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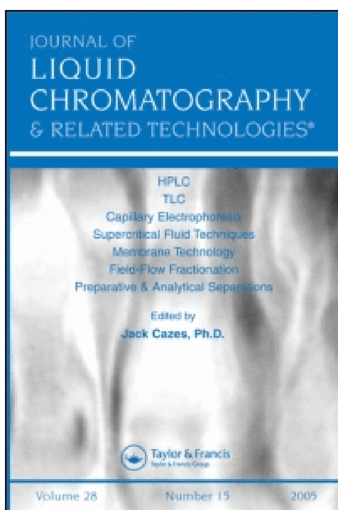

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Analyte Recovery from Deproteinized Serum for HPLC

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Analyte Recovery from Deproteinized Serum for HPLC

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Abstract: Serum deproteinization is often used as a rapid method for analysis of drugs and small molecules in serum in HPLC. When the recovery of these compounds is calculated based on peak height of aqueous standards treated also by the acetonitrile similar to serum, a high recovery is obtained. However, when these drugs treated with the acetonitrile are calculated based on aqueous standards diluted in the mobile phase (pump solvent) a low recovery is obtained. Acetonitrile in the sample decreases the peak height and limits the amount of sample to be injected on the column in drug analysis in serum due to formation of a short gradient leading to a non-symmetrical peak shape with a decrease in the plate number. We present two general mixtures for serum deproteinization as an alternative to acetonitrile: trichloroacetic acid-methanol and zinc sulfate-methanol. Both of these solvents allow the injection of larger sample volume without the decrease in peak height or affecting too much the plate number.

Keywords: Acetonitrile, Capillary electrophoresis, Deproteinization, Drugs, Endogenous substances, Proteins, Recovery, Serum

INTRODUCTION

Unfortunately, serum can not be injected directly on the HPLC columns with exception of small amounts injected on special columns such as

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shielded hydrophobic phase, wide pore, and molecular sieve columns. The serum contains close to 70,000 mg/L of proteins, which can adsorb, denature on the inlet, and clog the small pores of the column. In order to analyze drugs and endogenous small molecules present in serum, these compounds have to be extracted either by liquid or solid phase. Both extraction methods avoid the proteins, concentrate the analytes, and yield clean chromatograms, but they are laborious procedures especially for routine analysis. A more common procedure for drug analysis in serum is to remove the proteins by the addition of two volumes of acetonitrile and occasionally by the addition of acids or heavy metal salts.[1–7] Acetonitrile is a common method for deproteinization not just for HPLC but also in capillary electrophoresis (CE). It requires two volumes of acetonitrile to 1 volume of serum to completely remove serum proteins.[8] The advantages here are the simplicity and speed of deproteinization by the acetonitrile. In CE the acetonitrile present in the sample leads to sample concentration on the capillary known as stacking.[9,10] About a 30 fold increase in sensitivity can be obtained by acetonitrile treatment, which is based on transient-pseudo isotachophoresis.[9,10] However, in HPLC, as we show here, the reverse occurs where the sample volume, which can be injected on the column becomes limited before band broadening and peak shape deterioration occur.

As more rapid methods of analysis are sought, shorter columns are getting more common as a means of rapid analysis. Also, Organic solvents are getting more expensive, and more difficult to dispose and store. Thus, more polar columns such CN and C8 are becoming more used. All these new trends worsen the effects of the acetonitrile in the sample on the peak shape and height.

Here we investigate the recovery of few drugs, as an example, using columns with different dimensions and polarity. We show the recovery can be deceiving depending on the method of calculation. We show that many variables such as column dimensions, sample volume, and solvent concentration in the sample affect the peak height (detection limit) and indirectly the recovery. We describe alternative mixtures for serum deproteinization which improve the detection limits.

EXPERIMENTAL

Deproteinizing Agents

Trichloroacetic acid (TCA)-Methanol

Methanol (10 mL) is mixed with water (10 mL) and 0.5 g TCA (Stable at room temperature).

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$ZnSO_4$-Methanol

One volume of $ZnSO_4$ (0.1 M) is mixed with one volume of methanol (Stable at room temperature).

Acetonitrile

Deproteinization Procedure

Serum, 100 μL, was vortex-mixed for 10 s with 200 μL of the above deproteinization agents and centrifuged at 13000 X g for 30 s. The supernatant was injected onto the column.

Instrument

A model 110 Pump (Beckman Instruments, Fullerton, CA) was used to deliver the different solvents (isocratic) at 1 mL/min. A detector (Model 441 Waters Associates, Milford, MA) was used to measure the absorbency. The pump solvent and test conditions were dependant on the drug as described below.

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Test Conditions

Caffeine: (Microsrb C8 column, 5 μm particle size 4.6 × 150 mm, Varian, Palo Alto, CA); Phosphate buffer 6 mmol/L, pH 2.7 at wavelength 280 nm.

Voriconazole: (Nova-Pak CN-HP cartridge column 100 × 3.9 mm, CN, 4 μm average particle size, Waters Associates, Milford, MA); Phosphate buffer 24 mmol/L pH 2.0, with 0.05% n-butyl amine at wavelength 254 nm.

Lamotrigine: (Microsorb C18 column, 5 μm particle size, 4.6 × 150 mm, Varian; 17% acetonitrile with phosphate buffer 18 mmol/L, pH 6.2 at wavelength 313 nm.

Pentobarbital: (Microsorb C18 column, 4.6 × 150 mm, 5 μm particle size, Varian, 25% acetonitrile; Phosphate buffer 18 mmol/L pH 6.2 at wavelength 210 nm.

Apparent Recovery Calculation

Peak height of the drug in deproteinized serum/peak height of the drug (in water) treated with the same deproteinizing solvent used for serum × 100.

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RESULTS AND DISCUSSION

Acetonitrile is often used as a deproteinization agent for analysis of drugs and different endogenous small molecules in serum in both HPLC and also in CE. Figure 1 shows an example of how the sample volume affects the peak shape in HPLC. As long as the sample injection volume is small (~10 μL), the peak height of a drug like pentobarbital is the same, regardless if the sample is prepared in acetonitrile or simply diluted in the mobile phase (pump solvent). On the other hand, when the volume is increased to 50 μL the peak height drops greatly for the sample prepared in acetonitrile, although the area under the peak remains similar, Figure 1. When the peak is examined closely, it becomes clear that the peak shape is broad and nonsymmetrical for the 50 μL injection prepared in acetonitrile. The peak starts to elute rapidly, or prematurely, in the first part, different from its second part. Occasionally, the peak has a shoulder or even splits into more than one peak.[11]

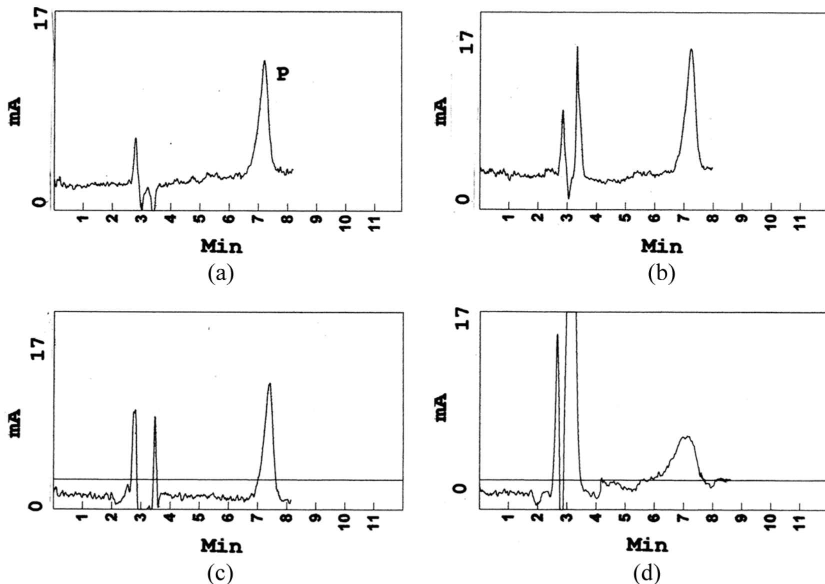

Figure 1. Effect of the acetonitrile in the sample on peak height of pentobarbital (P). Injection of 10 μL of pentobarbital standard (P) 100 mg/L dissolved in: A- mobile phase, B- 66% acetonitrile. Injection of 50 μL of Pentobarbital 20 mg/L dissolved in: C- mobile phase and D- dissolved in 66% acetonitrile. Solvent 25% methanol in phosphate buffer 18 mol/L, pH 6.2, Column C18, 5um 4.6×25 cm.

Basically, acetonitrile present in the sample forms a short gradient causing a partial and early elution of the analyte. The peak shape is distorted and the plate number drops. Furthermore, immediately after injection of a sample prepared in the mobile phase, the reinjection of 66% acetonitrile without any analyte leads to the same peak distortion, indicating again that the distortion effect is due to formation of the short gradient. Large volume injection of the sample can also cause sample overloading and a decrease in plate number. However, by injecting different volumes of the sample dissolved in the mobile phase on the column, as we see in Figure 2, sample overloading is not a major problem here. The injection of large volumes of sample is done usually in pursuit of enhancing the detection limits or for compounds with low absorbency, provided sample overloading is avoided. This observation of decrease in peak height by acetonitrile is not peculiar or limited to pentobarbital but occurs in the analysis of most compounds under different analysis conditions such as caffeine, lamotrigine, and voriconazole, Figure 3.

The affect of the deproteinization agent on peak height is modulated by several variables such as the concentration and the hydrophobicity of the organic solvent in both the sample and also in the mobile phase, as well as the column dimensions and polarity too, as expected, Figure 3, Figure 4. Smaller columns are affected more than large columns since the compound does not adsorb for too long on the column, Figure 4. However, the sample volume remains to be a critical factor regardless of the column size, Figure 4. Compounds, which elute with a lower concentration of acetonitrile, are more affected by the organic solvent in the

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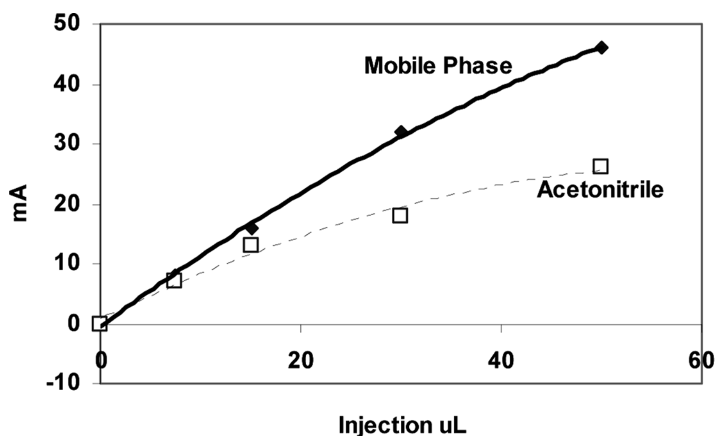

Figure 2. Injection for different volumes of lamotrigine standard (10 mg/L) diluted either in the mobile phase (pump solvent) or 66% acetonitrile.

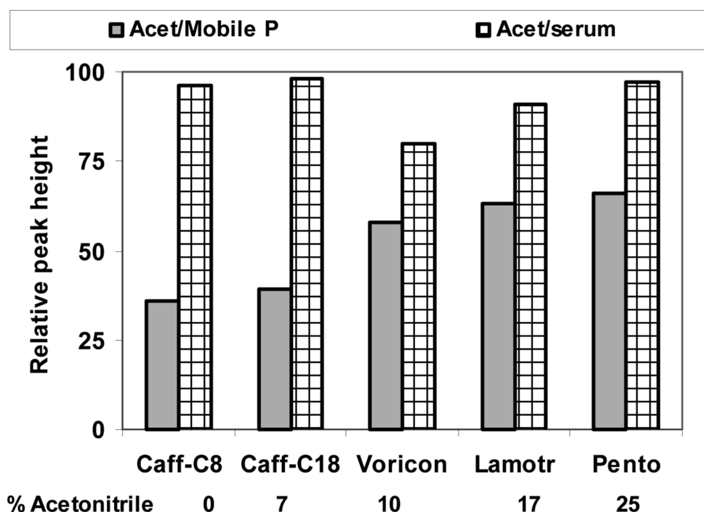

Figure 3. Relative peak height of different drugs dissolved in acetonitrile relative to that dissolved in the mobile phase (Acet/Mobile P) and apparent recovery of serum deproteinized with acetonitrile (Acet/Serum); 50 μL injection; as function of the percentage of acetonitrile in the mobile phase (Caff = Caffeine, Vor = Voriconazole, Lamot = lamotrigine, Pento = pentobarbital). (see Apparent Recovery Calculation).

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sample, Figure 3. When recovery of the drug in the serum is calculated based on the standard prepared also in acetonitrile, a high misleading value close 100% can be obtained, Figure 3. This high recovery essentially

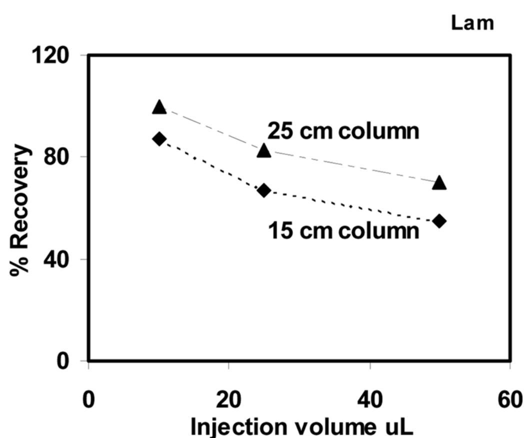

Figure 4. Effect of injection volume and column length on peak height of lamotrigine in acetonitrile relative to that in the mobile phase.

indicates that the drug is freed from the serum and recovered in the acetonitrile, but it does not describe if the peak shape or the plate number are deteriorating. The plate number in this case is decreasing since the area under the peak remains the same.

Since the decrease in peak height is mainly due to the high amount of organic solvent in the injection loop several options are possible to decrease this effect; for example, using smaller sample volumes, increasing the column length, choosing a more hydrophobic column, or decreasing the hydrophobicity of the deproteinizing agent. For practical reasons it is easier to manipulate the latter option. For example, methanol can be used in place of acetonitrile. Unfortunately, the methanol is not as good a deproteinizing agent as the acetonitrile. It requires about five volumes of methanol for serum deproteinization, leading to further sample dilution. Proteins can be removed with acids such as trichloroacetic acid (TCA), and heavy metal salts. Acids have high absorbency at low wavelength. TCA by itself has been used previously as deproteinizing agent.[8,12]. The zinc sulfate – methanol or zinc sulfate acetonitrile has been used also in some cases to precipitate whole blood, but with a higher ratio of solvent to blood than is used here.[7,13–16] We investigated, here, using a mixture of methanol-TCA and also a mixture of methanol-zinc sulfate. These mixtures simply decreases both the relative contaminate peaks in these solvents and decrease the hydrophobicity, while improving the deproteinization ability of methanol, removing all the serum proteins much better than methanol alone Figure 5.

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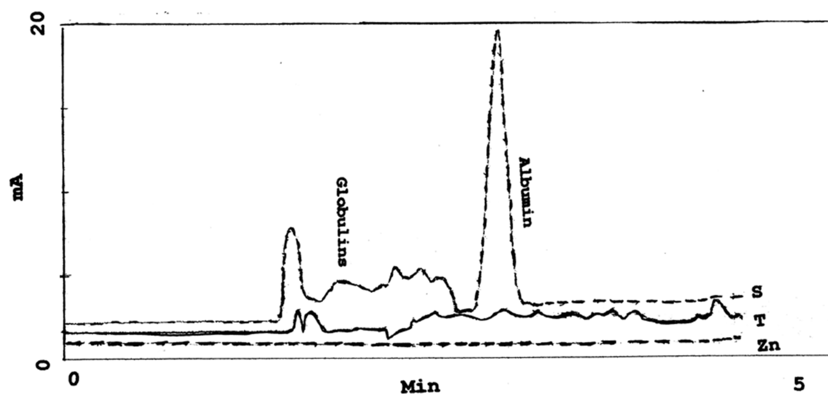

Figure 5. Capillary electrophoresis of: (S) serum diluted 40 times without deproteinization and electrophoresed; (T) supernatant of TCA-methanol deproteinization without dilution; and (Zn) supernatant of zinc sulfate- methanol deproteinization of the serum without dilution.

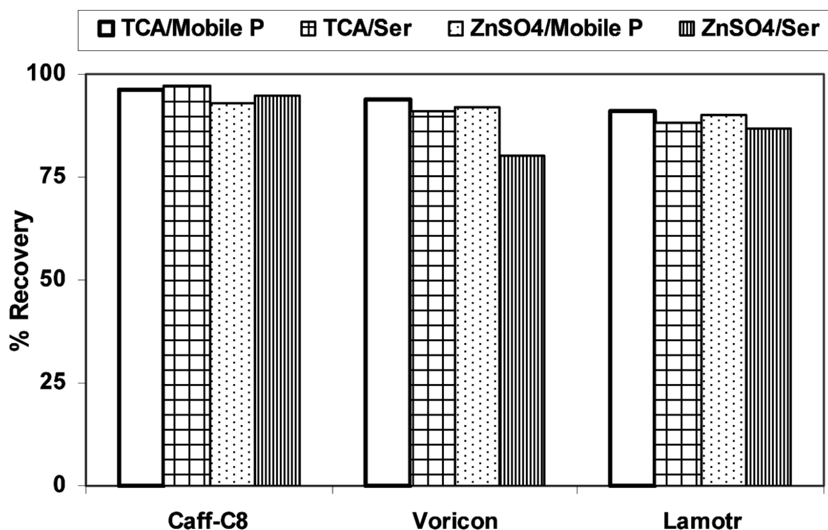

Figure 6. Relative peak heights of caffeine, voriconazole, and lamotrigine in the two new deproteinizing agents at 50 μL injection: TCA-methanol agent relative to that dissolved in the mobile phase (TCA/Mobile P), and the apparent recovery from serum (TCA/Ser); also, for the ZnSO4-methanol agent ($ZnS0_4$/Mobile P) and its apparent recovery ($ZnS0_4$/Ser).

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The relative peak heights of the different drugs in both of these mixtures are much better than the peaks in the acetonitrile. This is because of the lower hydrophobicity of these solvents compared to acetonitrile. Using large injection volumes (~50 μL), these deproteinizing agents give the peak height of an average of about 90% of those in the mobile phase, Figure 6 compared to only 45% for those prepared in acetonitrile, Figure 3. The TCA-Methanol has more background absorption compared to the methanol-zinc sulfate but gives better serum recovery for voriconazole compared to the zinc sulfate-methanol (91 vs. 80%). This may reflect the better solubility of this basic drug in the acid. In general, both of these solvents allow larger volumes of sample to be injected on the column, improving the sensitivity of the analysis for many compounds, provided sample overloading is avoided, especially with the short columns.

CONCLUDING REMARKS

Recovery of drugs in serum is mostly calculated in relation to standards prepared in a similar way, i.e., using the same deproteinizing agent used for serum. If the serum is prepared in 2 volumes of acetonitrile, the

standard is also treated the same way. Usually, this calculation gives a high recovery, over 90%, without consideration to change in the peak shape. It indicates that the drug has been freed from the serum binding proteins and now is present in the acetonitrile. However, if the drug in serum is deproteinized by acetonitrile and compared to standard dissolved in water or in the mobile phase the relative peak or (recovery) can be much lower than expected, as seen in Figures 1 and 2, due to a decrease in the plate number.

This study shows that acetonitrile in the sample changes the peak shape, limiting the amount of sample to be injected on the column for drug analysis in serum due to formation of a short gradient leading to a non-symmetrical peak shape with a decrease in the plate number. This effect depends on several factors, among them the column dimensions as well as the hydrophobicity of both the mobile phase and the amount of organic solvent in the injection loop. To overcome this problem we describe two general deproteinization mixtures, which are effective in removing serum proteins and enhancing the amount of sample which can be loaded on the column, thus improving the detection limit while keeping the sample preparation simple.

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