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ANALYTICAL METHODOLOGY TO DETERMINE STABLE ISOTOPICALLY LABELLED AND UNLABELLED THEOPHYLLINE IN HUMAN PLASMA USING CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A method is described for the simultaneous determination of [1,3-^{15}N]theophylline and unlabelled theophylline in human plasma using gas chromatography–mass spectrometry. Plasma samples were subjected to extractive alkylation and the stable isotopically labelled and unlabelled forms of the drug were analysed as their N-pentafluorobenzyl derivatives on an SE-52 fused-silica capillary column. Quantitation was made by selected-ion monitoring employing as the internal standard 3-isobutyl-1-methylxanthine. The method has been used to study the absorption kinetics and bioavailability of a sustained release formulation of the drug when co-administered to human volunteers with a conventional formulation of the drug labelled with the stable isotope.

INTRODUCTION

Theophylline (1,3-dimethylxanthine) is used extensively as a bronchodilator in the treatment of asthma and other reversible obstructive airway disorders. Maximal bronchodilation with minimal toxicity is seen with plasma concentrations in the range 10–20 μg/ml, and a range of side-effects of increasing severity is seen with sustained plasma levels of >20 μg/ml. The substantial inter- and intra-patient variation in theophylline clearance, its rapid absorption and short elimination half-life all make it difficult to maintain plasma levels within this narrow therapeutic range with conventional tablet preparations. To attempt to

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overcome these problems, theophylline is nowadays generally used in sustained release formulations, a substantial number of which are commercially available in most countries.

The literature contains a number of methods for the determination of the absorption kinetics and bioavailability of a drug from a sustained release formulation [1, 2]. All of these require a knowledge of the pharmacokinetic behaviour of the drug in the same panel of subjects after the oral administration of a conventional formulation, or its intravenous infusion. In the case of theophylline, such approaches are hampered by the known intra-subject lability of its pharmacokinetics (for example, its elimination rate constant, K_{el}, may vary up to 60% between two administrations three to four days apart [3]) rendering the use of reference data obtained in separate studies problematical [4]. To overcome these problems, we have administered stable-isotope-labelled [1,3-^{15}N]theophylline concurrently with a widely used sustained release formulation of this drug (Uniphyllin; Napp Labs., U.K.), and distinguished the two forms (^{14}N and ^{15}N) of the drug in the plasma by means of capillary gas chromatography (GC) with selected-ion monitoring (SIM) mass spectrometry (MS). This permits the simultaneous determination of the pharmacokinetic behaviour of both test and reference formulations and establishes unequivocally the absorption properties and bioavailability of the drug from the sustained release formulation.

EXPERIMENTAL

Standards and reagents

[1,3-^{15}N]Theophylline was obtained from CEA Saclay (France). Non-labelled theophylline and 3-isobutyl-1-methylxanthine were purchased from Aldrich (Gillingham, U.K.). Stock standard solutions of these reference compounds prepared in water at a concentration of 1 mg/ml were stored at 0°C. Working standards were prepared by dilution of these solutions before use. β-Hydroxyethyltheophylline (Sigma, Poole, U.K.) was prepared at a concentration of 20 μg/ml in 10% (w/v) trichloroacetic acid and was also stored at 0°C. [^{14}C]Theophylline (specific activity 38 mCi/mmol) was obtained from Amersham International (Amersham, U.K.). All solvents were BDH (Poole, U.K.) Analar grade and were used without further purification.

Tetrabutylammonium hydrogen sulphate (TBAH, Aldrich) was prepared as a 0.5 *M* solution in 1.0 *M* sodium hydroxide. Pentafluorobenzyl bromide was also obtained from Aldrich.

Gas chromatography–mass spectrometry

GC–MS analyses were carried out on a 70-70 VG Analytical double-focusing instrument interfaced to a Carlo Erba HRGC 5160 Mega Series gas chromatograph. The SE-52 coated fused-silica capillary column (20 m × 0.32 mm I.D., film thickness 0.25 μm) used for the analyses was prepared in the authors' laboratory. Samples were introduced either by cold on-column injection or by split injection into a 1 m × 0.32 mm I.D. deactivated fused-silica capillary which was attached to the analytical column by means of a Valco butt connector. The helium carrier

gas flow-rate was 2 ml/min measured at a column temperature of 200°C. The column was maintained at 80°C for 1 min, then programmed at 30°C/min to 280°C. The mass spectrometer was operated in the electron-impact mode with an ionisation potential of 70 eV, trap current of 200 μA and an accelerating voltage of 4 kV. The source temperature was 220°C and the GC–MS interface temperature 280°C. SIM at 0.1 s per mass channel was carried out using the *m/z* 326 in the spectra of tri(pentafluoroethyl)-S-triazine (Fluorochem, Glossop, U.K.) as the lock mass. The ion intensity was recorded using a Rikadenki Series DBE-6 multi-pen recorder.

Gas chromatography with nitrogen–phosphorus detection (NPD)

The instrument used for the GC determinations was a Carlo Erba Fractovap 4160 equipped with an NPD-40 nitrogen–phosphorus detector. The analyses were carried out on a 20 m×0.32 mm I.D. SE-52 fused-silica capillary operated under similar conditions as for GC–MS analyses. Samples were introduced by split injection (10:1 split ratio).

High-performance liquid chromatography (HPLC)

The HPLC equipment (Waters Assoc. Milford, MA, U.S.A.) consisted of a Model 6000A pump, a Model 720 system controller and a Model 440 UV detector. Samples were injected with a WISP 710B autoliquid injector. The column (25 cm×5 mm I.D.) packed with 5-μm ODS Hypersil (Shandon Southern Products, Cheshire, U.K.) was operated isocratically. The mobile phase consisted of 1.28 g/l sodium acetate trihydrate containing 5.3% (w/v) acetonitrile and 0.5% glacial acetic acid, pH 4. The column flow-rate was 1.5 ml/min and each run time was 15 min.

Extraction and derivatization procedure for GC–MS and GC–NPD analysis

Plasma (100 μl) contained in an Eppendorf disposable microcentrifuge tube was spiked with 300 ng of the internal standard 3-isobutyl-1-methylxanthine and 500 μl hexane were added. After vortex-mixing and centrifugation (2 min at 5600 *g*) the hexane layer was removed and discarded. To the remaining plasma were added 50 μl of 1 *M* sodium hydroxide, 50 μl of TBAH solution, 1 ml of methylene chloride and 10 μl of pentafluorobenzyl bromide. The mixture was vortexed for 10 s and then placed on a rotary mixer for 45 min. After centrifugation (5 min at 5600 *g*) the upper aqueous layer was removed and the remaining solvent layer transferred to a clean Eppendorf centrifuge tube. The solvent extract was washed with 400 μl water and then taken to dryness under nitrogen at room temperature. The dried extract was finally taken up in 50 μl dried ethyl acetate for analysis by GC–MS and GC–NPD.

Quantitation of the drug in the plasma sample was made by reference to a standard calibration curve determined from the analysis of 100-μl aliquots of drug-free plasma spiked with internal standard (300 ng) and with added unlabelled theophylline (0–600 ng) and [1,3-^{15}N]theophylline (0–600 ng). For the analysis by GC–MS with SIM the ratios of the peak heights of the molecular ions of the derivative of theophylline (*m/z* 360) or of [1,3-^{15}N]theophylline (*m/z*

362) to the ion $(M-(CH_3)_2{=}CH_2)^+$ of derivatized 3-isobutyl-1-methylxanthine (m/z 346) were plotted against the concentration of labelled and unlabelled theophylline in the plasma. For the analysis by GC–NPD calibration lines were obtained by plotting the peak heights theophylline/3-isobutyl-1-methylxanthine against the combined concentrations of labelled and unlabelled theophylline.

Sample preparation for analysis by HPLC

Plasma (100 μl) contained in an Eppendorf microcentrifuge tube was spiked with the internal standard solution [100 μl of 20 μg/ml solution of β-hydroxyethyltheophylline in 10% (w/v) trichloroacetic acid]. After vortex-mixing and centrifugation (10 min at 15 600 g) the supernatant was removed and a 50-μl aliquot taken for HPLC analysis. Quantitation was achieved by measuring the peak-height ratio of absorption at 280 nm of theophylline to the internal standard and relating this to calibration curves constructed over the concentration range of theophylline, 0–20 μg/ml.

Drug administration and sample collection

After four days abstention from dietary methylxanthines, seven healthy male volunteers aged 21–38 years received an oral dose of 400 mg unlabelled theophylline in a sustained release formulation and 100 mg [1,3-^{15}N]theophylline dissolved in water. Blood samples were collected from an indwelling venous cannula immediately before and 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24, 26, 28 and 30 h after administration. After centrifugation plasma samples were stored at $-20\,^{\circ}C$ until analysed.

RESULTS AND DISCUSSION

Theophylline and the internal standard 3-isobutyl-1-methylxanthine can be chromatographed underivatized on a well deactivated capillary column but the GC properties of the two compounds are considerably improved by N-alkyl derivative formation. Plasma levels of the drug have been determined by GC [5–16] and by GC–MS [17, 18] after derivatization using a number of different alkylating agents. The N-pentafluorobenzyl derivatives employed in the method described in this paper are chemically very stable and have excellent GC properties. Also, their electron-impact mass spectra (see Fig. 1) exhibit intense ions at high mass which are ideally suited for SIM.

Extractive alkylation using the ion-pair reagent TBAH provided a convenient quantitative method for the simultaneous extraction and derivatization of the drug and the internal standard from plasma. The recovery of the ^{14}C-labelled drug from human plasma after extractive N-pentafluorobenzylation in six experiments was $86.7 \pm 2.1\%$ (mean $\pm$ S.D.).

Fig. 2 shows a typical SIM trace from an extract of plasma from a human subject given a single combined oral dose of the unlabelled and ^{15}N-labelled theophylline. The tracings obtained from the analysis of over 126 plasma samples from seven human subjects receiving the combined drug formulation were free from interference from endogenous compounds. The sensitivity of the method

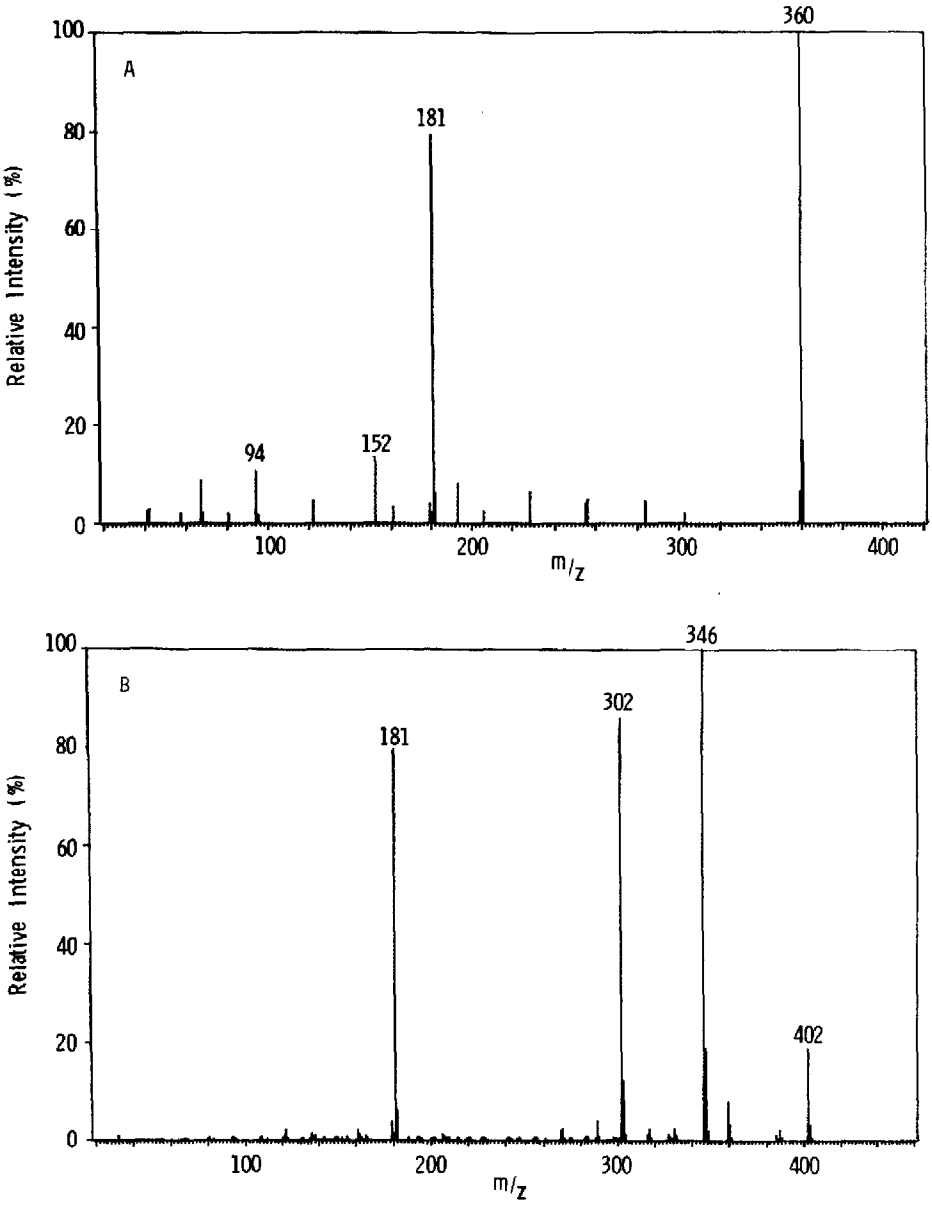
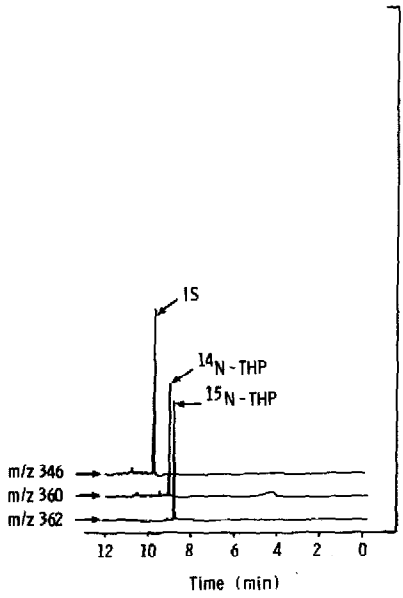


Fig. 1. Electron-impact mass spectrum of the N-pentafluorobenzyl derivative of (A) unlabelled theophylline and (B) 3-isobutyl-1-methylxanthine.

allowed the unlabelled and labelled drug to be quantitated in plasma down to levels of 10 ng/ml. Although the precision and accuracy of the GC–MS method would have been improved by the use of an isotopically labelled form of the drug (e.g. [2-^{13}C]- or [$^{2}H_3$]theophylline) as an internal standard, by employing the analogue 3-isobutyl-1-methylxanthine as the internal standard quantitation of the combined levels of the two drug formulations could be carried out on the same



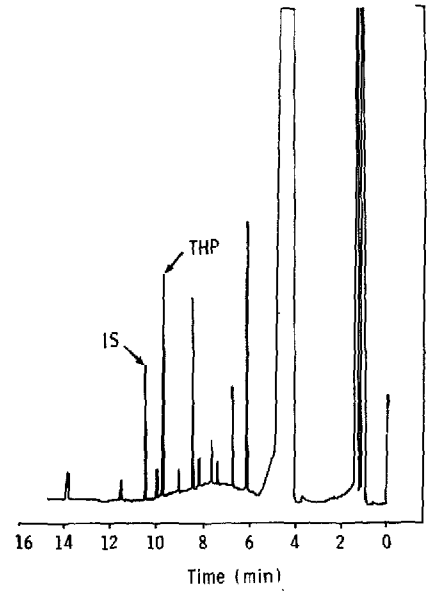


Fig. 2. SIM trace (m/z 346, 360, 362) from the analysis of a plasma sample from a human volunteer after a combined oral dose of unlabelled theophylline (^{14}N-THP, 400 mg) and labelled theophylline (^{15}N-THP, 100 mg); IS = internal standard (3-isobutyl-1-methylxanthine). For GC–MS conditions see text.

Fig. 3. GC-NPD profile from the analysis of a plasma sample from a human volunteer after dosage with the combined drug formulation (THP); IS = internal standard (3-isobutyl-1-methylxanthine). For GC conditions see text.

plasma extracts by GC–NPD. Fig. 3 shows a typical GC–NPD tracing from a plasma sample from one of the volunteer subjects.

The precision and accuracy of the GC–MS method was determined from replicate recovery experiments of the unlabelled and ^{15}N-labelled drug added to drug-free plasma. The calculated recoveries at two different concentrations (1 and 6 μg/ml) levels are given in Table I.

Plasma samples from the subjects involved in the pharmacokinetic study were also analysed by GC–NPD and by HPLC. The GC–NPD analyses were carried

TABLE I

CALCULATED RECOVERIES OF [^{14}N]- AND [^{15}N]THEOPHYLLINE ADDED TO DRUG-FREE PLASMA DETERMINED BY GC–MS

Added (μg/ml)	Recovery (%)			
	[^{14}N]Theophylline ($n=6$)		[^{15}N]Theophylline ($n=6$)	
	Range	Mean ± S.D.	Range	Mean ± S.D.
1	91.2–103.5	99.0 ± 4.23	91.7–104.8	101.7 ± 4.41
6	98.8–104.3	101.5 ± 1.51	98.6–102.8	101.2 ± 1.39

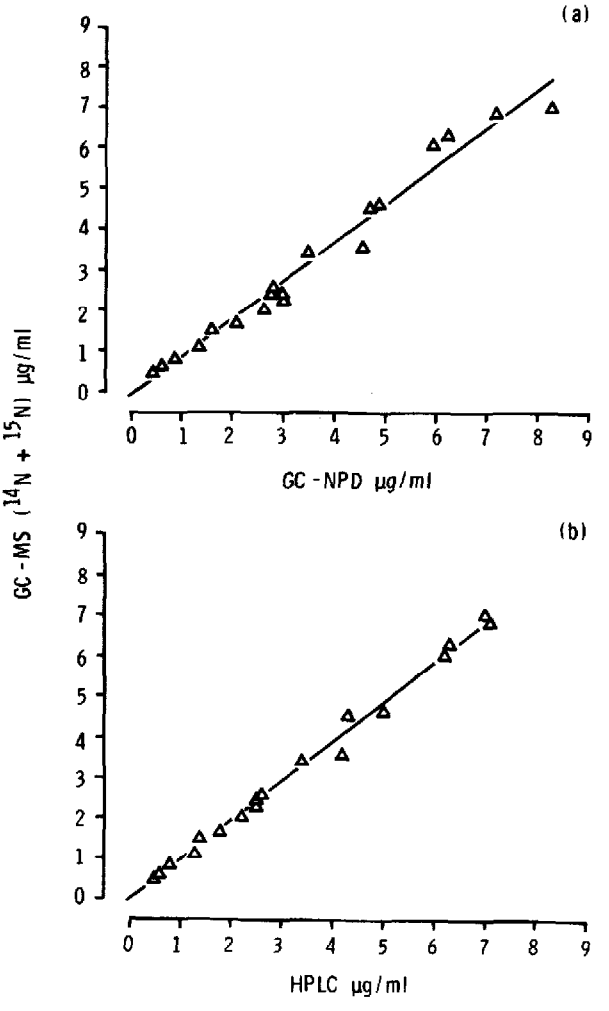


Fig. 4. Sum of [^{15}N]- and [^{14}N]theophylline concentrations in plasma determined by GC–MS plotted against the total theophylline concentrations determined by GC–NPD (a) and by HPLC (b).

out on the same extracts of plasma as used for the GC–MS determinations whereas the HPLC analyses were done on separate aliquots of plasma and using the internal standard β-hydroxyethyltheophylline. Fig. 4 shows a comparison of the concentration levels found in the plasma samples from one of the subjects using the different methods of quantitation. The combined concentrations of [^{15}N]theophylline and [^{14}N]theophylline determined by GC–MS were plotted against the total concentration measured by GC–NPD (Fig. 4a) and by HPLC (Fig. 4b). The slope of the regression line GC–MS on GC was 0.935 ± 0.035 with $r^2 = 97.5\%$ and the slope of the regression line GC–MS on HPLC was 0.981 ± 0.019 with $r^2 = 99.3\%$. Fig. 5 shows the time course of [^{15}N]theophylline and [^{14}N]theophylline levels in the plasma of this subject.

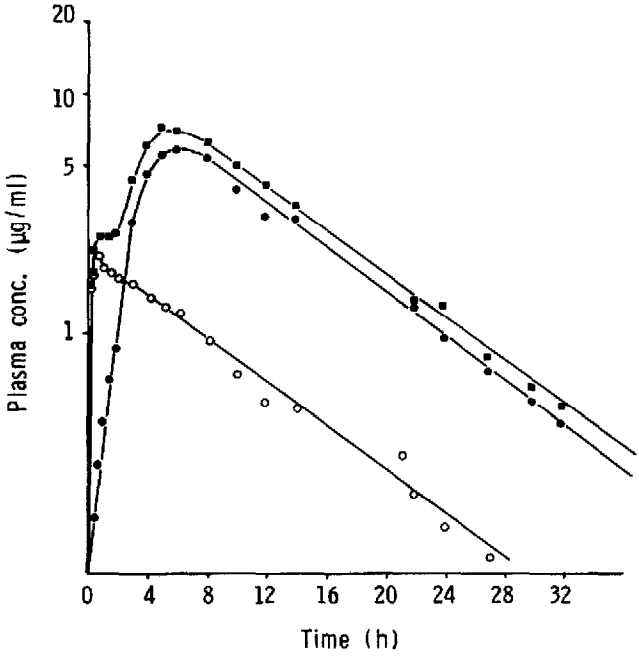


Fig. 5. Plasma concentration–time profile in a healthy adult volunteer following the simultaneous oral administration of 400 mg unlabelled theophylline and 100 mg [^{15}N]theophylline. (●) [^{14}N]Theophylline; (○) [^{15}N]theophylline; (■) [^{14}N]- and [^{15}N]theophylline (measured by HPLC).

Application

The application of this methodology to the determination of the absorption kinetics and bioavailability of sustained release theophylline formulations will be described in full in a separate publication.

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