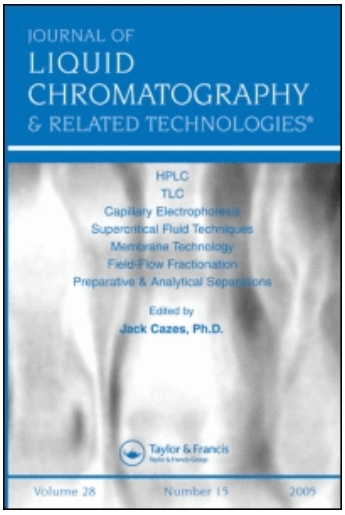

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Augustijns, P. and Verbeke, N.(1992) 'A Microassay Method for the Determination of Theophylline in Biological Samples Using HPLC with Electrochemical Detection', Journal of Liquid Chromatography & Related Technologies, 15: 8, 1303 — 1313
To link to this Article: DOI: 10.1080/10826079208018289
URL: http://dx.doi.org/10.1080/10826079208018289

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(8), 1303-1313 (1992)

A MICROASSAY METHOD FOR THE DETERMINATION OF THEOPHYLLINE IN BIOLOGICAL SAMPLES USING HPLC WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A micro assay method is presented to measure theophylline plasma levels by HPLC with electrochemic detection.
Plasma samples of 10 µl were sufficient to determine theophylline levels down to 0.2 µg/ml. Intraday and interday variations were lower than 4% and 6% respectively.
This method was evaluated by comparison with an immun assay and correlation was excellent.
The method is sensitive enough to allow blood samplin by the finger prick method in order to perform monitoring of drug levels.

INTRODUCTION

Theophylline is endowed with several pharmacological actions: stimulation of the central nervous system, diuretic effects, inotropic action on

cardiac muscle and smooth muscle relaxation. Therapeutic uses are essentially based on the effects on CNS and on smooth muscle. Theophylline is useful to prevent apnoea in neonates, to cure acute asthmatic attacks and for prophylaxis of bronchial asthma.

Plenty of structurally related compounds (diprophylline, bamiphylline, proxyphylline, etc...) have been developed to improve the bioavailability and kinetic properties of theophylline. A lot of salts of theophylline (ethylenediamine, choline, sodium glycinate, calcium salicylate) have been prepared for the same reasons. However, theophylline is still commonly used for chronic therapy, especially since the development of slow release preparations with reliable and complete absorption characteristics.

It is essential to monitor theophylline plasma levels in order to avoid adverse effects and life threatening toxicity (1). Plasma levels of theophylline are influenced by various factors which affect clearance : age, smoking, enzyme induction, drug interactions, liver pathology, etc...

Various assay methods have been described. The main choice lies between chromatographic methods (2-6), offering high selectivity and avoiding in particular interference with metabolites and other xanthines (caffeine, theobromine), and immunological competition methods (RIA, EMIT, FPIA) offering high sensitivity and speed (7).

The present work is an attempt to conciliate selectivity and sensitivity requirements : concentrations down to 200 ng/ml and the use of small plasma samples of no more than 10 μl. A theophylline assay method needs to be suitable for samples taken from newborn children or in adults by the fingerprick method.

MATERIALS AND METHODS

Reagents

Methanol, acetonitrile, dichloromethane and isopropyl alcohol were obtained from Carlo Erba (Milano, Italy). Potassium dihydrogenphosphate was purchased from UCB (Leuven, Belgium). Theophylline, diprophylline (= internal standard), theobromine and caffeine were supplied by Federa (Brussels, Belgium). 3-Methylxanthine was obtained from Sigma (Deisenhofen, Germany).
All reagents were analytical grade and were used as received.

The water we used throughout the procedure was purified by a Millipore Milli-Q system (Bedford, USA).

Apparatus

HPLC analyses were carried out with a Waters Assoc. (Milford, USA) chromatography system consisting of a Model 6000A pump, connected to a U6K-injector. A double detection system was used consisting of an electrochemical detector (ESA Coulochem Model 5100 A, Bedford, U.S.A.) equipped with an analytical cell (Model 5010) and a guard cell (Model 5020) in line with a UV detector (Lambda Max Model 480, Waters Assoc).

The output signals of the electrochemical and the UV detector were recorded with Chromatopac C-R1B integrators (Shimadzu, Kyoto, Japan).

Chromatographic conditions

Chromatographic separation was carried out on a NovaPak C-18 column (Waters Assoc.) under radial

compression. The mobile phase consisting of 0.01 M KH_2PO_4, CH_3CN and CH_3OH (900-25-90) was degassed in an ultrasonic bath. The flow rate of the mobile phase was 2 ml/min. All separations were done at room temperature.

Detection

For detection of theophylline, the electrochemical detector was used in the oxidative screen mode. The analytical cell is composed of two serial electrodes. Each one can be adjusted at a different potential. The first electrode (= screen electrode) was set at a potential of 0.68 V, and the second (= the measuring electrode) at 0.98 V.

The guard cell, placed before the injector, was set at a potential of 1.0 V.

No convenient internal standard with similar extraction, chromatographic and electrochemical properties as theophylline is available. Diprophylline was used as internal standard, which was monitored with a UV detector set at 270 nm.

Extraction procedure

Plasma samples (10 µl) were diluted with 300 µl KH_2PO_4 buffer (0.1 M, pH 6.0). After adding 100 µl of a solution of 10 µg/ml internal standard, the mixture was extracted with 2 ml chloroform:isopropyl alcohol (1/1). Test tubes were vortexed during 30 seconds and centrifuged for 10 min at 2000 RPM. After centrifugation, the water layer was removed by vacuum aspiration. The organic layer was transferred to a dry test tube and evaporated to dryness at 40 °C under a gentle stream of air. The residue was dissolved in 100

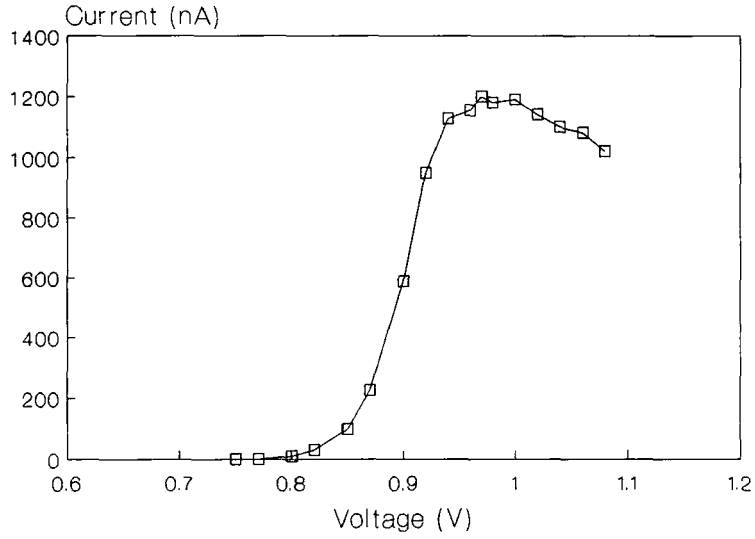

FIGURE 1 : Current-voltage curve of theophylline

µl of mobile phase and 25 µl was injected into the chromatographic system.

Calibration

Concentrations of theophylline were determined using a calibration graph in which peak height of the drug (EC-detection) over the peak height of the internal standard (UV-detection) was plotted against concentration.

The standard solution contained 1.00 mg/ml of theophylline in CH_3OH. Theophylline concentrations ranging from 2.5 to 20 µg/ml were prepared by dilution in plasma.

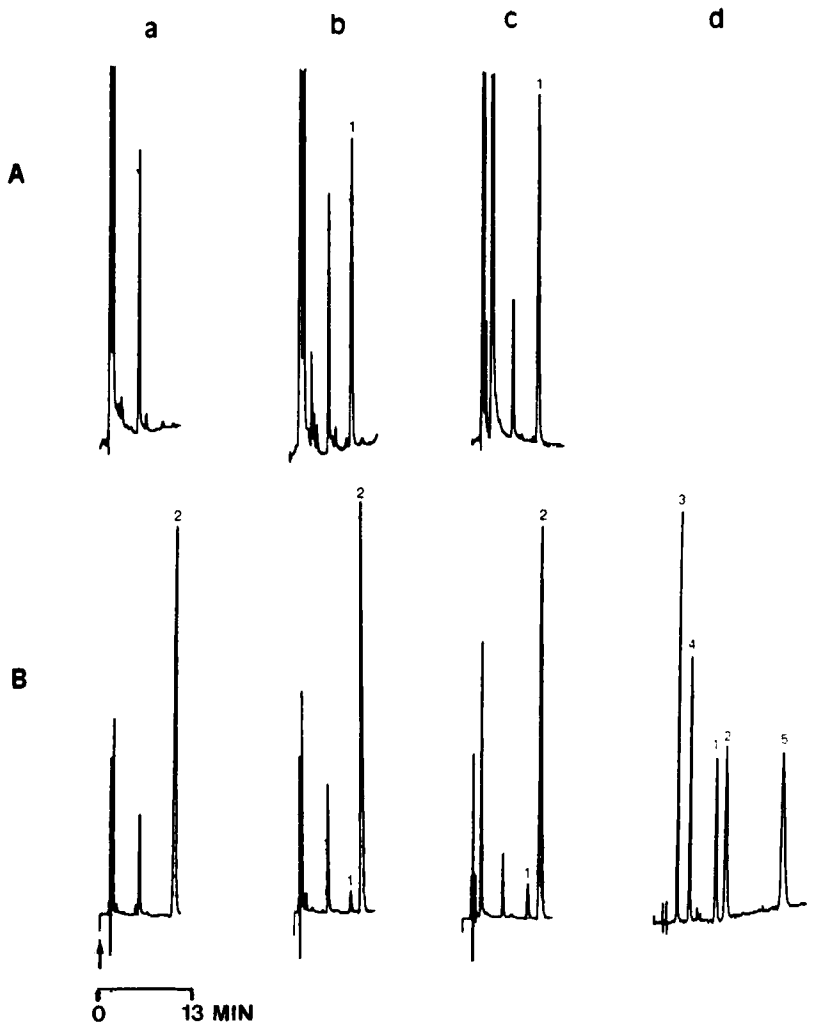

FIGURE 2 : Representative chromatograms of HPLC assay: A : chromatograms obtained by EC detection, B : corresponding chromatograms recorded by UV detection. (a) blank blood sample; (b) plasma spiked with 10 μg/ml of theophylline; (c) human plasma sample after an oral dose of theophylline; (d) test solution (recorded by UV detection) containing theophylline (1), diprophylline (2), 3-methylxanthine (3), theobromine (4) and caffeine (5).

TABLE 1

	Concentration added (μg/ml)	Concentration found ± S.D. (μg/ml)	RSD
Interday variation (n=8)			
	2.50	2.54 ± 0.14	5.39
	5.00	4.97 ± 0.13	2.69
	10.00	9.87 ± 0.24	2.48
	20.00	19.70 ± 0.36	1.83
Intraday variation (n=10)			
	2.50	2.54 ± 0.09	3.82
	5.00	5.12 ± 0.19	3.79
	10.00	10.03 ± 0.27	2.72
	20.00	20.29 ± 0.37	1.84

RESULTS AND DISCUSSION

The current-voltage curve of theophylline, obtained by repeatedly injecting the same amount of theophylline, increasing the potential after each injection, is shown in figure 1. At a potential of 0.68 V no oxidation of theophylline occurs, so the first electrode of the analytical cell was put at 0.68 V in order to remove all impurities that could be oxidized up to 0.68 V. The second electrode, the measuring one, was put at 0.98 V, the maximum of the current-voltage relationship. The guard cell, placed before the injector, was set at a potential of 1.0 V, so all impurities of the mobile phase that could interfere with electrochemical detection at 0.98 V were removed.

Figure 2 shows representative chromatograms of spiked plasma samples for theophylline with corresponding chromatograms for the internal standard.

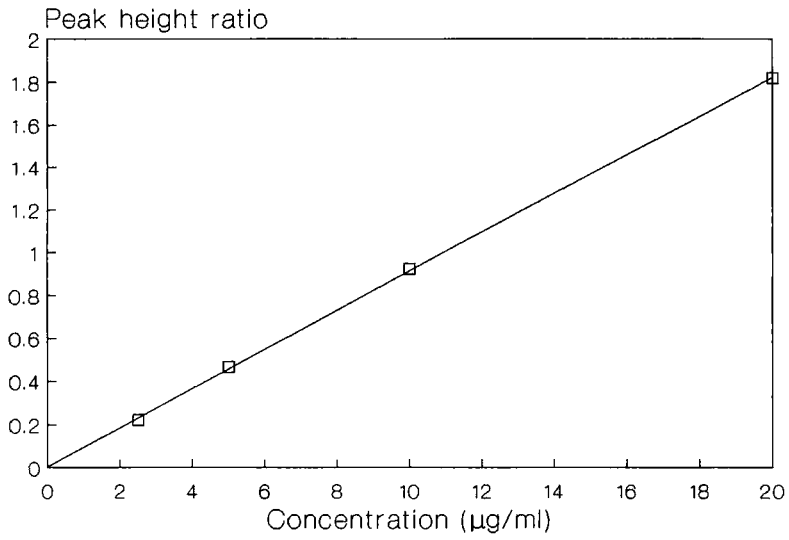

FIGURE 3 : Calibration curve for theophylline in plasma

The extraction yield of theophylline and diprophylline from plasma was determined at a concentration of 10 µg/ml, by comparing the results of a full analysis with the results of a standard solution in the mobile phase. Recovery amounted to 102 ± 2 % for theophylline and 89.3 ± 1.8 % for the internal standard.

The intraday (n=10) and interday (n=8) reproducibility of the assay were determined at a concentration of 2.5, 5, 10 and 20 µg/ml theophylline. Variations, expressed as the relative standard deviation (RSD), were lower than 4% and 6% respectively. Results are reported in table 1.

The standard curve for theophylline showed good linearity and passed nearly the origin (r=0.999). A typical standard curve, ranging from 2.5 to 20 µg/ml theophylline is shown in figure 3.

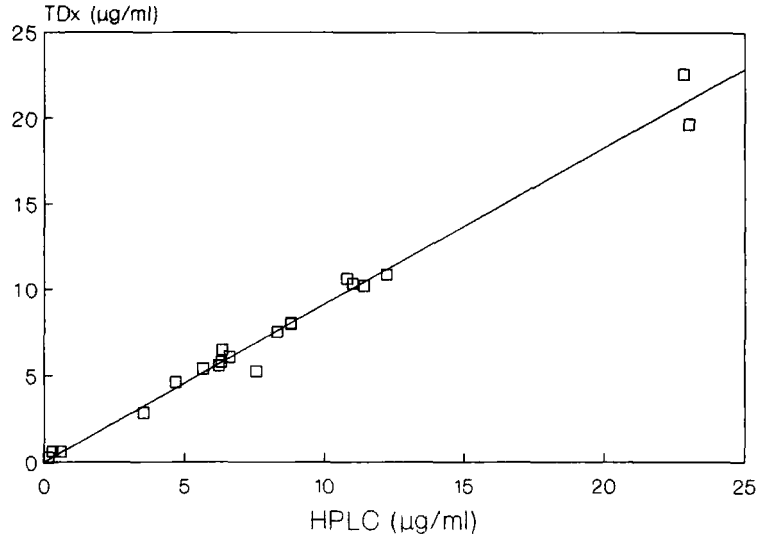

FIGURE 4 : Correlation of plasma levels measured in patient samples by the HPLC method and by TD_x.

The limit of sensitivity of the assay, estimated as corresponding to a signal to noise ratio of 3/1, was 0.2 µg/ml.

The method described here was evaluated in comparison with a fluorescence polarized immunoassay (TD_x, Abbott). The correlation coefficient amounted to 0.992, as illustrated in figure 4.

No interference of caffeine and theobromine, often present in plasma samples, could be observed. 3-methylxanthine, a major metabolite of theophylline, did also not interfere with our HPLC method.

CONCLUSION

A specific method was developed for the determination of theophylline using as little as 10 µl of plasma.

By the use of electrochemical detection, a 5 times lower limit of sensitivity could be obtained as compared with UV detection.

Acknowledgment

The authors wish to thank Dr. R.Verbesselt (Laboratory of Pharmacology, O&N, Gasthuisberg, 3000 Leuven) for the determination of theophylline plasma levels by FPIA (TD_x, Abbott).

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Received: August 16, 1991
Accepted: October 29, 1991