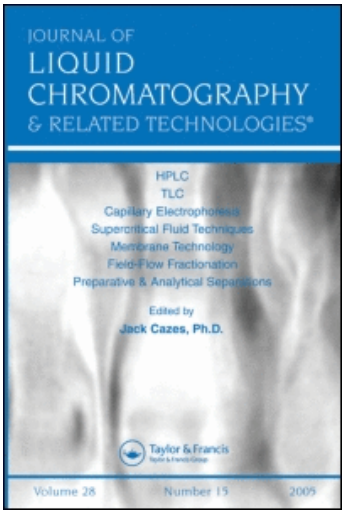

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Serum Injection on the HPLC Column for Pentobarbital Assay

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To cite this Article Shihabi, Z. K. , Dyer, R. D. and Scaro, J.(1987) 'Serum Injection on the HPLC Column for Pentobarbital Assay', Journal of Liquid Chromatography & Related Technologies, 10: 4, 663 — 672
To link to this Article: DOI: 10.1080/01483918708069017
URL: http://dx.doi.org/10.1080/01483918708069017

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(4), 663-672 (1987)

SERUM INJECTION ON THE HPLC COLUMN FOR PENTOBARBITAL ASSAY

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ABSTRACT

Serum is injected directly on a polymeric PRP-1 (5 μm) column for pentobarbital assay and eluted with 12% acetonitrile in 15 mmol/L phosphate buffer, pH 11.8. The pentobarbital peak is well separated from other endogenous substances and common drugs in the serum.

Serum protein precipitation is shown to depend on several factors, such as, the concentration of acetonitrile in the mobile phase, the pH of the buffer, and the amount of sample injected. Serum protein precipitation can be minimized for the weakly acidic organic compounds, such as, pentobarbital and phenobarbital by injecting a small volume of sample onto a polymeric reversed-phase column at a highly basic pH. Column resolution and pressure did not change after 300 injections.

The method is simple enough for stat work and for full automation. This method can be extended to the assay of other weakly acidic organic compounds.

INTRODUCTION

Direct serum injection on the HPLC column eliminates the need for an extraction step, thereby simplifying the technique and rendering it more suitable for full automation. Furthermore, the reproducibility of the assay is improved. However, direct serum injection leads to rapid pressure build-up and deterioration of the column resolution due to protein precipitation.

Here we describe one approach to avoid or minimize protein precipitation that is applicable to certain a class of substances, particularly the weakly acidic organic compounds. These compounds are eluted off from a polymeric reversed-phase column by a very basic buffer containing a minimum amount of organic solvent, thereby avoiding serum protein precipitation.

Here we use the pentobarbital determination as an example to illustrate how the method can be simplified. Pentobarbital is a short acting, hypnotic barbiturate. It is often given in high doses to lower intracranial pressure in cerebral accidents (1,2,); however, the serum level has to be regulated in a narrow range (3,4).

MATERIALS AND METHODS

We used a chromatograph consisting of a Beckman 110 A pump (Beckman Instruments, Fullerton, CA), with a Rheodyne injection valve (Rheodyne, Cofafi, CA) connected to a Micromeritics variable wavelength detector, Model 785 (Micromeritics, Norcross,

GA) set at 0.005 nm. The developmental work was performed on a 150 x 4.6 mm column packed by the slurry technique with 10 µm PRP-1 particles (Hamilton, Reno, NV). For routine clinical work, we used a 150 x 4.6 mm column packed with 5 µm PRP-1 particles.

In the routine analysis of pentobarbital, 2 µL of serum was injected on the column and eluted with freshly prepared mobile phase that contain 12% acetonitrile in 15 mmol/L phosphate buffer at pH 11.8

Nephlometry was performed on a Perkin-Elmer Model 91 amylase-lipase analyzer (Coleman Instruments Division, Oak Brook, IL).

RESULTS AND DISCUSSION

Serum proteins precipitate with increasing concentrations of organic solvents (Fig. 1). Acetonitrile more effectively precipitates serum proteins than methanol, especially at concentrations greater than 30%. This precipitation depends on the pH especially at neutral pH, which is close to the pI of the majority of serum proteins (Fig. 1). Certain buffers (5,6), such as acetate buffer, are more effective as precipitant for serum protein. Furthermore, this precipitation is a function of time (Fig. 2), and the amount of sample used.

The most important factor in avoiding serum protein precipitation is to minimize the concentration of organic solvent used in the mobile phase. Pentobarbital requires 40%

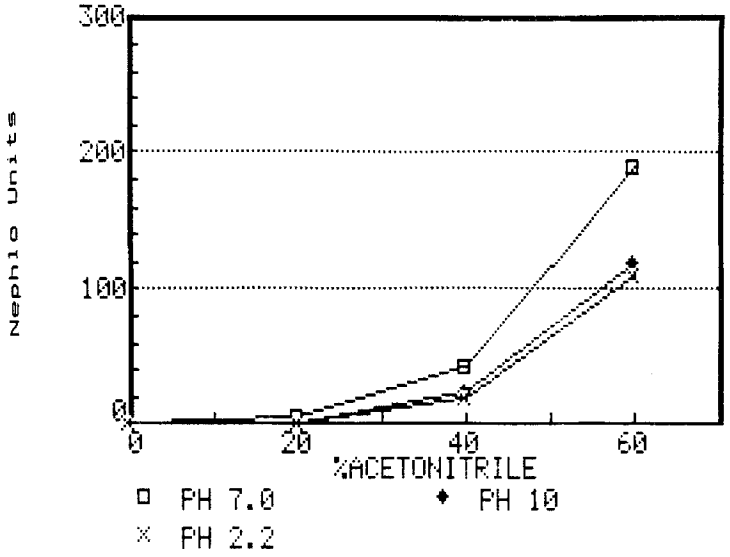

Figure 1. Serum turbidity as measured on a nephlometer. Serum 50 µL was added to 3 ml of different concentrations of acetonitrile made in buffers (25 mmol/L) of different pH: pH 7, phosphate; pH 2.2 phosphate buffer; and pH 10, NH_4OH/Na_2CO_3.

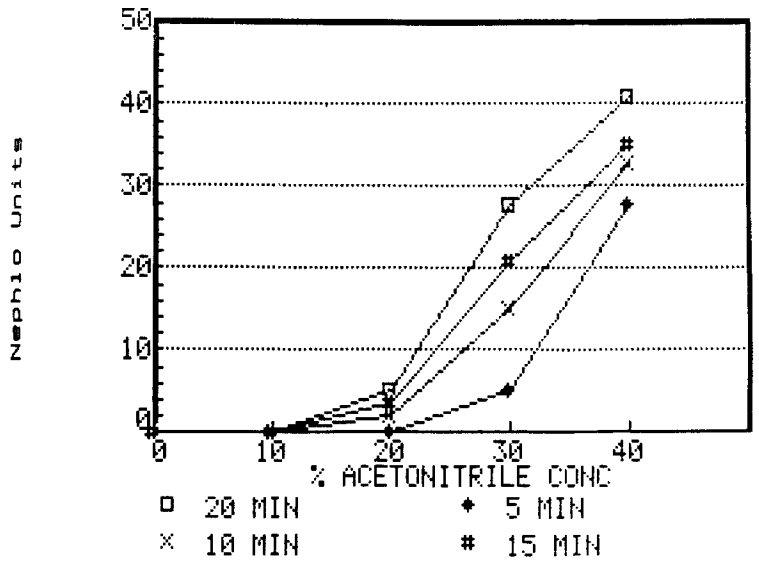

Figure 2. Effect of time on serum turbidity at different acetonitrile concentration made with phosphate buffer 50 mmol/L, pH 7.0.

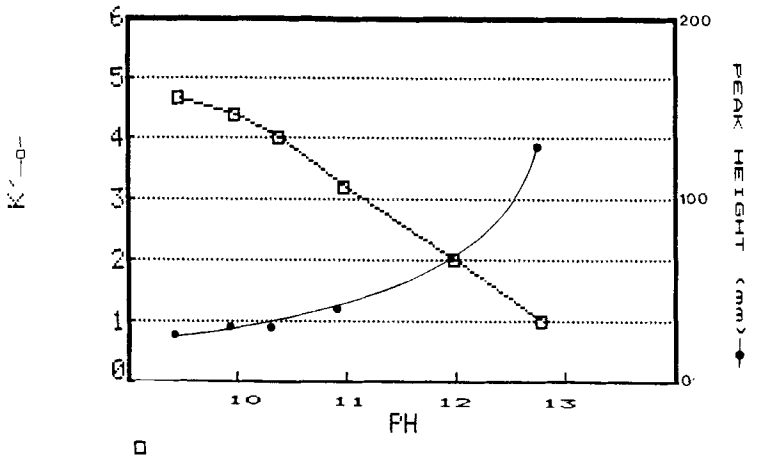

Figure 3. Effect of pH on the capacity factor K' and peak height for pentobarbital.

acetonitrile at pH 6.7 for elution, but only 12% acetonitrile at pH 11.8 (Fig. 3 and Fig. 4). Consequently, it is advantageous to elute this drug with a highly basic buffer (i.e., pH 11.8) in the mobile phase to avoid the use of a high concentration of organic solvent which causes serum protein precipitation. Under these conditions, silica-based columns deteriorate rapidly, however, the polymeric resins such as PRP-1 can withstand such effects.

Barbiturates in general have a much stronger absorption at 254 nm in basic buffers (7) compared to acidic buffers (Fig. 4), thus less expensive detectors can be used for this assay. Also a smaller amount of sample is injected on the column (2 μL), leading to much longer column life. If an acidic pH is used it is not possible to detect the therapeutic concentraitons at 254

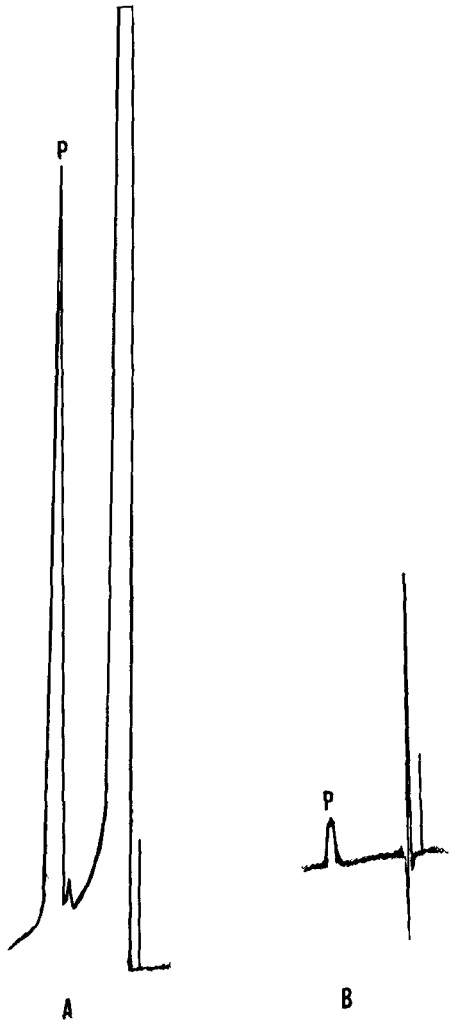

Figure 4. Pentobarbital aqueous standard (50 mg/L) injected directly on the column with detection at 254 nm eluted in: A - with 12% acetonitrile in phosphate buffer 15 mmol/L at pH 11.8 and B - in 40% acetonitrile in phosphate buffer 15 mmol/L pH 6.7 (P, pentobarbital peak, time for P = 6 min).

nm with direct serum injection. Instead, absorption at 200 nm is needed, but at that wavelength the pentobarbital peak is masked by many interfering compounds present naturally in the serum.

For routine analysis, we found that pentobarbital by direct serum injection on the column at pH 11.8 is well separated from other compounds in the serum (Fig. 5). Furthermore, we did not find any interferences from other barbituates or from other common drugs such as phenytoin, tegretol, theophylline and carbamazepine. The test is linear between 5-50 mg/L. The between-run coefficient of variation over a 30-day period of time is 8.6% (mean 19.8; N = 21). Since there is no sample preparation, the test is suitable for emergency work as well as for full automation.

In addition to the problem of protein denaturation in the column with organic solvents repeated injection of serum proteins directly on the column eventually results in adsorption of some of the serum proteins, as well as nonpolar compounds, on the inlet of the column. We tried several methods to remove the build-up of these proteins, all of which seemed to work well. For example, every 50 samples, or whenever there is increased column pressure, the first few millimeters of the resin of the inlet is removed and replaced with fresh particles. We also tried to reverse the direction of the column every 50 injections. A pre-column would also be effective in trapping serum proteins. After 300 sample injections, we did not experience any pressure build-up or deterioration in column performance.

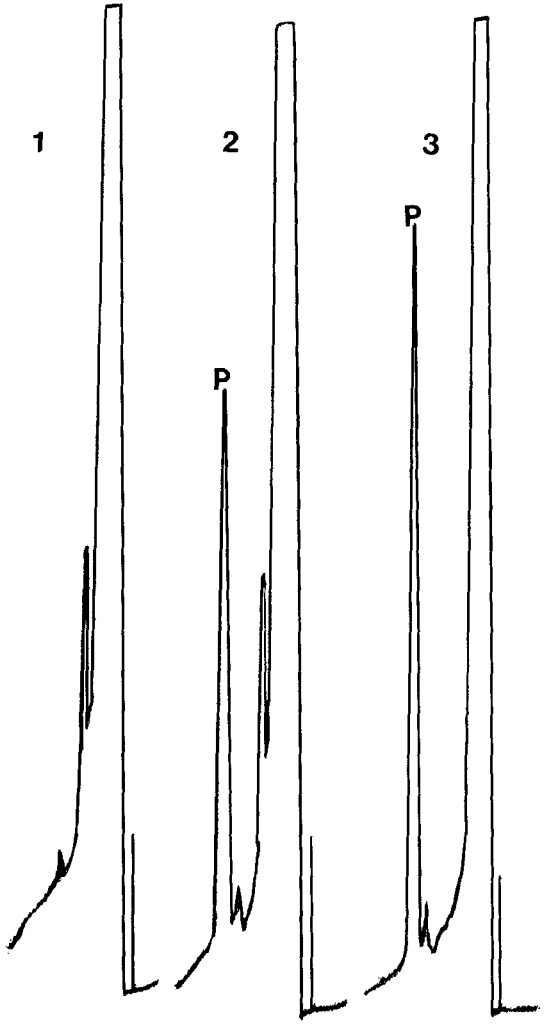

Figure 5. Representative chromatogram of 1: patient serum free from pentobarbital, 2 - patient treated with pentobarbital, and 3 - aqueous standard 50 mg/L. (P = pentobarbital peak; time for P = 6 min). Serum, 2 µL was injected directed onto the column, eluted as described with detection at 254 nm.

This method can be extended to analysis of other compounds. For example, phenobarbital shows similar changes in the capacity factor as compared to pentobarbital. However, for routine assay of phenobarbital we used an elution solvent of 5% acetonitrile in 15 mmol/L phosphate buffer at pH 10.4. We found the regression analysis for phenobarbital between this method and an immunoassay (TDX, Abbott Laboratories, Irving, TX) of $y = 1.055x - 0.1197$; $r = 0.982$.

ACKNOWLEDGMENT

The authors thank Dr. Willie L. Hinze and Dr. Michael L. O'Connor for reviewing this manuscript.

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