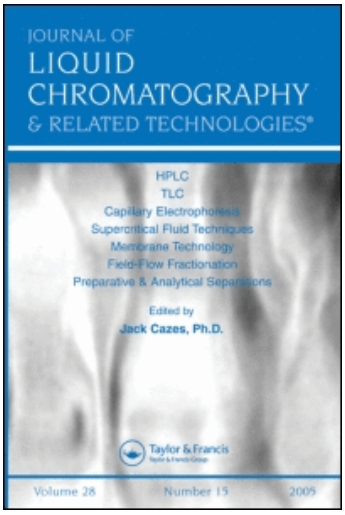

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THE ANALYSIS OF THEOPHYLLINE BY HPLC

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ABSTRACT

Theophylline is a drug used in the treatment of asthma. Its therapeutic value is dependent upon its concentration in the blood with levels greater than 20 mg/l causing side affects, sometimes severe. Various people metabolize the drug at different rates, so a method for analyzing serum Theophylline is necessary. We have developed a rapid, quantitative HPLC method for the analysis of serum Theophylline. No pretreatment of the sample is necessary, the method uses direct injection of the serum sample onto a guard column.

INTRODUCTION

Theophylline (1,3-dimethylxanthine) is used as a bronchodilator in the treatment of asthma. Its effect as a therapeutic agent appears to be dependent upon its concentration in the bloodstream, with levels of 10 to 20 mg/l having the desired effect, and levels greater than 20 mg/l having undesirable side effects, sometimes severe. Studies have shown that the rate of theophylline metabolism varies greatly among individuals. Because of this, a rapid, quantitative analysis for theophylline is a necessity.

Previously, spectrophotometric methods have been used for determining theophylline concentrations. Using this procedure has a drawback because any other xanthines present interfere with the analysis. Gas chro-

matographic methods, which separate theophylline from the other xanthines, have been used but require extraction and derivatization, which are time consuming steps.

Several researchers have used high performance liquid chromatography to determine serum theophylline levels with good success.[1-20] However, the methods used have required some form of sample preparation, such as extraction, or protein precipitation and centrifugation to remove the solid materials. We have now developed a rapid, precise HPLC analysis method, which requires only 10 μl of serum and uses direct injection of the serum sample onto a guard column, containing Co:Pell ODS, a pellicular bonded reverse phase. The actual theophylline separation is done with a Partisil-10 ODS-2 microparticulate reverse phase column.

MATERIALS

A Liquid Chromatograph was assembled using a Constrametric II G pump, a Spectro Monitor II detector, with an 8 μl flow cell, model #1201, and a model #3401 chart recorder, all from Laboratory Data Control (Riviera Beach, Florida). A Rheodyne valve injector, model #7105, with a 175 μl loop(Berkeley, California) was used for sample introduction. Columns used were Partisil-10 ODS-2, 10 μ reverse phase column, 4.6mm I.D. x 25cm length, and a Co:Pell ODS guard column, 2.1mm I.D. x 7cm length, from Whatman, Inc.(Clifton, New Jersey). Theophylline, xanthine, chlorophenylalanine, uracil and amino uracil were obtained from Nutritional Biochemical(Cleveland, Ohio). Theobromine was obtained from Baker Chemical(Phillipsburg, New Jersey) and dyphylline and hypoxanthine were obtained from ICN(Plainsview, New York). 8-Chlorotheophylline was obtained from Chemical Dynamics Corporation(South Plainfield, New Jersey).

Packing is replaced in the Co:Pell ODS guard column as follows: A fitting is removed and the packing is pushed out with a solvent delivery pump. The column is then rinsed with acetone and dried. The Co:Pell ODS can then be poured into the column through a funnel while the column is lightly tapped on a counter top. The top fitting is then replaced and the column is ready for reuse. 10 grams of material was supplied with the guard column, enough to repack it twenty times. The cost of the guard column packing averages $.22 per sample, using the described procedure.

METHODS

In arriving at a system to be used for serum theophylline analysis, several parameters were investigated to optimize the separation. Among the areas of investigation were various mobile phases, salt buffers, salt concentration, pH and temperature. The system which was determined to be best suited to the analysis was Methanol and .025 M KH_2PO_4. The KH_2PO_4 was adjusted to pH 2.5 with Phosphoric acid. The methanol and buffer were mixed in a 35/65 ratio. This solvent was degassed with water aspiration for ten minutes, immediately before use, to remove any dissolved gases which might cause detector problems.

Ambient temperatures were used throughout the analysis. A slight increase in efficiency was noted at elevated temperatures, but this was offset by the advantages of running at room temperature and the good chromatograms obtained.

In any quantitive system, there are potential day to day differences which could affect results. In this system, slight changes in mobile phase composition, or minor temperature variation could have some effect on peak heights and retention times. To offset the

differences, an internal standard, 8-chlorotheophylline, was used. Standard solutions of theophylline and 8-chlorotheophylline in methanol/water, 50/50, were analyzed. The concentrations used were 2, 5, 10, 15, 20, 25, 30, and 35 mg/l. A graph of peak height versus concentration was drawn for both samples to show linearity and proportionality of absorbance for the two compounds. (Figure 1). This range covers the useful therapeutic levels of the drug, 10 to 20 mg/l, and a wide range on each side. The use of 8-chlorotheophylline has one drawback. Dramamine®, a drug used for motion sickness, contains some 8-chlorotheophylline and could interfere with results.[4]

pH has a dramatic effect on the location of the 8-chlorotheophylline peak. By changing pH, the peak can be moved throughout the chromatogram. No changes are noted in the other peaks. A possible explanation for this effect, based on pK_a values of the sample components is suggested by Hill.[10]

Theophylline has its maximum absorbance at 273nm. However, a large number of detectors used in LC register absorbance at 254nm. Due to this fact, 254nm was used throughout the study. Though the absorbance of theophylline at 254nm is only about 35% of the absorbance at 273nm, good results can still be obtained at the lower wavelength.[4,7,9] The detector had an 8μl cell and was set at .04 A.U.F.S.

For serum analysis, a Co:Pell ODS guard column was placed in line between the injector and the Partisil-10 ODS-2 column. This column collected the protein and impurities present in the serum samples and prevented them from destroying the analytical column. Up to 30 serum samples were run before the packing in the guard column needed replacement. At a flow rate of 1.5 ml/min., the pressure gradually increased from 1,500 psi to 2,800 psi after 30 injections. When the guard column

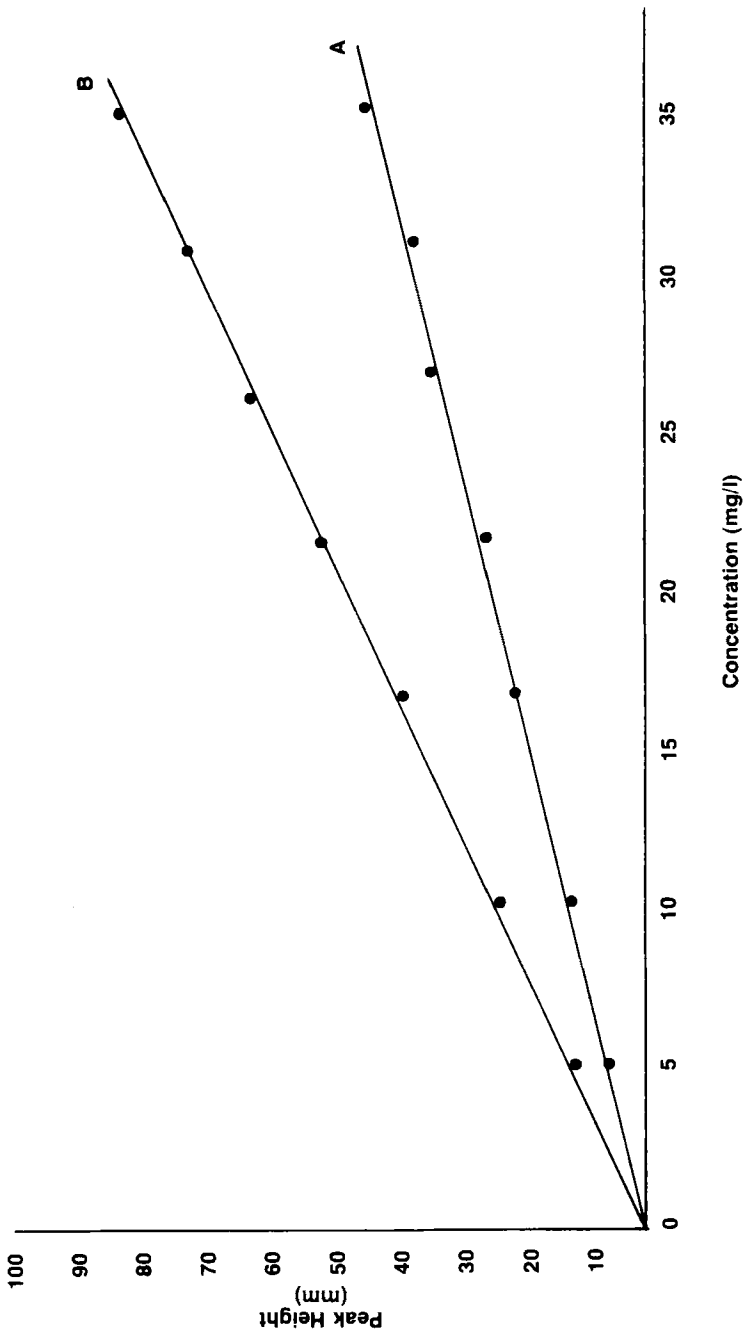


Figure 1: Peak height vs concentration. A. 8-chlorotheophylline. B. Theophylline.

was repacked, a simple ten minute tap packing procedure requiring only 0.5 gram of material, the pressure returned to 1,500 psi. No frit was used at the inlet end of the guard column, as the frit clogged after only a couple of injections.

The sample was mixed with the standard in a unique manner. 10 μl of sample were injected with a syringe into a loop injector. This was followed by a 10 μl injection of the standard, 8-chlorotheophylline, 15 mg/l. When the loop was turned, both samples were injected onto the column. The guard column, because of the large particle size, aided in the mixing of the samples. After each injection, the loop was flushed with at least 200 μl of distilled water to insure that no contamination interferred with the next sample.

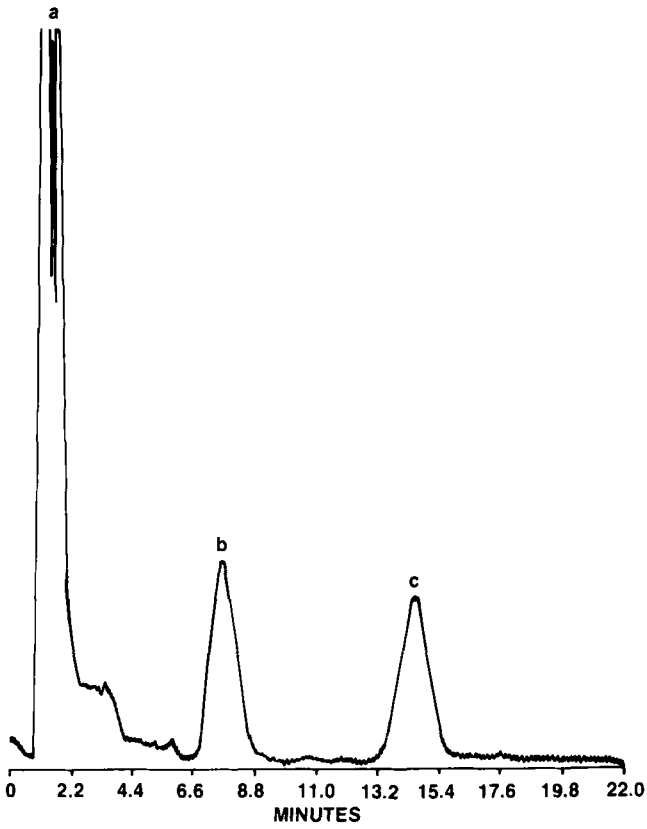
Chromatogram #1 shows an actual serum theophylline separation. The good resolution of the theophylline and 8-chlorotheophylline peaks from the rest of the sample can be clearly seen.

In order to determine whether any peak overlap, which would interfere with the analysis, had occurred, several other xanthines and derivatives were analyzed including xanthine, chlorophenylalanine, uracil, amino uracil, theobromine, dyphylline, hypoxanthine and methyl xanthine. Twenty five serum samples from patients who had received theophylline were also analyzed. No interferences were found.

After every 120 to 130 serum samples, 60 ml of methanol were run through the Partisil-10 ODS-2 column to remove any impurities which might have been absorbed onto the column.

DISCUSSION

The repetitive analysis of serum theophylline has shown the procedure to be quite reproducible. At one point, five injections of a 20 mg/l sample were made. Peak heights ranged from 4.9cm to 5.1 cm,



Chromatogram #1. Separation of Blood Serum-peaks: a)impurities, b)theophylline, and c)8-chlorotheophylline. Columns: PXS-1025 ODS-2, 4.6mm x 25cm(analytical), and Co:Pell ODS, 2.1mm x 7cm (guard column); column temperature: ambient; mobile phase: 0.025M KH_2PO_4/MeOH(65:35) (pH 2.5); pressure: 2000 psi, flow rate: 1.5 ml/min.; and detection: UV @ 254 x .04 aufs.

a difference of $\pm$2%. Using this method, as little as 2.0×10^{-8} grams of theophylline could be detected.

Since no sample preparation is necessary, and no premixing of sample and standard has to be done, as little as 10μl of serum has been used with satisfactory results. Small sample size is especially important when dealing with pediatric patients.

Due to the high efficiency of the ODS-2 column, placement of the guard column before the analytical column had little effect on the separation. The use of the guard column in place of other preparation techniques has many costs and time saving advantages. A technician, instead of spending his or her time with one of the preparation techniques, can simply inject the serum and move on to something else.

CONCLUSION

A rapid, quantitive analysis for serum theophylline analysis, using HPLC, has been developed in which no sample preparation is required. The method is accurate throughout the therapeutic range of the drug. Sample preparation is eliminated by the use of a Co:Pell ODS guard column. Premixing of the sample and standard is eliminated by the use of a loop type injector.

Recent investigations show that the guard column procedure could possibly be used in other serum analysis and possibly even with whole blood.

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