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AUTOMATED THEOPHYLLINE ASSAY USING GAS CHROMATOGRAPHY AND A MASS-SELECTIVE DETECTOR

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SUMMARY

An automated gas chromatographic—mass spectrometric assay for theophylline is described. Theophylline is extracted from plasma or urine (50 μl) and transformed into an N-pentyl derivative. The internal standard used for quantitation is [1,3-^{15}N, 2-^{13}C]theophylline. The detection is performed by monitoring the molecular ions 250 for theophylline and 253 for the internal standard with a quadrupole mass specific detector HP 5790 A. The system has been fully automated: injection, calibration, assay, calculation. The method shows excellent analytical parameters: linearity between 2 and 40 μg/ml; day-to-day reproducibility 1.82% for a concentration of 15 μg/ml; repeatability 0.75% (15 μg/ml) and 0.33% (30 μg/ml). Accuracy is also excellent. Due to the use of an internal standard labelled with stable isotopes, the specificity and high analytical quality of the method make it useful as a reference method to compare with routine theophylline assays.

INTRODUCTION

Theophylline (1,3-dimethylxanthine) is widely used to treat asthma, apnea of prematurity and obstructive lung diseases. To be effective, theophylline plasma levels must be within a narrow therapeutic range (7—20 mg/l) [1, 2].

Many factors have been reported to cause variations in the pharmacokinetics of this drug, among them age [3], nutrition [4], diseases [5, 6], administration of other drugs or xenobiotics [7—9]. Because of the narrow therapeutic index

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and numerous sources of kinetic variations, theophylline levels have to be monitored carefully to insure safe and effective use of this drug.

Current methods for measuring theophylline in plasma or serum include spectrophotometry, liquid chromatography and several immunoassay techniques.

We describe here a method using capillary gas chromatographic (GC) separation and detection with a mass specific detector with automatic monitoring of the analytical procedure. This method can deliver one assessment every 16 min using theophylline labelled with stable isotopes (^{15}N, ^{13}C) as an internal standard.

EXPERIMENTAL

Materials

Theophylline (1,3-dimethylxanthine) was purchased from Sigma (St. Louis, MO, U.S.A.). The internal standard, [1,3-^{15}N, 2-^{13}C]theophylline, was synthesized by C.E.A. (Saclay, France). Tetramethylammonium hydroxide, N,N-dimethylacetamide, iodopentane, isopropanol, ethyl acetate and chloroform (analytical-grade reagent) were purchased from E. Merck (Darmstadt, F.R.G.) and used without further purification. In order to check the analytical quality of the method, standard sera containing various known concentrations of theophylline were obtained from Biotrol (Paris, France) and Syva Biomérieux (Lyon, France).

The stock solution of internal standard (100 μg ml^{-1}) was prepared in ethanol. It was stored at 4°C and could be used within a month. The working solution was prepared by a ten-fold dilution in water (10 μg ml^{-1}). The extraction solvent was chloroform—isopropanol (95:5). Before extraction, serum or plasma was buffered with acetate buffer (pH 5.2).

Extraction

To 50 μl of serum or plasma in a 20-ml conical centrifuge tube were added 200 μl of acetate buffer (pH 5.2) and 50 μl of internal standard solution (10 μg ml^{-1}). After mixing and addition of 2 ml extraction solvent, the sample was extracted for 1 min on a vortex mixer. After centrifugation, the organic phase was transferred to another tube and evaporated to dryness under a stream of nitrogen at 40°C.

Derivatization of the NH groups of both theophylline and internal standard was performed according to the alkylation procedure described by Greeley [10] for barbiturates and adapted to theophylline by Johnson et al. [11] for N-butyl derivatives and by Lowry et al. [12], Berthou et al. [13], Joern [14] for N-pentyl derivatives, and for methylxanthines as we described previously [15]. A 50-μl volume of N,N-dimethylacetamide and 25 μl of a 0.1 *M* solution of tetramethylammonium hydroxide were added to the drug residue and thoroughly mixed for 10 sec. Then 25 μl of iodopentane were added to the solution which was shaken and allowed to stand at room temperature for 10 min. The organic phase was transferred to another tube and evaporated to dryness. The sample was then ready for injection.

Chromatographic separation

The gas chromatograph used was a Hewlett-Packard Model 5790 designed for capillary column chromatography. This apparatus was equipped with an automatic injector HP 7672 A. The capillary column used was a cross-linked dimethylsilicone silica column, 12 m × 0.23 mm. Samples were automatically injected into the chromatograph according to the splitless mode. The splitless valve time was 1 min. The injector temperature was set at 250°C. Oven temperature was programmed from 115°C (1 min) to 195°C (5 min) at 15°C/min. The transfer line temperature was 260°C. Helium was used as carrier gas. Before splitless injection, the drug extraction residue was dissolved in 70 μl of a toluene—ethyl acetate mixture (50:20, v/v). The injected volume was 1 μl.

Detection and measurements

A mass-selective detector Hewlett Packard 5970 A was used for detection. The monitored ions were *m/z* 250 for theophylline and *m/z* 253 for the internal standard. The dwell time was 75 msec for each ion. The detector was operating between 7 and 8.5 min, the retention time of theophylline being 7.76 min in the described conditions. Areas were integrated in the horizontal mode with a 5% slope sensitivity and a rejection area of 150.

Analytical-quality parameters measurements

Standardization and linearity were checked from Syva standard sera using the following concentrations: 2.5, 5, 10, 20 and 40 $\mu g\ ml^{-1}$. In order to check reproducibility two samples containing theopylline at 7 and 15 $\mu g\ ml^{-1}$ (Biotrol) were extracted and measured once a day for ten days. Precision was measured by the determination of theophylline concentrations ten times a day from standard sera (Biotrol) containing 7.5, 10 or 30 $\mu g\ ml^{-1}$ theophylline.

Accuracy was determined by comparison of measured concentrations and true concentrations from fifteen different commercial standard sera ranging between 2.5 and 40 $\mu g\ ml^{-1}$. Each of these sera was extracted twice and extraction residues injected twice.

The method was then validated by the routine determination of 100 samples for therapeutic drug monitoring and the results were compared with those obtained by the enzyme multiplied immunoassay technique (EMIT).

RESULTS

Mass spectra and fragmentograms

Fig. 1 shows the mass spectra of the N7-pentyl derivatives of theophylline (Fig. 1A) and [1,3-^{15}N, 2-^{13}C]theophylline (Fig. 1B) used as internal standard. Molecular ions are *m/z* 250 and 253, respectively, and base peaks *m/z* 180 and 183, respectively. They correspond to the M—pentyl (M-70) fragment ion. Because of slight interference in patient's serum or plasma at *m/z* 180 due to a fatty acid fragment, molecular ions were used for mass fragmentography. The relative abundance of these ions was large enough (45%) to ensure a good analytical quantitation. Typical fragmentograms are shown in Fig. 2a for a standard serum and in Fig. 2b for a patient's serum. They correspond to the monitoring of ions 250 and 253. The retention time of the corresponding chro-

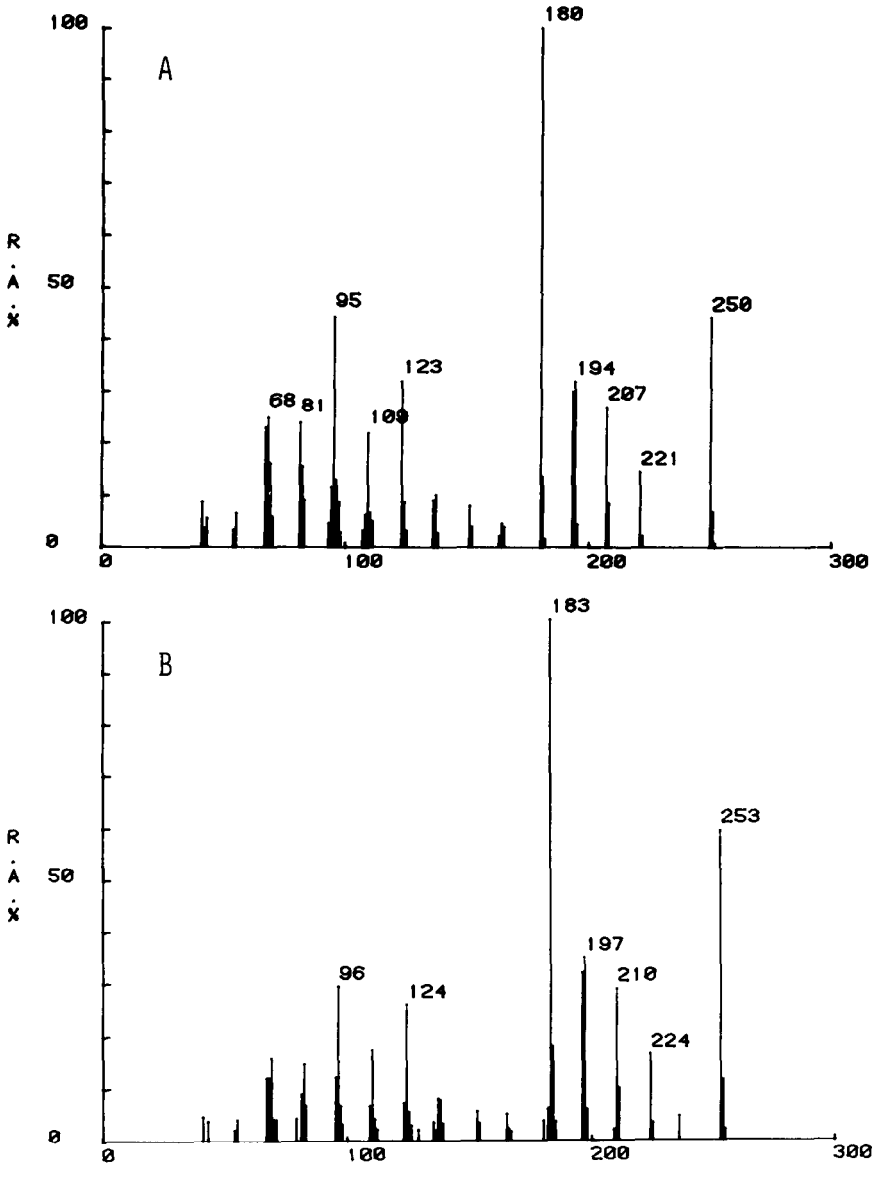

Fig. 1. (A) Mass spectrum of N-7-pentyl derivatives of theophylline. (B) Mass spectrum of N-7-pentyl derivatives of [1,3-^{15}N,2-^{13}C]theophylline.

matographic peak, in the described conditions, is 7.76 min. The fragmentogram of a blank sample does not show any peak at this retention time.

Linearity

A standard curve obtained under the conditions described above is shown with its confidence interval in Fig. 3. Each point is the result of a duplicate injection. The regression analysis gave the following results: slope 0.1108; intercept 0.035, r = 0.997; residual error $1.7 \cdot 10^{-3}$. Samples for another

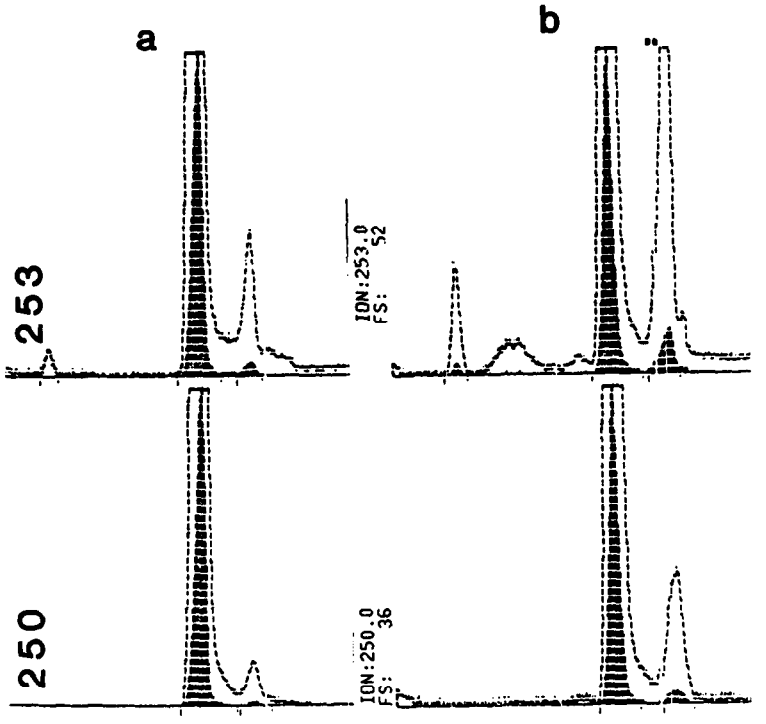

Fig. 2. Typical fragmentograms of ions 250 and 253 (a) from a standard serum, (b) from a patient's serum.

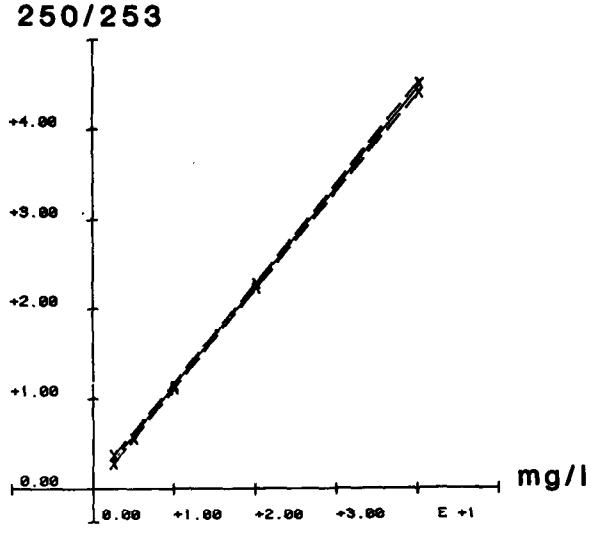

Fig. 3. Calibration curve with confidence interval.

standard curve were extracted and injected only once on day 0 and then on day 6. The results shown in Table I indicate that linearity is very good in the range 2.5—40 μg ml^{-1} and that extracted samples can be stored at 4°C before measurement without any loss.

TABLE I

LINEAR REGRESSION OF A STANDARD CURVE MEASURED ON DAY 0 AND DAY 6

	Day 0	Day 6
Slope	0.1059	0.1056
Intercept	$4.8 \cdot 10^{-2}$	$6.3 \cdot 10^{-2}$
r	0.99993	0.99994
Residual error	$2.34 \cdot 10^{-4}$	$3.25 \cdot 10^{-4}$

Day-to-day reproducibility

The day-to-day reproducibility measured by ten assays corresponding to a ten-day period gave the following results for the coefficient of variation: 1.93% for 7 μg ml^{-1} and 1.82% for 15 μg ml^{-1}.

Repeatability

Ten determinations of the same concentration on the same day gave the following coefficients of variation: 1.87% (7.5 μg ml^{-1}), 0.76% (15 μg ml^{-1}) and 0.33% (30 μg ml^{-1}).

Accuracy

The comparison of measured concentrations against true concentrations in the range 2.5—40 μg ml^{-1} for fifteen different standard sera gave the following results: slope 0.992; intercept 0.035, $r = 0.9999$; residual error $1.7 \cdot 10^{-3}$.

One hundred sera from patients treated with theophylline have been assayed with this method and the results compared with the EMIT. Concentrations ranged between 0 and 25 μg ml^{-1}. The parameters of the correlation (X = EMIT, Y = this method) are: slope 0.951; intercept 0.188; $r = 0.9997$. Results of the two methods are in good agreement.

CONCLUSIONS

This automatic method for theophylline assay exhibits good parameters in terms of analytical quality and practicability. Due to these qualities it can be used as a "reference method" to test other kinds of theophylline assays. Moreover, the automation of the analytical procedure affords a high degree of safety and quality for routine analysis. Finally, a mass-selective detector connected to a capillary gas chromatograph and the use of internal standard labelled with stable isotopes and automatic analytical procedures produce a flexible, practical and versatile tool which can be adapted to many other drug assays as well as therapeutic and biological profiles.

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