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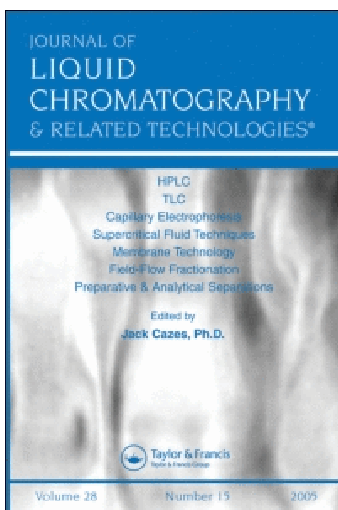

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THEOPHYLLINE IN THE PRESENCE OF CAFFEINE IN BLOOD SERUM AND PHARMACEUTICAL FORMULATIONS

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ABSTRACT

A reversed phase high-performance liquid chromatographic (HPLC) method has been established for the separation and quantitative determination of the alkaloid theophylline in the presence of caffeine -internal standard- in blood serum and in pharmaceutical preparations. The separation was performed on Spherisorb-5 RP-18 5μm reversed phase column using methanol: 0.038 M ammonium acetate: acetonitrile (38: 57:5) at a pH of about 7.20. The eluted alkaloids are detected at 272 nm. The retention time is 3.09 min for theophylline and 3.85 min for caffeine. The correlation of the integrated peak area with the concentration of theophylline showed a linear relationship between 0.05 to 5.0

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ppm per 10μl injection. The proposed technique was applied to the analysis of theophylline in blood serum, tablets, sprinkles, syrups, suppositories and injectable solutions.

INTRODUCTION

Theophylline is a substituted xanthine (1,3-dimethylxanthine). This compound is a naturally occuring xanthine alkaloid found in the leaves of the plant "Thea sinensis" from which the beverage tea is prepared. Theophylline is potent bronchodilator and widely used in the treatment of asthma and neonatal apnoea. This compound is a powerful, smooth muscle relaxant used primarily in the treatment of chronic obstructive pulmonary disease where dilation of the bronchi and pulmonary vasculature are warranted. The pharmacological activity of theophylline has been shown to be highly correlated with its concentration in plasma. However, because of the large inter-individual variation in the clearance of the drug, dosages must be individualized in order to optimize therapy. Besides, large amounts of theophylline may also be absorbed by the consumption of tea, coffee, cola-flavoured drinks, cocoa and chocolate[1-5].

Typical therapeutic dosages result in blood levels in the range of 10-20 μg/ml, but above these levels the toxic side effects may occur. Because of this potential danger, an acceptable analysis method in pharmaceutical formulations and in plasma samples is required to confirm that the drug administration program is under control. The bronchodilator effect of theophylline is proportional to the logarithm of the serum concentration over the range of 5-20 μg/ml. As serum concentration increases, greater bronchodilator effect is observed, with optimal response often described by serum concentrations over 10 μg/ml. Therefore with an at least 90% theophylline absorption and maintenance of a blood serum theophylline concentration within normal therapeutic least toxic risk range of 8 to 20 ng/μl would solve a significant problem[6-9].

In the past, the main technique used for the analysis of theophylline was ultraviolet spectroscopy, but interference from caffeine was possible.

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During these days High Performance Liquid Chromatography (HPLC) has been used as an acceptable technique because of the limitations of interferences, the advantages in speed, ease and sensitivity. This technique gives the analyst the opportunity to have quantitative results in a few minutes.

Over the past several years, much effort has been directed toward increasing both speed and efficiency in HPLC. The technique has been used for monitoring of serum theophylline concentrations.

Schumann et al[10] developed a high performance liquid chromatographic method for the determination of theophylline in blood serum. The sample was directly applied to a Hypersil ODS analytical column. In order to maintain an adequate performance it was necessary to change the analytical column after about forty injections of serum samples. This technique is less suitable due to the relatively short life time of the column for routine analysis of serum theophylline.

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Maijub and Stafford[11] presented an HPLC technique for quantitative determination of theophylline. However, this procedure requires silica column deactivation and extraction from plasma with low recovery for theophylline.

Valia et al[12] proposed an HPLC procedure for the same purpose, but this technique requires large blood serum volume (1 ml), has a long chromatogram run time about 12 min and is not very sensitive. This method also requires a long sample preparation time, it needs 30 min for one sample.

Wenk et al[13] developed an HPLC method for the analysis of theophylline in blood serum, but this technique requires a large sample volume 0·5 ml, a long sample preparation and long chromatogram run time about 16 min.

Apart from these techniques, there is in literature a number of assays for theophylline in samples of clinical interest[14-20], but a lot of these suffer from interferences, of similar components such as caffeine (1,3,7-trimethylxanthine).

In the present paper a simpler, fast and sensitive method for the determination of theophylline, in the presence of caffeine, in blood serum, tablets, sprinkles, syrups, suppositories and injectable solutions is developed.

EXPERIMENTAL

Apparatus: The high performance liquid chromatograph employed was a ternary gradient system Spectra Physics SP 8800 pump a Spectra Physics spectra chrom

100 variable wavelength UV-Vis detector operated at 272 nm and a sensitivity setting of 0.001 absorbance units full scale (A.U.F.S). A Rheodyne 7125 injection valve was fitted with a 10 µl loop. A Spherisorb-5 C18 reversed-phase column 5 µm particle size, 220x4.6 mm of I.D. stainless steel from Spectra Physics. The flow rate was 1.0 ml/min at a pressure of 2400 psi.

Computations were performed using a PC 1512 AMSTRAD computer.

Materials: Theophylline anhydrous (powder, Ph. Eur.), was supplied from Bristol Hellas S.A., Athens. Caffeine was from BDH Chemicals Ltd., Poole, England. These reagents were both used, as provided, without further purification. All standard solutions of these compounds were prepared by dissolving the appropriate amounts in distilled water. HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt Germany). Ammonium acetate pro analysi reagent was also from Merck. All other reagents used were of analytical grade and glass-distilled water was used throughout.

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Mobile phase: The mobile phase consisted of methanol: 0.038 M ammonium acetate: acetonitrile (38: 57: 5) at a pH of about 7.20. This eluent system was selected through a number of mobile phases on the basis of their relative polarities and low absorption at 272 nm. It was ultrasonically degassed under vacuum before use for about 15 min and by using helium during the elution time.

System suitability: The Spherisorb-5 RP-18 5 µm reversed phase analytic column was equilibrated with mobile phase at a flow rate of 1 ml/min. After a stable line was achieved, the standard and the sample solutions were injected into the column. The two peaks appeared over the increased retention time. The resolution factor, R_t, was calculated between the two peaks from the equation:

$$R_t = 2\left[\frac{t_{R_2} - t_{R_1}}{W_1 + W_2}\right],$$

where t_{R2} and t_{R1} are the retention times of the two peaks, W_1 and W_2 are the peak widths at the base of the two respective peaks. The resolution factor R_t was 2.17 signifying complete separation. This is illustrated in Figure 1.

The relative standard deviation of eight replicate injections of a standard was no more than 12.5%.

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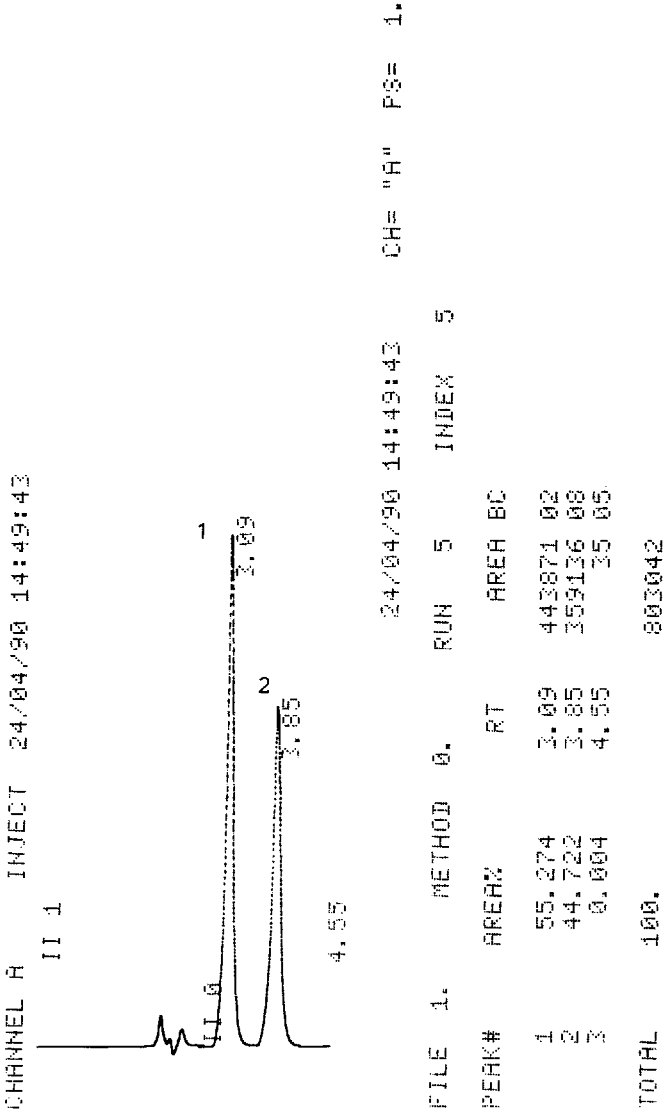

Figure 1. High-performance liquid chromatogram of Theophylline (1) using Caffeine (2) as internal standard.
Chromatographic conditions as described in text.

Detection limit: Pure anhydrous theophylline and caffeine were accurately weighed and subsequently dissolved in distilled water to give theophylline and caffeine solutions at concentrations of 1000 ng/μl. These stock solutions were diluted and 10 μl of each dilution were injected onto the HPLC system. The limit at which theophylline could be detected, as represented by a peak height, twice the size of background noise on this HPLC system was considered to be the detection limit.

Standard calibration curves for the determination of theophylline in aqueous solutions: Calibration curves for the determination of theophylline in aqueous samples were constructed both with and without caffeine as internal standard. Pure reagents were accurately weighed and dissolved in distilled water to give theophylline and caffeine solutions at concentrations of 1000 ng/μl. These stock solutions were serially diluted by a factor of ten to give theophylline concentrations in the range of 0.05 to 5.0 ng/μl and caffeine, as internal standard, at a concentration of 2.0 ng/μl. Aliquots of 10 μl of each dilution were injected on to the HPLC system, and the relative peak area of theophylline to caffeine was recorded in each case. Results taken are given in Table 1. Samples were similarly prepared as above except that no caffeine was used. In this case calibration curves were constructed by plotting the peak areas produced by the injection of 10 μl of each theophylline solution. These procedures were repeated eight times for statistical evaluation.

Standard calibration curves for the determination of Theophylline in methanol after solid phase liquid extraction. Determination of Theophylline in blood serum: Calibration curves for the determination of theophylline in methanol were constructed both with and without caffeine as internal standard. Methanolic solutions of theophylline were treated employing solid phase liquid extraction. As extraction column a Bond Elut C18 was used and methanol as extraction solvent. A syringe with an adaptor was used to apply positive pressure to the cartridge.

The cartridges were conditioned with 3x1.5 ml methanol applying positive pressure with the syringe, then the columns were washed with distilled water. Methanol and water were discarded.

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Table 1

Peak Area Ratio of Theophylline to Caffeine over the Concentration Range 0.05 - 5.0 ppm in Aqueous Solutions, equivalence quantity injected 0.5 - 50 ng

Concentration of Theophylline (ppm)	Equivalence Quantity Injected (ng)	Peak area ratio on Theophylline to internal Standard Caffeine								Mean Value	SD	RSD
		1	2	3	4	5	6	7	8			
5.0	50	3.336	3.095	3.259	3.222	3.678	3.562	3.330	3.136	3.327	0.188	5.65
1.0	10	0.538	0.558	0.562	0.560	0.548	0.543	0.562	0.613	0.560	0.021	3.75
0.25	2.5	0.127	0.122	0.151	0.114	0.119	0.114	0.141	0.146	0.129	0.013	10.0
0.10	1.0	0.056	0.056	0.054	0.054	0.060	0.048	0.055	0.054	0.054	0.003	5.55
0.05	0.5	0.036	0.035	0.035	0.040	0.030	0.028	0.028	0.027	0.032	0.004	12.5

The next step was the addition to the cartridge of 250 μl standard solution of theophylline or the same volume of blood serum. Then the cartridge was washed with distilled water and 3 ml of 1 M HCl. Theophylline was eluted with 5x250 μl of methanol, to this eluent a proper volume of a caffeine solution was added and then the final solution was evaporated to dryness on a water bath at 40°C in a gentle stream of dry nitrogen. The residue was redissolved in 80 μl of methanol and aliquots of 10 μl were injected on to the column. The relative peak area of theophylline to caffeine was recorded in each case.

As standard solutions of theophylline were used the aqueous solutions over the range of 0.05-5.0 ng/μl, 250 μl of these solutions were used and the final concentrations in the 80 μl of methanol ranged from 0.15 to 15.6 ng/μl. This procedure was repeated eight times for statistical evaluation.

Results taken are given in Table 2.

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Determination of Theophylline in pharmaceutical preparations: The proposed technique was applied to the analysis of theophylline in pharmaceutical formulations such as: tablets, syrups, suppositories and injectable solutions.

Preparation of tablets and determination of Theophylline: Tablets of theophylline 300 mg were prepared by pressing on a Korsch tableting machine, using a rectangular TM punch and die, (Courtesy of Bristol Hellas. S.A., Athens).

Theophylline and cellulose were mixed and then granulated with a water solution of hydroxypropylcellulose, to prepare granules of 0.8-0.9 mm size. Granules were dried for over 24 h, at 55°C. Granulometric analysis showed 19% fines (<100 microns). Finally, granules were pressed to give tablets of 320 mg of weight and a hardness of 20 Kp[21].

Ten tablets were weighed and the average tablet weight was determined, and found to be 315 mg. The tablets were finely powdered and a portion of 10 mg was weighed and quantitatively transferred into a 1000 ml volumetric flask. The content was diluted to volume with distilled water and gave a concentration of 9.52 ng/μl. From this stock solution 5 ml was taken and transferred into a 50 ml volumetric flask. To this solution 1 ml of a 100 ng/μl caffeine solution was added and the final solution was diluted to 50 ml. Aliquots of 10 μl of this solution were injected onto the HPLC column.

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Table 2

Peak Area Ratio of Theophylline to Caffeine over the Concentration Range 0.15-15.6 ppm in Methanol after solid phase liquid extraction, equivalence quantity injected 1.5 - 156 ng.

Concentration of Theophylline (ppm)	Equivalence Quantity Injected (ng)	Peak area ratio of Theophylline to internal Standard Caffeine								Mean Value	SD	RSD
		1	2	3	4	5	6	7	8			
15.6	156	3.923	3.616	4.159	3.877	3.812	3.931	3.840	4.045	3.900	0.150	3.84
3.1	31	0.509	0.573	0.664	0.564	0.593	0.601	0.541	0.569	0.576	0.042	7.29
0.78	7.8	0.182	0.190	0.160	0.178	0.184	0.191	0.167	0.174	0.178	0.010	5.61
0.31	3.1	0.084	0.075	0.072	0.077	0.074	0.079	0.081	0.074	0.077	0.004	5.19
0.15	1.5	0.038	0.041	0.038	0.032	0.036	0.039	0.042	0.032	0.037	0.003	8.10

Determination of Theophylline in sprinkles: Ten sprinkles were weighed and the average sprinkle weight was determined, and found to be 176 mg. The content of the sprinkles was pulverized and an amount of 10 mg was weighed and quantitatively transferred into a 1000 ml volumetric flask. The content was diluted to volume with distilled water and gave a concentration of 7.1 ng/µl according to labelled amount of theophylline which was 125 mg. From this stock solution 5 ml was taken and transferred into a 50 ml volumetric flask. To this solution 1 ml of a 100 ng/µl caffeine solution was added and the final solution was diluted to 50 ml. Aliquots of 10 µl of this final solution were injected onto the HPLC column.

Determination of Theophylline in suppositories: Six theophylline suppositories were weighed and the average suppository weight was estimated and found to be 1194 mg. The suppository was placed in a glass beaker and 25 ml methanol was added. The solution was vortexed with a magnetic stirrer for about 2h, then the content of the glass beaker was transferred in a 500 ml volumetric flask and diluted to volume with methanol and distilled water (1:1). The volumetric flask was placed in an ultrasonic for 15 min. The fatty matter of the suppository formed a solid layer on the surface of the volumetric flask. These excipients were trapped and discarded. 0.5 ml of this solution was diluted to 100 ml and with the proper addition of the internal standard caffeine gave a solution of 1 ng/µl in theophylline and 2 ng/µl in caffeine. Aliquots of 10 µl of this final solution were used for analysis by HPLC.

Determination of Theophylline in syrups: Syrup (100 µl) was tranferred to a 100 ml volumetric flask and diluted to volume. According to labelled amount the concentration of this solution was 10 ng/µl. 5 ml of this solution and 100 µl of a 1000 ng/µl solution of caffeine were transferred to a 50 ml volumetric flask and diluted to volume. This solution was 1 ng/µl in theophylline and 2 ng/µl in caffeine. Aliquots of 10 µl of this final solution were injected onto the HPLC column.

Determination of Theophylline in injections: An aliquot of 20 µl of the injectable solution was transferred to a 500 ml volumetric flask. To this flask 1 ml of a 1000 ng/µl caffeine solution was also added, then the solution was diluted to 500 ml with distilled water. According to labelled amount of the injections the final

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concentration of theophylline was 2.4 ng/µl and the concentration of caffeine 2 ng/µl. From this solution aliquots of 10 µl were used for HPLC analysis.

RESULTS AND DISCUSSION

The areas of the chromatographic peaks for both compounds, Theophylline and caffeine were linearly related to concentration. The linear regression equations were found to be:

STATISTICAL EVALUATION

Samples of Theophylline	Regression Equation	Correlation Coefficient
Aqueous	$y=0.6695x-0.0354$	0.9991
Methanolic	$y=0.2232x-0.0268$	0.9982
Blood	$y=0.4826x+0.7469$	0.9896

Where y = peak area ratio of the theophylline to that of caffeine, x = concentration of theophylline in ng/µl.

Figure 1 illustrates the separation of theophylline and caffeine in under four minutes time by reversed phase high-performance liquid chromatography. From the solvent eluting systems examined the most suitable was found to be methanol: 0.038 M ammonium acetate: acetonitrile (38: 57: 5) at a pH of about 7.20. This system elutes theophylline within 3.09 min at a pressure of 2400 psi. with a detection at 272 nm.

A number of compounds were examined as internal standards, of those the most suitable proved to be caffeine which is a trimethylxanthine.

Using aqueous and methanolic, after solid phase liquid extraction, solutions of the alkaloids, the recommended procedure yielded a linear relationship between the concentration of theophylline and the relative peak area ratio of theophylline to caffeine. The range of linearity was found to be from 0.5 to 50 ng for aqueous and 1.5 to 156 ng for methanolic solutions.

Experimental results for the determination of theophylline using caffeine as internal standard are given in Table 3.

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TABLE 3

Experimental Results for the Determination of Theophylline in Aqueous Samples using Caffeine as Internal Standard After Separation by HPLC

Compound	Retention Time (min)	Added (ng)	Found[a] (ng)
Caffeine	3.85		
		2.5	2.4±0.21
Theophylline	3.09	5.0	5.2±0.19
		10.0	9.8±0.28

[a]Average value of eight determinations ± standard deviation.

TABLE 4

Experimental Results for the Determination of Theophylline in Methanolic Samples using Caffeine as Internal Standard After Separation by HPLC.

Compound	Added (ng)	Found[a] (ng)
Caffeine		
	10.0	9.6±0.29
Theophylline	30.0	27.9±0.41
	50.0	46.2±0.38

[a]Average value of eight determinations ± standard deviation.

TABLE 5

Experimental Results from Determination of Theophylline in Pharmaceutical Preparations by Reversed-Phase HPLC

Sample	Labelled Amount[a]	Found[b]	RSD (%)
Tablets	300	308±20	6.49
Sprinkles	125	128±6	4.59
Suppositories	0.100	0.0600 ± 4.10^{-4}	0.74
Syrups	150	149±4	3.14
Injection	0.24	0.280 ± 9.10^{-3}	3.04

[a] mg/Tablet, mg/Sprinkle, g/Suppository, mg/15 ml Syrup, g/4 ml Injection

[b] Mean value of five determinations ± standard deviation.

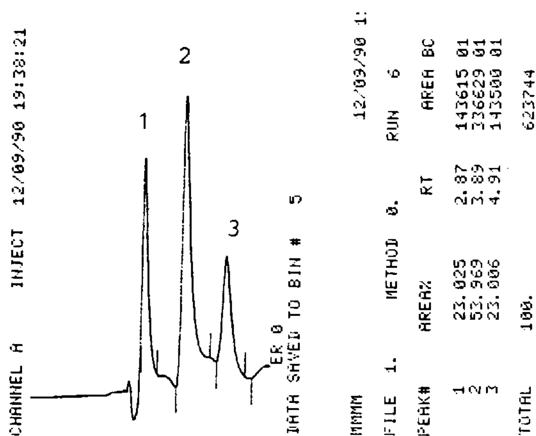

Figure 2. High-performance liquid chromatogram of Theophylline in Pharmaceutical preparations-Injections.
Peaks : 1 = Unknown , 2= Theophylline , 3= Caffeine

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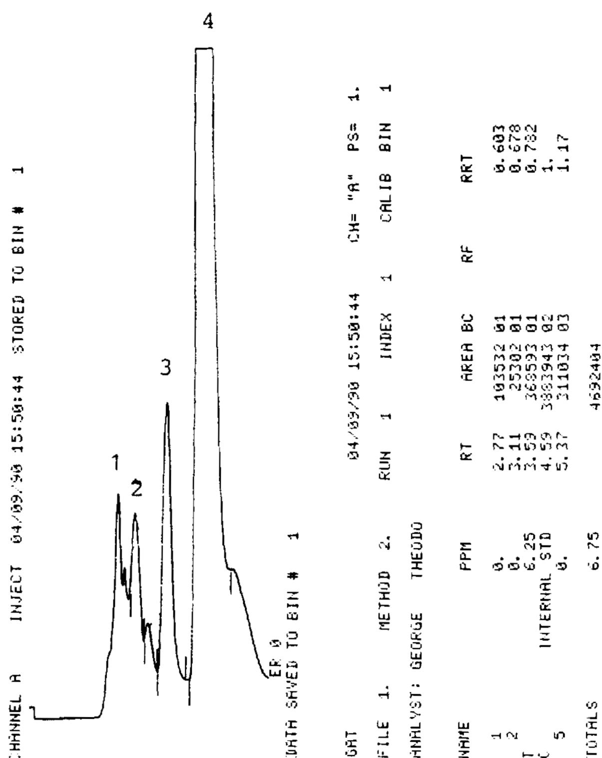

Figure 3. High-performance liquid chromatogram of Theophylline extracted from Blood.
Peaks: 1,2= Unknown , 3= Theophylline , 4= Caffeine

Results taken for the analysis of theophylline in methanol using caffeine as internal standard, after solid phase liquid extraction are presented in Table 4.

The results of the quantification of theophylline in different pharmaceutical formulations are laid out in Table 5.

These results were in accord with the labelled amount and there are not observed interferences from the excipients in the chromatograms. An example of the HPLC analysis of injections is given in Figure 2.

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TABLE 6

Determination of Theophylline in Blood Serum by HPLC using Caffeine as internal standard.

Drug	Added (ng)	Found[a] (ng)	Recovery %
	20	20.3±0.14	101.5
Theophylline	50	51.5±0.23	103.0
	100	98.2±0.41	98.2

[a] Mean value of ten determinations ± standard deviation.

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The proposed technique was also applied to the determination of theophylline, using caffeine as internal standard, in blood serum. Employing the standard addition technique it is possible to determine theophylline in 250 μl blood serum samples in under five minutes.

The method applied to the analysis of theophylline in blood samples which was collected from ten volunteers. Theophylline was determined by using caffeine as internal standard, in nine cases. One volunteer had consumed coffee and the chromatogram was dramatically changed.

In this case theophylline was successfully determined employing the procedure described without using internal standard. The chromatogram is given in Figure 3.

The experimental results taken for the analysis of theophylline in blood serum are given in Table 6.

CONCLUSION

Because of its high specificity, accuracy, precision and save of time the described HPLC method appears to be very useful for the routine analysis of

theophylline in blood serum, tablets, sprinkles, syrups, suppositories and injectable solutions.

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