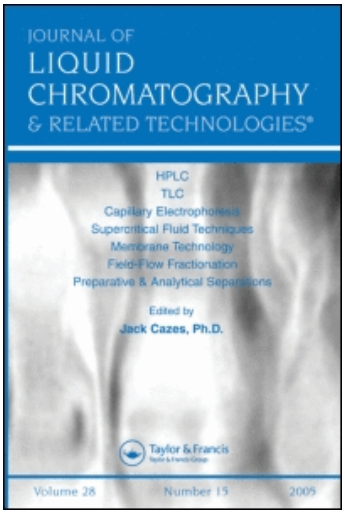

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Kapke, Gordon F. and Franklin, Ray B.(1987) 'A Direct Liquid Chromatography Method for Serum Caffeine Analysis', Journal of Liquid Chromatography & Related Technologies, 10: 2, 451 — 463
To link to this Article: DOI: 10.1080/01483918708066728
URL: http://dx.doi.org/10.1080/01483918708066728

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(2&3), 451-463 (1987)

A DIRECT LIQUID CHROMATOGRAPHY METHOD FOR SERUM CAFFEINE ANALYSIS

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ABSTRACT

A rapid sensitive method for serum caffeine analysis is reported. One hundred microliters of serum is mixed with 50 microliters of the acetonitrile/internal standard mixture, centrifuged, and 10 microliters of the supernatant is injected into the liquid chromatograph. Caffeine and internal standard are eluted at 6.09 and 7.17 minutes respectively. The procedure is linear to at least 20 micrograms/milliliter. The within run precision was 2.3% and 3.8% (N=10) respectively for mean concentrations of 7.77 and 15.73 micrograms/milliter. Between day precision was 11.0% and 8.6% respectively for mean concentrations of 7.63 and 15.55 micrograms/milliliter. Recovery of caffeine over the analytical range averaged 104%.

INTRODUCTION

Methylxanthines (including caffeine, theophylline, and the rarely used theobromine) have a number of important therapeutic effects. These compounds are used

to relax bronchial smooth muscle in the treatment of asthma and other obstructive lung diseases (theophylline is most potent in this action) and to stimulate the central nervous system to increase performance, decrease fatigue, and to enhance respiration (caffeine is most potent in these effects) (1).

The principle indication for caffeine therapy is neonatal apnea. While theophylline was the methylxanthine originally used for this disorder (2), recently the advantages of caffeine have become widely acknowledged (3,4).

Breathing disorders are common problems in preterm infants. Periodic breathing (intermittent breathing with intervals less than 10 seconds (5)) has been noted in approximately 50% of all premature infants (6) and the incidence increases with greater prematurity, approaching 90% of neonates of 28-29 weeks gestation (5). Further, it is estimated that at least half of periodic breathing infants (25% of all premature infants) progress to at least one episode of apnea (cessation of respiratory movements for greater than 20 seconds) (7). The sequelae of apneic episodes and the resulting hypoxia and hypotension may be serious. Bacola et al. (8) reported a high incidence of neurologic abnormalities, including mental retardation, among infants with apneic episodes of two minutes or

longer. Jones and Lukeman (9) reported a 24% incidence of "a major handicap" in infants surviving recurrent apnea. In many cases, periodic breathing is secondary to a specific underlying disease. Disorders which are associated with apnea include intracranial hemorrhage, patent ductus arteriosus, respiratory distress syndrome, pneumonia, seizures, hyperthermia, hypoglycemia, hypocalcemia, anemia, maternal oversedation and gavage feeding (5). While apnea secondary to these syndromes will respond to therapy of the underlying disease, there remains a large number of infants with breathing disorders for whom no specific etiology (other than prematurity) has been established and for whom chemical intervention is indicated.

Caffeine has a number of practical therapeutic advantages over theophylline in the treatment of neonatal apnea. Caffeine is more potent in central nervous system stimulation and has fewer peripheral effects, especially the undesirable cardiovascular effects which may complicate theophylline therapy. Toxic reactions to caffeine are much less frequent than to theophylline due to caffeine's favorable therapeutic index. At serum concentrations of greater than 50 micrograms/milliliter (therapeutic range being 6 to 12 micrograms/milliliter (10,11)) toxic signs including "jitteryness" (an exaggerated response to stimuli) and

tachycardia (200 to 260 beats per minute) may be observed in the neonate treated with caffeine (3,4). Fatal reactions to caffeine are distinctly rare. In contrast, theophylline toxicity is relatively common and death may ensue from too rapid administration of even a therapeutic dose (1). Plasma theophylline concentrations just slightly greater than the therapeutic range may induce nausea and seizures (1). In addition, theophylline is metabolized to caffeine in the neonate (12), and therefore it has been suggested that both theophylline and caffeine be monitored in the neonate when theophylline is administered (13).

Caffeine is metabolized slowly and unpredictably in the neonate (3); the plasma half-life of caffeine ranges from 60 to greater than 150 hours. By six months of age, the half-life has decreased to approximately four hours. This progression is apparently due to the maturation of the infant's hepatic microsomal drug metabolizing enzymes. The prolonged half-life in the neonate is advantageous in that it allows therapeutic concentrations to be maintained with a single daily dose after an initial loading dose. Because the metabolism of caffeine is unpredictable, it is important that it is monitored to avoid the undesirable effects of toxic or subtheraputic concentrations.

MATERIALS

Lyophilized caffeine standards and the internal standard, beta hydroxypropyl theophylline, were obtained from Sigma Chemical Company, St. Louis, MO. Chromatography grade acetonitrile and methanol were obtained from Mallinckrodt, Paris, KY. The liquid chromatography column, obtained from Supelco Inc., Bellefonte, PA, was a Supelcosil, 3 micron, 15 centimeter LC 18 column fitted with a 3 centimeter guard column filled with LC 18 pellicular packing. An IBM 9533 HPLC with a 9523 variable UV detector and a 9533 operator station, from IBM Instruments, wasused for the chromatography. The detector output was recorded and integrated using a Hewlett Packard 3390A integrating recorder. Disposable 0.45 micron filters were obtained from Universal Scientific Inc., Atlanta, Ga. A vacuum pump (model 00A-V141-AA, Gast, Benton Harbor, MI) was used for degassing and vacuum filtration of solvents. Samples were centrifuged using the Abbott TDX centrifuge from Abbott Laboratories, Dallas, TX. All injections were made with a 50 microliter Hamilton syringe (Hamilton Company, Reno, NV) into a 20 microliter loop of the injection valve.

METHODS

Internal standard was prepared by adding beta hydroxypropyl theophylline to acetonitrile to a final concentration of 30 micrograms/milliliter. Mobile phase solvent was prepared by taking 200 ml of 0.5% acetic acid (5 ml of glacial acetic acid to a volume of 1 liter) and adding 50 ml of methanol. The mobile phase was degassed by stirring under a vacuum produced by the Gast vacuum pump and filtered under vacuum through a 0.45 micron filter prior to being placed on the liquid chromatograph. The LC 18 column was equilibrated with the mobile phase for 30 minutes at a flow rate of 1 ml/minute. The column flow rate was increased to 1.5 ml/minute for the caffeine analysis. Column operating back pressure was approximately 350 psi.

Caffeine standard solutions were prepared from lyophilized caffeine standards by adding 10 milliliters of the mobile phase to a vial of lyophilized standard. The assay was routinely calibrated using a standard of approximately 10 micrograms/milliliter. The reconstituted standards were mixed by inversion and allowed to sit at room temperature for 15 minutes prior to use. Standards were stored at 4 degrees centigrade and used until depleted which was approximately 3 months.

Samples were prepared for analysis by pipetting 100 microliters of sample (standard or serum) into a 1.5 ml disposable polypropylene centrifuge tube followed by 50 microliter of the acetonitrile/internal standard mixture. The sample/internal standard mixture was vortexed for 60 seconds and allowed to sit at room temperature for 15 minutes. The samples were then centrifuged for 2 minutes at 9,500 x g using the Abbott TDX centrifuge.

Ten microliters of the sample/internal standard mixture were injected into the liquid chromatograph. The column effluent was monitored at 273 nanometers (lambda maximum for caffeine (14)) with a detector sensitivity of 0.005 absorbance units full scale. The chromatograms were recorded and integrated using a Hewlett Packard recording integrator with settings of: attenuation, 4; peak width, 0.16; and threshold 4. The column was washed for 30 minutes with an equal mixture of methanol/water following the sample analysis and stored in the methanol/water mixture.

RESULTS

The results of the chromatography of standards and patients are shown in figure 1. The chromatogram

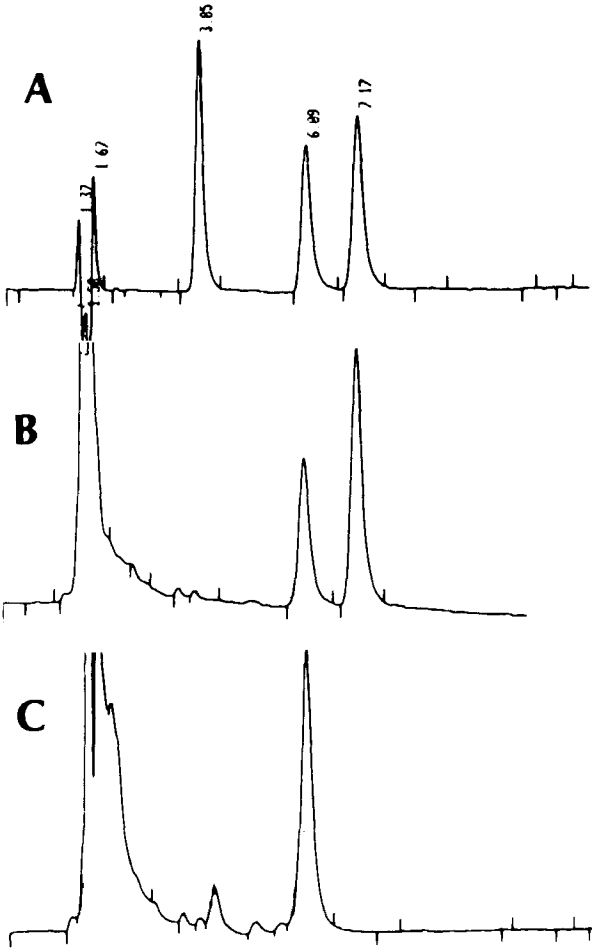

FIGURE LEGEND

Typical chromatograms are shown in figure 1. Chromatogram A is of a mixture of theophylline, caffeine and internal standard beta-hydroxypropyl theophylline with respective retention times of 3.85, 6.09, and 7.17 minutes. Chromatogram B is of a typical neonatal sample after the addition of internal standard and centrifugation. Chromatogram C is of a typical neonatal sample after the addition of internal standard solvent, acetonitrile, and centrifugation. All patient samples have a very large peak eluting at the solvent front.

labeled A in figure 1 shows the elution of theophylline (3.85 minutes), caffeine (6.09 minutes) and the internal standard beta hydroxypropyl theophylline (7.17 minutes). Theobromine, a potential metabolite of caffeine (15), also elutes prior to theophylline. The chromatogram labeled B illustrates the results obtained on a typical patient sample. The chromatogram labeled C is from a patient sample which has had no internal standard added.

The recovery of caffeine from drug free serum averaged 104%. Recovered concentrations of caffeine were 2.20, 5.41, 9.63, 16.39, 24.79, and 32.47 micrograms/milliliter with theoretical values of 1.90, 4.68, 9.14, 17.45, 25.04, and 32.00 micrograms/milliter. These values represent recoveries of 115%, 115%, 105%, 94%, 99%, and 101% respectively. The samples with caffeine concentrations greater than 20 micrograms/milliter were analyzed using serum samples which had been diluted 1 to 2 with distilled water prior to mixing the sample and the internal standard. The obtained value was then multiplied by the dilution factor of 2.

The within run coefficient of variation of the assay was 2.3% and 3.8% (N=10) at a mean concentrations 7.77 and 15.73 micrograms/milliliter. The between day coefficient of variation of the assay was 11.0% and 8.6% (N=10) at mean concentrations of 7.63 and 15.55 micrograms/milliliter.

DISCUSSION

The results demonstrate that caffeine can be effectively quantitated in human neonatal serum without an extraction step by liquid chromatography. This assay has demonstrated acceptable precision and accuracy. While there are multiple drugs utilized in the neonatal population, this caffeine assay has performed well during the past half year. No significant interfering substances have been observed during the analysis of 40 neonatal specimens over the past 6 months. In addition, in the process of determining chromatography conditions, 10 neonatal samples, chosen at random, where chromatographed after mixing 100 microliters of serum with 50 microliters of acetonitrile and no significant interfering peaks were observed.

Caffeine analysis in neonatal serum samples generally do not present difficulties in this system. However, if a sample is not analyzed for a prolonged period after centrifugation and the sample becomes turbid due to protein denaturation, the chromatogram may have a very broad initial peak starting at the solvent front. This broad peak may not interfere with the caffeine quantitation but continuous injection of such samples may lead to decreased column life. Turbidity due to protein denaturation can be removed by recentrifugation of the specimen.

The recovery data indicate that caffeine is quantitatively recovered from serum samples using this method. The average recovery of 104%, rather than 100%, probably represents analytical error. Most of the over-recovery occurred at the low caffeine concentrations where small absolute differences in observed values result in relatively large percentage differences in the average recovery.

The minimum sensitivity of this assay has not been extensively investigated. Samples containing as little as 1 microgram/milliliter of caffeine produce acceptable chromatography peaks for integration.

In summary, this method offers the advantages of direct sample injection following addition of internal standard and centrifugation, small sample size, acceptable precision, and excellent recovery. Without an extraction step, this procedure is also very rapid and convenient allowing for a single sample to be analyzed in 60 minutes. This procedure is acceptable for the routine monitoring of serum caffeine concentrations in neonates.

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