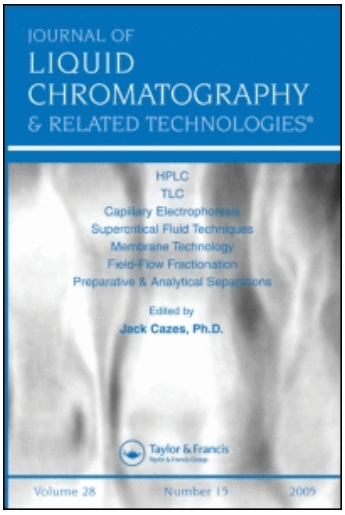

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J. P. Sommadossi[a]; C. Aubert[a]; J. P. Cano[a]; A. Durand[a]; A. Viala[a]
[a] I.N.S.E.R.M. SCN N° 16 Laboratoire de Pharmacocinétique, MARSEILLE, France

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DETERMINATION OF THEOPHYLLINE IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

J.P. Sommadossi, C. Aubert, J.P. Cano, A. Durand and A. Viala
I.N.S.E.R.M. SCN N° 16
Laboratoire de Pharmacocinétique et de Toxicocinétique
27, boulevard Jean Moulin
MARSEILLE 13385, France

ABSTRACT

We describe a rapid and highly specific method for determining theophylline in plasma. Following addition of ammonium sulfate and β-Hydroxy-ethyl-theophylline as internal standard, theophylline is extracted into a mixture of chloroform hexane (70 : 30) and evaluated by high performance liquid chromatography, using Microporasil "Waters" 10 µm as stationnary phase and N-hexane-ethanol (76 : 24) mixture as mobile phase. Absorption at 280 nm is monitored. The method has a good precision (coefficients of variation between 3 % and 4 % for 1 mg/l and 10 mg/l) and its sensitivity is about 0.25 mg/l. No interferences from endogenous compounds, metabolites of theophylline, or from drugs commonly co-administrated with theophylline have been encountered. This technique can be used in analytical toxicology, and also for therapeutic controls and pharmacokinetic studies.

INTRODUCTION

The determination of theophylline in blood is of interest for both the therapeutist and the toxicologist for several reasons. This medicine, used in the treatment of asthma and, more recently, that of apnea in new-born children, actually requires precise monitoring of its concentrations in plasma in order to best match the therapeutics to the individual clinical response. In addition, the therapeutic index is narrow due to the fact that the toxic and efficient doses are close to one another.

Since the findings of Schack and Waxler (1), based on the use of ultraviolet spectrophotometry, technology for the determination of theophylline in blood has considerably progressed. Thus, besides a few immuno-assays (2), most of the methods have relied on gas chromatography (3-5) and high pressure liquid chromatography (HPLC), the only two techniques fulfulling the desired specificity and sensitivity criteria.

The gas chromatographic techniques are applied after extraction by several solvents. They include the use of an internal standard and, in most cases, the formation of one derivative. Usually, detection is performed by flame ionization or, at times, by electron capture. Sheehan and al. (6) have recommanded its use in combination with mass spectrometry. These processes are rather easy to implement but prove inadequate, as a rule, due to the lengthy operations they require, particularly at the extraction and derivative formation stages.

Using the liquid chromatography techniques, the preliminary extraction is simplified even eliminated. One resorts either to adsorption (7-9) or to reverse phase chromatography (10-21) or to chromatography by ion-pair (22-23). According to the apparatus, the UV spectrometry detectors use a fixed (254 nm) or variable wavelength (in this latter case they operate on 275 or 280 nm) and also electrochemical detection (24-25) is employed. In view of the foregoing, we have preferred high performance liquid chromatography with internal calibration and detection by UV spectrometry at 280 nm. The method we have developed is presented below.

MATERIALS and METHODS

Apparatus and Operating Conditions

A Hewlett-Packard HPLC Model 1084 B with automatic injector and recorder/integrator 79850 ALC H.P. terminal is used. The

chromatograph is equiped with a column (30 cm long, 3.9 mm, μPorasil, 10 μm, Waters Associates).

The mobile phase is a mixture Hexane/Ethanol (76 : 24) with a flow rate of 1.5 ml/mn. Absorbance is monitored at 280 nm.

A Mass Spectrometer, Model 5980 A, with data system 5934 A (Hewlett-Packard) was also used to establish identity and purity of the theophylline liquid chromatography peak.

Reagents

All solvents and reagents (ethanol, n-hexane, chloroform and ammonium sulfate) are analytical grade.

The stock solutions of theophylline (N° T 1633 crystallin anhydrous "Sigma Chemical Company"), β-hydroxy-ethyl-theophylline ("Boehringer Ingelheim"), 1-methyl-xanthine (N° 69720 "Fluka"), 3-methyl-xanthine (N° 69722 "Fluka"), and 1,3-dimethyl-uric acid (N° D 2889 "Sigma Chemical Company"), prepared in ethanol, contain 1 mg per ml. Standard solutions are prepared by dilution in the same solvent.

Operating Procedure

The blood samples are collected in oxalated tubes (Venoject T 200 x F 105) and then centrifuged for 15 min at 2000 rpm and + 4° C ; at this time, the plasma should immediately be frozen until analysis.

Insert 0.5 ml of plasma into a centrifuge tube of suitable volume and stopper. Add 0.5 g of ammonium sulfate, 2 ml of double-distilled water and 1 to 10 μg of internal standard (according to the anticipated theophylline quantity to be determined in the sample). Mix the contents on a vortex-type mixer for 15 s after each new addition of a different product. Add 15 ml of the chloroform-hexane mixture then mechanically agitate for twenty minutes. Centrifuge at 4000 rpm during 5 minutes at + 4° C. Recover the organic phase, dry-evaporate at 60° C under nitrogen stream and pick up the residue in 100 μl of ethanol while vortex-mixing for 15 s. Then inject a 20 μl sample of this solution into the chromatograph for analysis.

Under the conditions defined above, the retention times of the theophylline (I) and the internal standard (II) were 6.88 and 10.33 minutes, respectively (Figure 1).

The ratio of the I/II peak areas were calculated with an integrator. Refer to calibration curves (Figures 2-3) plotted from I/II peak area ratios obtained after sample analysis of test sample plasmas to which increasing quantities of theophylline were added (0.25 to 2.00 µg/ml or 2.5 to 40 µg/ml) and a constant quantity of internal standard (1 µg or 10 µg) as well.

RESULTS and DISCUSSION

Internal standard

β-hydroxyethyltheophylline was chosen as an internal standard. This substance is structurally very similar to theophylline ; their maximum absorption wavelength is the same and their extraction conditions are alike. In addition, β-hydroxyethyl-theophylline is neither a drug nor a theophylline metabolite.

Specificity

Maximum absorbance of theophylline occurred at 272 nm with the instruments used. However we have preferred to operate at 280 nm since the base line is better at this wavelength and the technical capabilities are improved. Actually, between 270 and 275 nm, interferences due to substances existing in the plasma have been noticed. This phenomenum is particularly apparent whenever the theophylline quantities to be determined are small.

No interferences were observed from uric acid or the theophylline metabolites (1 methylxanthine, 3 methylxanthine and 1.3 dimethyl uric acid). Their retention times are 3.40, 7.53, 8.68 and 21.39 minutes, respectively.

In regards to specificity, no interference exists from caffeine or theobromine, even if the levels of these components are significant (Figure 4). The same holds true for

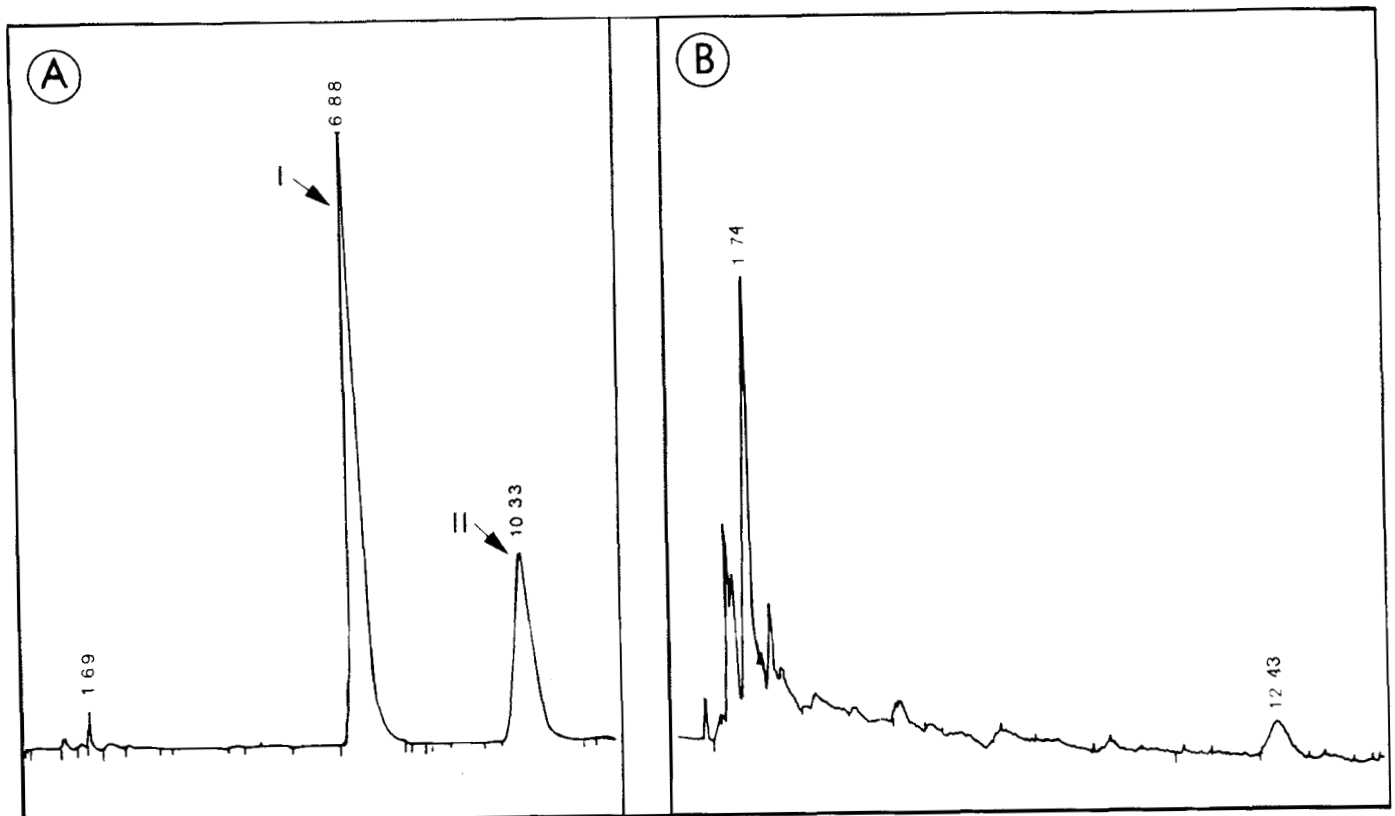
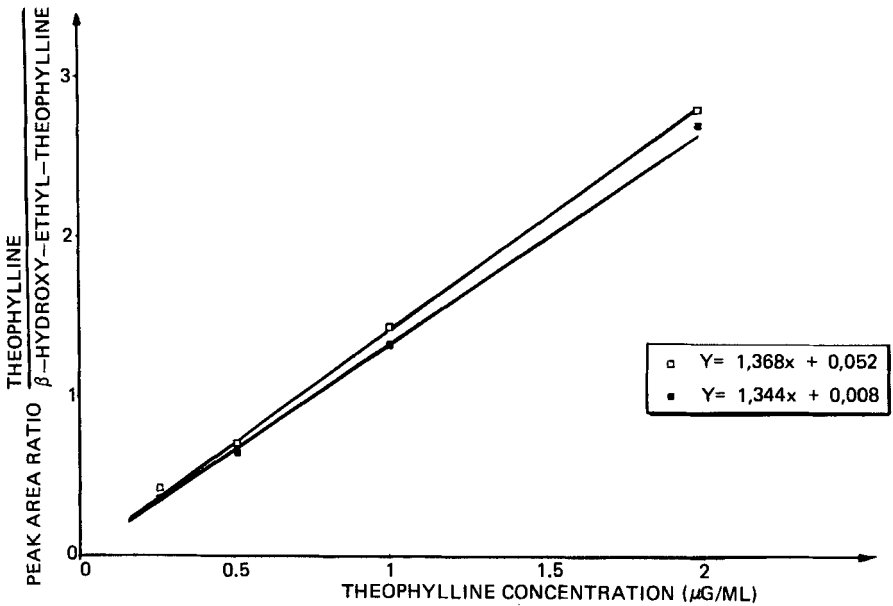


FIGURE 1. (A): Chromatogram of a patient's plasma, with internal standard, containing 12 µg/ml of Theophylline. I, theophylline; II, β-hydroxy-ethyl-theophylline (internal standard). (B): Plasma control after extraction.



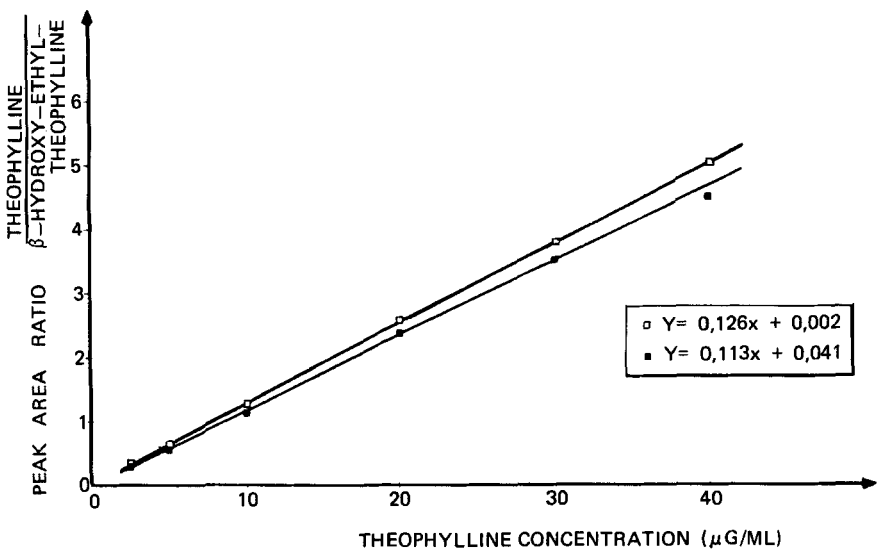
2. Standard curves for the quantitative analysis and determination of the relative recovery to the internal standard of Theophylline with low values

   □ Theophylline and Internal Standard were chromatographed directly (r = 0,99)

   ■ Both Theophylline and Internal Standard were extracted from plasma (r = 0,99).

drugs such as salbutamol (Ventoline^{R}) and terbutaline (Bricanyl^{R}) used as relays for theophylline and corticoids. But we note an interference with Bactrim^{R} (association of sulfamethoxazole and trimethoprim).

Moreover, plasma from a patient has been checked using mass spectrometry to show that the liquid chromatography peak with a retention time of 6.88 minutes is actually imputable to the theophylline itself by its molecular ion (M-181) (Figure 5). For this work, the drug-containing fraction was collected and introduced into the mass spectrometer via the direct insertion probe after evaporation of the solvent.



3. Standard curves for the quantitative analysis and determination of the relative recovery to the internal standard of Theophylline with high values

   □ Theophylline and Internal Standard were chromatographed directly (r = 0,99)

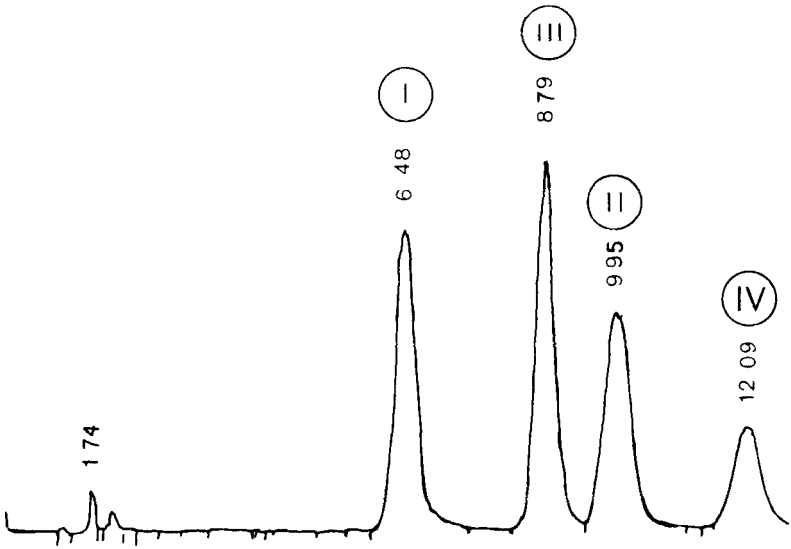
   ■ Both Theophylline and Internal Standard were extracted from plasma (r = 0,99)

Extraction Procedure

The performance of the proposed method is influenced by the extraction pH and the nature of the solvents. The best results have been obtained by performing the extraction with the chloroform/hexane mixture (7/3) at plasma pH, in presence of ammonium sulfate which precipitates serum proteins and reduces the significance of the interferences above mentioned. Under these conditions, the percent recovery determined for theophylline quantities from 0.25 to 40 μg was approximately 94.

Sensitivity, Reproducibility and Accuracy

Similarly, for routine assays, the quantitative limit of sensitivity is about 0.25 mg/liter.



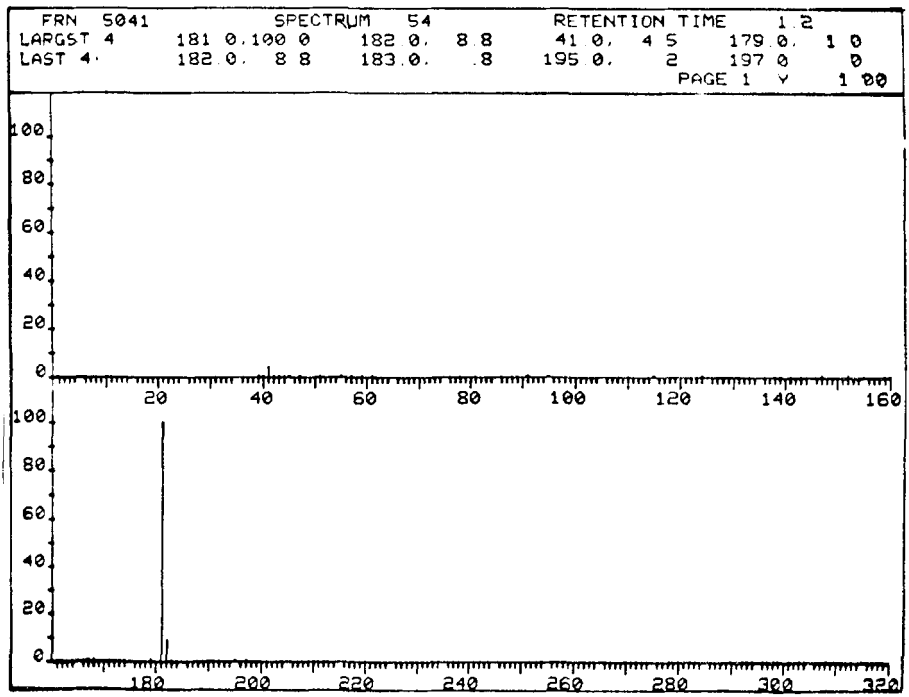
4. Chromatogram of a mixture of xanthines
I, theophylline ; II, Internal Standard ; III, caffeine ; IV, theobromine

Repeatability was investigated by analyzing a plasma pool containing 0.5 µg theophylline and 0.5 µg internal standard per 0.5 ml. The coefficient of variation within tests, for 6 successive extractions and assays was 3 %. With larger quantities (5 µg theophylline and 5 µg internal standard) in 0.5 ml plasma and 7 successive operations it was 4 %.

Injection repeatability was also considered. By injecting the same ethanol solution of theophylline and internal standard (1 and 1 µg), 6 times, the coefficient of variation was 1 %.

APPLICATIONS

The proposed technique is useful in toxicology, especially for the diagnosis of possible theophylline overdosage. It can also serve for performing various assays in the therapeutic



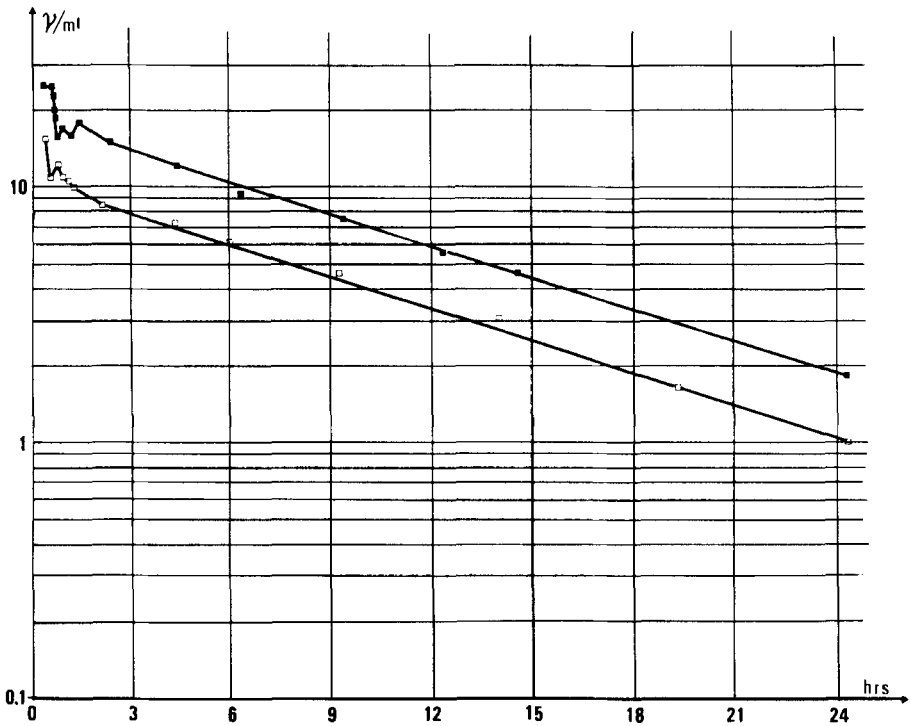
5. Mass spectrum for collected theophylline fraction (chemical ionisation mode ; $(M + 1)^+ = 181$) via the direct insertion probe into the mass spectrometer

and pharmacovigilance fields on both adults and children. Thus, as a routine procedure, it will be easy to check that the theophylline level is within the limits of the range generally recognized as efficient, between 10 and 20 mg/liter, and does not exceed the 25 mg/liter threshold beyond which toxicity would exist. The good sensitivity of the process also enables extension of its use to pharmacokinetics research.

We have studied the decrease, with time, of theophylline plasma levels of a patient having severe asthma crises, who had received, successively, at 72 h intervals, 240, then 480 mg by short infusion (15 mn). This application established the kinetic linearity in the zone of therapeutic concentrations.

The erratic aspect of the plasma concentration decreases between 15th mn and 1st H derives from a phenomenum of theophylline absorption on the catheter during the infusion. For the two dosages, 240 and 480 mg, the half-life varies from 9 to 7 h and plasmatic clearance from 2.2 to 2.5 l/h (Figure 6).

Using this method, we could individualize theophylline posology, by a previous pharmacokinetical identification, in view of increasing drug efficacity and decreasing toxic effects (26).



6. Theophylline plasma levels after I.V. infusion during 15 mn.

Dose administrated (□—□ = 240 mg ; ■—■ = 480 mg)

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