterioration of column performance is clearly evident.

mixtures with the SFC mobile phase. In checking the first possibility, recently introduced microfilters were used for filtering the methylene chloride extracts.

In a series of experiments, the use of microfilters would result in small interfering chromatographic peaks as shown by Fig. 3 A and B. In an attempt to eliminate these small peaks, microfilters were " washed '" with either n-hexane or methylene chloride overnight. However, this step did not eliminate the interference problem. Thus, the use of microfilter was discontinued.

Due to the rapid column deterioration as described above with the traditional liquid-liquid extraction, solid-phase extraction of phenobarbital was adapted from a procedure from AnalytiChem. In addition, a series of solvent and mixtures to be used for re-constitution later on, was checked for compatibility with the SF mobile phase. The solvents included: methylene chloride, methanol, n-hexane, n-pentane, and mixtures of these solvents. Conceptually, the open tubular capillary columns packing with polysiloxane may be regarded to be more comparable to normal phase than reversed-phase HPLC columns. Thus, the chosen solvent/mixture for reconstitution should be as non-polar as possible, with just the desired polarity of dissolving barbital and phenobarbital in the extracts, while preferentially " leaving behind " the polar components of the extracts. After a systematic study, the chosen mixture was n-pentane/methylene chloride (25:75).

In further protecting the column, a retention gap of deactivated silica was placed into the Injector adaptor, and connected to the column with a butt connector. The rationale of using the retention gap was recently reviewed by Lee et al. (17). The advantage may be the possible focusing effect - separating the analytes - the barbiturates, from the solvent. Further, the retention gap may also function as a guard column as in HPLC. In the event of retention gap contamination, our experience showed that periodic cutting of about 15-20 cm of the retention gap would most likely restore column performance.

By following the above protocols, the analysis of a drug-free extract, as shown by Fig. 4 shows a " clean " baseline, while Fig. 5 shows that a patient's phenobarbital peak corresponding to about 20 mg/L. That the protocol was adequate was verified further by the reproducible " test-mix " chromatograms before and after analyses of 12 serum extracts, as shown by Fig. 6A and B.

The precision of this procedure was established by repetitive SFC analyses of a series of samples containing phenobarbital. The precision data are

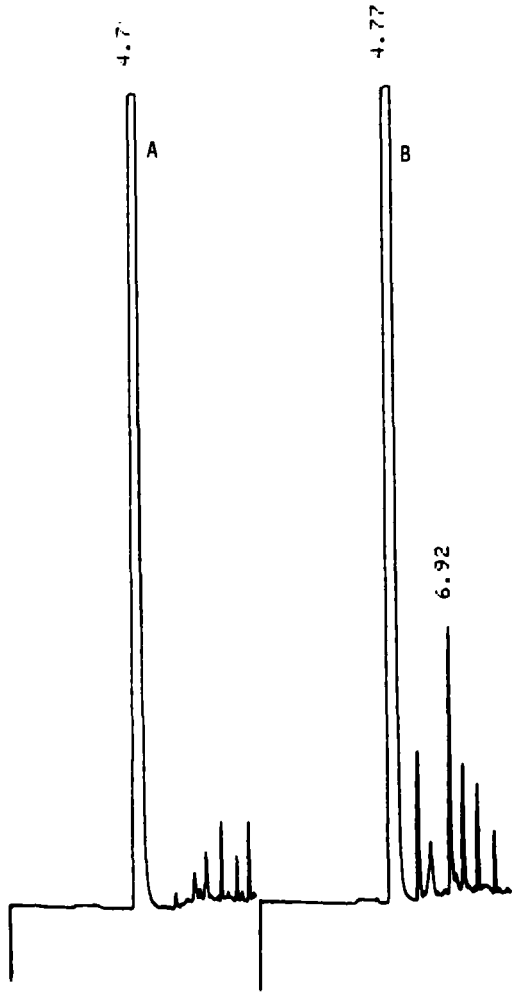

Fig. 3. Effect of microfiltration on SFC Chromatograms of extracts of: (A). drug-free serum, and (B). serum with about 20 mg/L of added phenobarbital and internal standard. Peak identification: phenobarbital, 6.92 min.

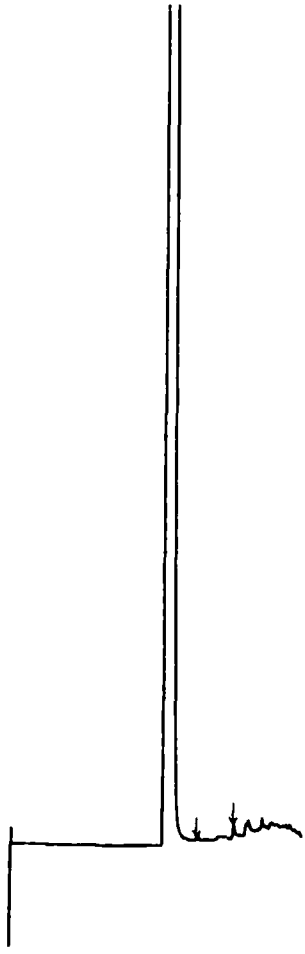

Fig.4. SFC Chromatogram of a drug-free serum extract obtained by solid-phase extraction, followed by SFC - density gradient analysis. SFC parameters - SB-Methyl-100, 120 ^{o}C, CO_2, 0.25 to 0.6 gm/mL at 0.02 gm/mL/min, and timed split injection. Arrows indicated the positions of the IS and phenobarbital peaks.

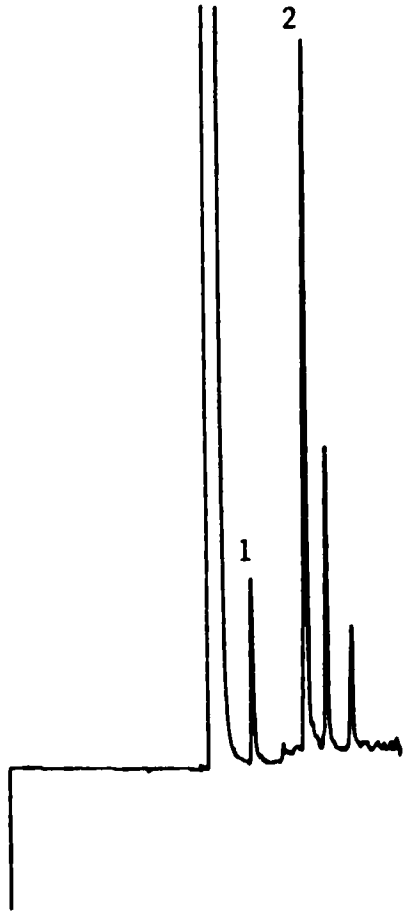

Fig. 5 SFC Chromatogram of a patient serum extract by solid-phase extraction. Peak identification: 1. IS, 5.6 min and 2. PB, phenobarbital (20 mg/L), 6.8 min.

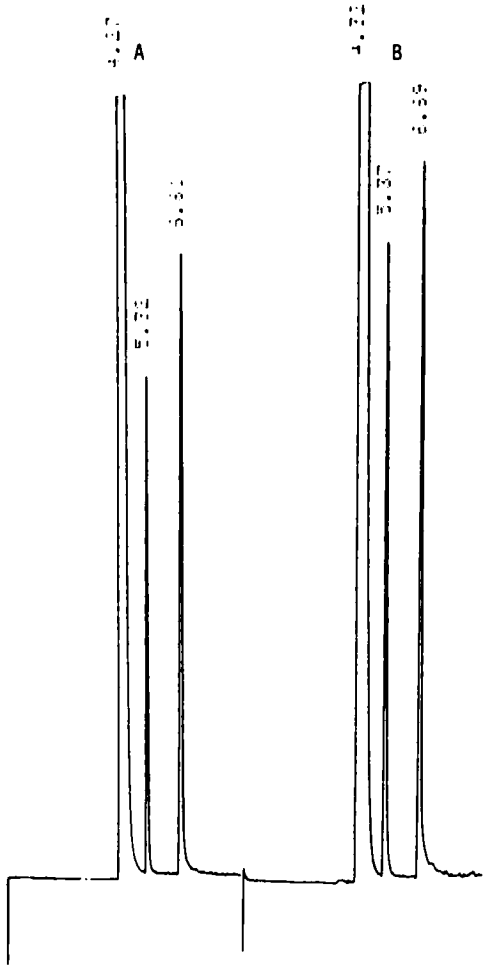

Fig. 6. SFC chromatograms of test-mix of IS, barbital, and PB, phenobarbital: (A). at the beginning, and (B). at the end of a run with a total of 12 serum extracts. Peaks identification: IS, 5.3 min and PB, 6.7 min.

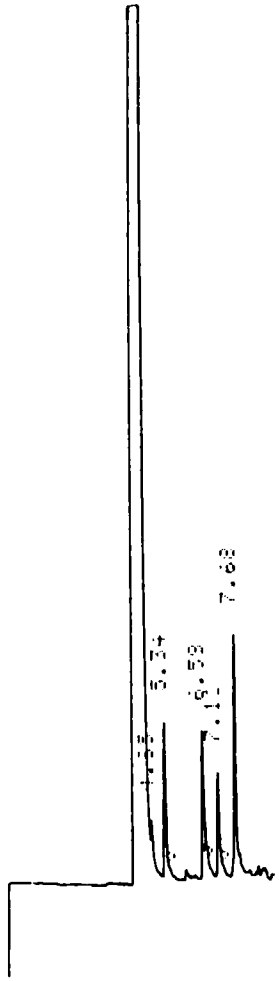

Fig. 7. SFC Chromatogram of serum extract of a commercial " QC " containing about 20 mg/L of phenytoin (7.68 min) and other antiepileptics - Extraction and SFC parameters similar to those of phenobarbital as in Fig. 5, except column temperature was 140 ^{o}C.

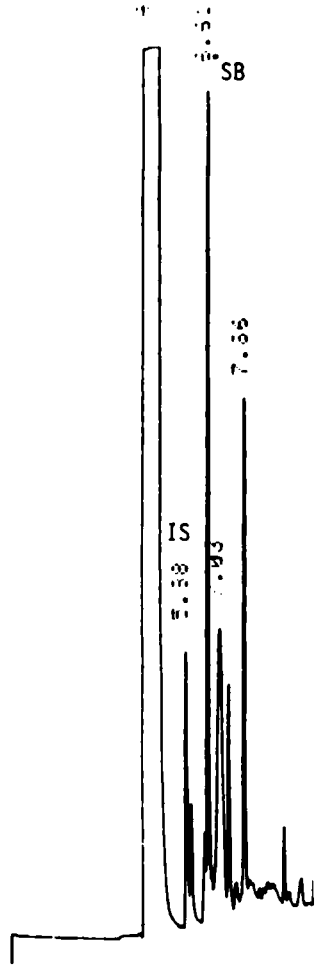

Fig. 8. SFC chromatogram of extract of a serum sample with added secobarbital. SFC parameters: SB-methyl-100, 120 °C, CO_2, 100 to 300 atom. at 20 atom./min., and split injection. Peak identification: IS - barbital, 5.9 min, and SB, secobarbital, 6.6 min.

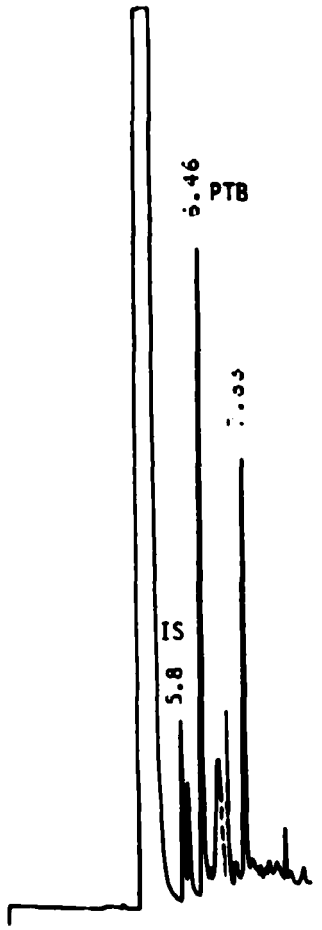

Fig. 9. SFC chromatogram of extract of a serum sample with added pentobarbital. SFC parameters: similar to those of Fig. 8. Peak identification: IS - barbital, 5.8 min, and PTB, pentobarbital, 6.5 min.

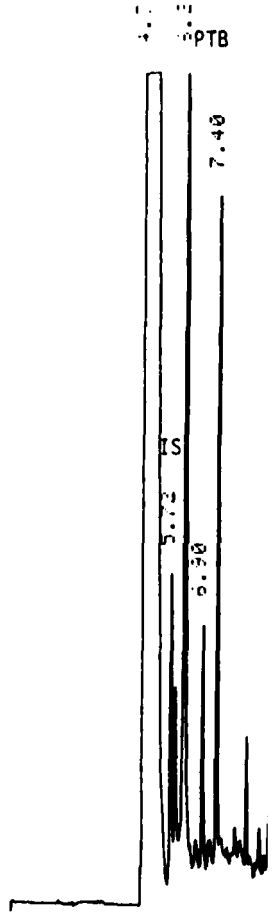

Fig. 10. SFC chromatogram of extract of a serum sample with added pentobarbital. SFC parameter: - similar to those of Fig. 8, except using density programming - 0.25 to 0.7 gm/mL at 0.05 gm/mL/min. Peak identification: IS - barbital, 5.7 min, and PTB, pentobarbital, 6.2 min.

Table I: Precision Studies for SFC Assay of Phenobarbital. (Concentration estimated by clinically used Fluorescence Polarization Immunoassay by TDx = 25.8 mg/L.).

Modes	Mean	SD	CV%	n
Within-run	25.8 mg/L	1.79 mg/L	6.9%	5
Day-to-day	23.8 mg/L	3.09 mg/L	12.9%	13

outlined in Table I. While the means are comparable to those of FPIA, the coefficients of variation were only marginally acceptable.

This procedure was used to carry out preliminary analyses of other anticonvulsants : phenytoin as shown by Fig. 7, secobarbital by Fig. 8, and pentobarbital by Fig. 9 and 10.

Conclusions

For drug analysis with SFC open tubular capillary columns, conventional liquid-liquid extraction of barbiturates in serum is not compatible, as shown by the drug interference peaks, and the rapid loss of column efficiency and selectivity. Our experience show that solid-phase extraction is compatible, and is thus recommended for the clinical drug analysis by SFC open tubular capillary column. The performance of the column of this study - SB-methyl-100, is normal-phase-like as in HPLC. This is important in the design of the extraction protocol, and in the maintenance of the column. For example, reconstituting with less polar methylene chloride/n-pentane mixture (75:25), as compared to the more polar methylene chloride, would minimize loading the column with polar extract-components, and possibly extending column life, and assay reproducibility. This may be useful for general biological and biomedical analysis. Guideline useful for routine SFC clinical drug analysis include:

1. Use test mix to check on the column performance before and after each run.
2.. Estimate the number of injections before cleaning injector, and cutting retention gap/column. Peak height ratios of the test-mix would serve as a useful guide.

Preliminary studies showed the use of microfilter introduced chromatographic interference peaks which are not " removable " by CH_2Cl_2 or hexane washing of the microfilters. Thus, its use is not recommended.

This preliminary study the feasibility of SFC analysis of phenobarbital analysis by open tubular capillary SFC column. It would be important to improve on the precision, and to carry out comparison of a large number of patient samples of at least 20 to 30. Further, preliminary studies also showed feasibility of SFC analysis of secobarbital, pentobarbital and phenytoin in serum.

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