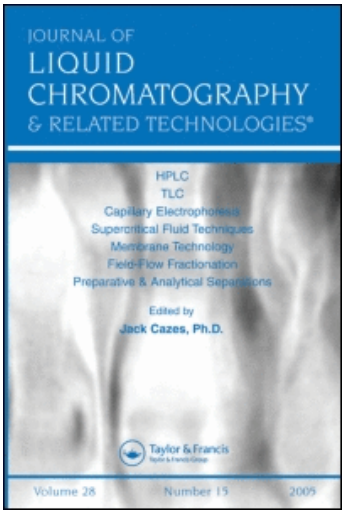

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Z. K. Shihabi[a]
[a] Department of Pathology, Wake Forest University The Bowman Gray School of Medicine, Winston-Salem, North Carolina

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REVIEW OF DRUG ANALYSIS WITH DIRECT SERUM INJECTION ON THE HPLC COLUMN

Z. K. Shihabi
Department of Pathology
Wake Forest University
The Bowman Gray School of Medicine
300 S. Hawthorne Road
Winston-Salem, North Carolina 27103

ABSTRACT

Contrary to the expectations of the past decade, routine therapeutic drug monitoring by HPLC failed to expand. Although more extensive to perform, immunoassays have dominated the market for drug analysis in the last few years. This is attributed to the lengthy sample preparation needed for HPLC. However, HPLC remains the main method for therapeutic drug monitoring of the uncommon drugs and their metabolites.

The method of direct serum injection on the column simplifies HPLC as an analytical technique and enables full automation. Advantages and disadvantages of this method of analysis are discussed. This review tries to summarize the different approaches for direct serum injection on the HPLC column: 1) Guard columns, 2) Micellar chromatography, 3) Internal surface reversed-phase chromatography, 4) Column switching, and 5) Wide-pore (protein) columns.

With direct serum injection, the pH of the mobile phase is very critical for separating the drug of choice from the endogenous substances found in serum and for attaining good recovery, especially for drugs which are tightly bound to

proteins. The percentage of the organic solvent in the mobile phase is another important factor in reducing serum protein precipitation at the column inlet.

Generally, direct serum injection decreases column life on one hand but saves labor time and cost on the other hand. Both the scientific literature and the commercial column supplies emphasize the protection of the column rather than the simplification of the procedure. However, declining column prices, especially for cartridge columns, might encourage direct serum injection. Further applied research in this area is needed.

INTRODUCTION

Because of its rapid growth over the last 15 years, therapeutic drug monitoring represents a significant and essential part of the clinical lab assays today. The continuous introduction of new drugs will keep this field constantly growing well into the future. Many drugs have a narrow therapeutic window which necessitates blood level monitoring to avoid toxicity. The difficulty of sample preparation prevented HPLC from attaining a large share of therapeutic drug monitoring in the clincal lab.

The aim of this review is to draw attention to the importance of this neglected area of applied research and to show the different approaches for direct serum injection on the HPLC column.

Historical:

Methodology for drug assays has changed greatly over the years. Twenty years ago, drug analysis, limited essentially to

phenobarbital and to phenytoin, was based on solvent extraction followed by ultraviolet spectrophotometry. In the early 70s, gas chromatography was commonly used as the number of assayed drugs greatly increased. In the mid-70s, HPLC became a common procedure for drug analysis. Ease of instrumentation and ease of sample preparation relative to that of gas chromatography were the main attractions to HPLC (1). A book completely devoted to drug analysis by HPLC has been published (2). However, the popularity of the HPLC for drug analysis did not last too long. In the last 7 years, immunoassays have become very popular and have replaced most HPLC methods for drug analysis. The driving forces behind this change are speed, automation, and the absence of sample preparation. Immunoassays have become attractive for stat work. HPLC is still used for analysis of the less common drugs where immunoassays have not been developed and is also used for the assay of drug metabolites in pharmaceutical research. HPLC will remain as a reference methodology.

Problems of Drug Analysis by HPLC:

Rapid analysis with minimum number of steps is of great importance in clinical labs. Lengthy procedures also tend to have greater imprecision. In the last few years, there has been great interest in speeding up the HPLC analysis by using shorter columns, e.g. 50 mm columns packed with 5 or 3 μm particles. However, sample preparation for the HPLC remains a major problem.

SAMPLE PREPARATION

Sample Extraction:

Liquid extraction followed by solvent evaporation remains the traditional method for sample preparation used in drug analysis. Recently, solid-phase extractions are being used more often in the labs. While the material of interest is absorbed to the solid-phase, many of the interfering substances can be selectively removed from the sample by using the appropriate mobile phase (3,4). Both clean-up and concentration of the sample are accomplished by this method. Solid-phase extraction for some compounds produces cleaner extracts when compared to liquid extraction. Furthermore, solid-phase extraction offers more choices for extraction and clean-up of the sample, e.g. reversed-phase, normal phase or ion-exchange chromatography.

Sample concentration by evaporation remains an essential element in this method. Sample extraction by either the liquid or solid-phase methods requires time and skill. Additionally, recovery can be very low, especially for drugs with basic characteristics and those with a low-boiling point. An automated system for sample solid-phase extraction has been described (5) along with a few commercial systems. In the long run, robotics may help in automating sample preparation for HPLC. These instruments are getting more widespread in industrial labs compared to clinical labs. At the present time, the expense and the complicated software needed for operating these instruments

are the two major drawbacks that inhibit the use of robotics in the clincical labs.

Serum Deproteinization:

Serum deproteinization before injection on the column is used frequently in drug analysis by HPLC because of the simplicity of this method. Deproteinization can be performed by alcohols and organic solvents such as methanol, ethanol, acetonitrile, and less commonly by acids, such as trichloroacetic or perchloric acid. Serum deproteinization adds extra steps and causes sample dilution. Co-precipitation of the drugs with proteins leads to low recovery (6).

Direct Serum Injection on the Column:

Serum can be injected directly on the HPLC column. However, proteins are present in serum at very high concentrations (60-80 g/L) relative to the serum concentrations of the majority of drugs (1-60 mg/L). Two main problems are associated with direct serum injecton on the column: First is the tight adsorption of some proteins to the column. Second is the rapid pressure buildup at the head of the column due to protein denaturation and absorption. Protein denaturation and precipitation occur as a result of the high concentration of organic solvents in the mobile phase used for elution from the reversed-phase columns, especially the C_{18} columns. Another condition favoring protein

denaturation and precipitation is a mobile phase pH close to the pK of the protein. Serum protein precipitation increases greatly in the pH range of 3-7. It is also time and temperature dependent. As a result of protein precipitation and adsorption, resolution is lost and column-life is greatly shortened (7,8). Chromatograms by direct serum injection on the column are not clean compared to those by solvent extraction; however, from a practical point of view, they are useful for quantitation.

Ion-exchange columns do not require organic solvent for elution. They are slightly less subject to protein adsorption and denaturation compared to the reversed-phase columns. Cation exchange columns were used for about 500 serum samples with direct serum injection on the column for theophylline assay (9).

Methods to Avoid Protein Precipitation on the Column:

Guard column: The use of guard column was one of the early attempts to lengthen the analytical column life (8). After 30-50 injections it was found that the inlet pressure increases from 1500 to 2800 psi. However, when the guard column is changed the pressure dropped back to 1500 psi (8). The precolumn traps the denatured, as well as the tightly adsorbed proteins.

Column switching: After direct serum injection on a precolumn or a different column, the segment of interest is switched to the analytical column (10,11). The recovery and reproducibility in this technique are good, however, this greatly

complicates the instrumentation. The details, advantages and disadvantages of this method have been reviewed recently (11,12). A manual switching technique has been recently described for the determination of anticonvulsant drug in which a small polymeric column is used in place of the injector loop to clean the sample. The type of column in the injector loop was very important in obstaining good recovery which was above 90% and provides results in about 5 min (13).

Internal surface reversed-phase: A special column where small molecules are allowed access into the inside small pores, 53 Å, while excluding the large molecules. The column has a hydrophilic diol-glycine groups on the external surface layer which absorbs very little protein and hydrophobic tripeptide partioning phase (gly-phe-phe) on the internal surface. Drug analysis by direct serum injection using this column has been described (14,15,16). The π electron interaction plays the major role in solute retention in this column (16). The drug seems to be released from the binding protein in the eluting solvent, especially in the presence of organic solvents with recovery close to 100% (16). The column is commercially available (Regis, Morton Grove, IL). It is more expensive than the common C_{18} column, and cannot be used with mobile phases containing more than 25% organic solvent.

Micellar liquid chromatography: Surfactants, such as sodium dodecyl sulfate at concentrations of about 20 mmol/L were used in

the moble phase to elute several drugs such as acetaminophen, propranolol, phenobarbital, chloroamphenicol quinidine using both fluorescence and ultraviolet detection (17,18). Surfactants at low concentrations have the property of solubilizing proteins thereby allowing direct serum injection on the HPLC column and also displacing the drugs from the binding proteins (17-20). Unfortunately, some surfactants give high absorbance readings at short wavelengths which limits their use. Also, we have experienced an increase in column pressure at a high concentration of surfactants. Drugs tightly bound to serum protein give more than a single peak unless the pH is carefully chosen (21). Under the proper conditions, the recovery of such drugs is close to 100% (21).

Wide-pore (protein) columns: Drugs, as well as other small molecules are traditionally analyzed on columns packed with particles containing small pores (80-100 Å). In general, these particles provide higher plate numbers than particles with large pores (200-300 Å) because of the increased surface area. However, the latter columns are more suited for separation of proteins and peptides due to the greatly improved mass transfer of such solutes (22). Pore size does not affect retention time or peak height for proteins (23). Large proteins can permeate through the large pores without being hindered. These columns characteristically have low inlet pressure and lower theoretical plate numbers than comparable ones packed with the small pore

particles. Of course, plate number can be increased simply by using either longer columns or smaller particle size. From a practical point of view, we found a 150 mm X 4.6 mm (I.D.) column packed with 6.5 μm particles, 300 Å pore size, to be adequate for most drug analyses (24). However, some drugs may require a longer column, e.g. 250 mm to achieve the separation. Figures 1 and 2 illustrates the assay of theophylline using a wide-pore column and the comparison to an immunoassay.

With direct serum injection on the column the pH of the mobile phase, the type of column resin, and the percentage of organic solvent are very critical factors which require special attention. For example, in the case of phenobarbital and of pentobarbital, a basic pH was found to enhance the absorption at 254 nm (25). In the case of quinidine elution with phosphoric acid, a pH of 2.2 enhances the absorption of this drug at 254 nm. Thus the drug will have enhanced sensitivity relative to the endogenous substances. To avoid unnecessary protein denaturation, the percentage of organic solvent is an important consideration, as well as the proper column type selected. For example, a C_1 column can be used for determination of highly hydrophobic compounds.

Drugs present in high concentrations (>1 mg/L), especially those with strong ultraviolet absorption (above 250 nm), are suitable for direct serum injection. This includes the majority of the common drugs that are monitored in the clinical lab, e.g.

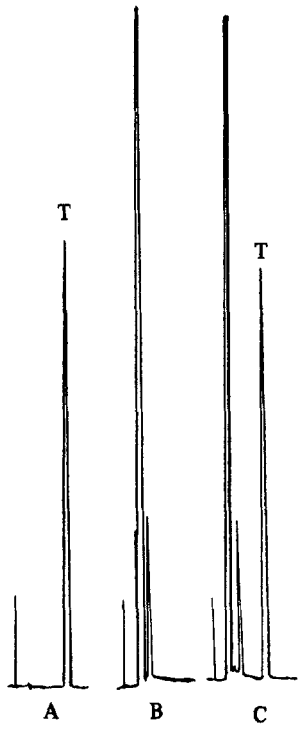

Figure 1. Representative chromatogram of direct serum injection for theophylline (T) assay: A- Theophylline std 20 mg/L. B- Serum sample from a patient free from theophylline. C- Serum sample from a patient on theophylline (time for T is 4 min).

The column is 150 mm X 4.6 mm packed with C_{18} 6.5 µm average particle size, 300 Å pore size (SynChrom, Inc, Linden, IN) and eluted with acetonitrile 60 ml/L of 32 mmol/L acetic acid. Absorbance was set at 280, 0.010 A.

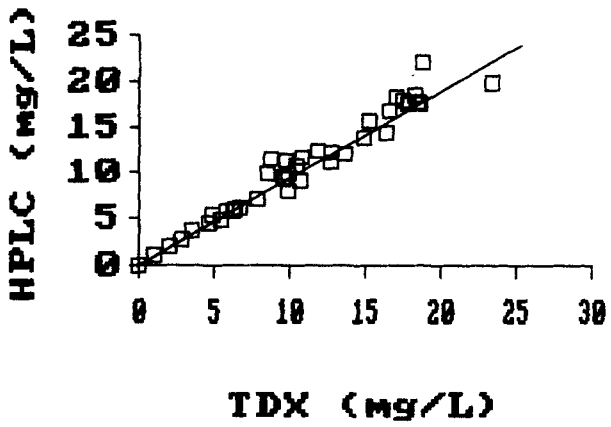

Figure 2. Comparison of theophylline (mg/L) assay by direct serum injection and a polarized fluorescence immunoassay method.

theophylline, dyphylline phenobarbital, pentobarbital phenytoin, carbamazepine, procainamide, quinidine, salicylate, and acetaminophen. Drugs which can give absorbances greater than 0.010 A by injection 5 µL of sample, at wavelengths above 250 nm, will be suitable for assay by direct serum injection. The optimum pH, column type, and organic solvent have to be ascertained for each drug. Of course, drugs which are present in very low concentrations (<1 mg/L) or have low absorptivities will require sample concentration or the use of different detectors such as fluoresence or electrochemical.

Direct serum injection on the column shortens column life. However, simple maintenance will increase and improve the column life. The steel inlet filter of the column periodically requires

sonication in concentrated acid to clean and remove precipitated proteins. The inlet filter of the guard column can be removed completely to avoid frequent clean up (8). The column requires washing with 100% acetonitrile every 50-100 injections. The use of a guard column with any of the previous approaches greatly increases column life (8).

In general, commercial protein columns are expensive because of supply and demand factors. However, such columns can be cheaply packed in the lab thereby lowering the cost of drug analysis by HPLC to $0.10 per sample compared to $1-3 per sample by immunoassays. Furthermore, an autosampler can be added to automate the analysis. Since there is no sample handling involved, internal standard addition is not essential but desirable. Under the proper assay conditions recovery of the drug is closed to 100%.

CONCLUDING REMARKS

Both the scientific literature and the commercial column suppliers emphasize the protection of the column rather than the simplification of the procedure. No doubt, the column life is shortened with direct serum injection; however, avoiding sample extraction saves money and time which will compensate for the price of the column. Probably in the future, as column prices decline further, more methods for drug analysis by direct serum

injection will be described. Further studies are needed for the combined effects of columns with wide-pore particles and mobile phases containing surfactant. Monitoring at two wavelengths will be helpful in direct serum injection to check for the peak purity. The substitution of ion-exchange and medium polarity columns for the more common C_{18} columns also probably will increase in the future. The ease of direct serum injection will justify future research in this area.

Direct sample injection on the column is not restricted to drugs or to serum. It can be used for endogenous substances, tissue homogenates (20), and different fluids, such as cerebrospinal fluid or urine (17). For example, we have successfully used direct urine injection for the routine assay of homovanillic acid in urine for several years without any problems (26).

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