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CAPILLARY GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETERMINATION OF STABLE ISOTOPICALLY LABELLED AND UNLABELLED THEOPHYLLINE IN SERUM AND URINE AND OF 1,3-DIMETHYLURIC ACID IN URINE

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SUMMARY

A capillary gas chromatographic–mass spectrometric method for the determination of [^{13}C,$^{15}N_2$]theophylline and unlabelled theophylline (TP) and of 1,3-dimethyluric acid (1,3-DMU), a major metabolite of TP, is described TP and the metabolite were extracted separately from serum or urine, purified by high-performance liquid chromatography and converted into alkyl derivatives (monoethyl-TP and dibutyl-1,3-DMU) The internal standards used for the respective determinations were [$^2H_6$]TP and [$^2H_6$]1,3-DMU Detection was performed by monitoring the molecular ions of the alkyl derivatives The method needed no complex corrections for contributions and provides good accuracy and precision

INTRODUCTION

Variability in an individual's clearance of theophylline (TP) is an important consideration when estimating bioavailability [1,2]. We have previously proposed a method for compensating for this problem, using the serum concentration of TP and the urinary excretion data on its major metabolites to make an estimation of the clearance after oral administration using the intravenous dose as a reference [3] Direct evidence for the validity of the proposed method can be provided by a stable isotope coadministration technique To apply this technique, we have developed an assay method for the quantification of TP and [^{13}C,$^{15}N_2$]TP in serum and urine and of 1,3-dimethyluric acid (1,3-DMU), a major metabolite of TP, in urine using gas chromatography–mass spectrom-

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etry–selected ion monitoring (GC–MS–SIM) The internal standards used were [$^{2}H_6$]theophylline (TP-d_6) and [$^{2}H_6$]1,3-dimethyluric acid (1,3-DMU-d_6) for TP and [^{13}C,$^{15}N_2$]TP and for 1,3-DMU, respectively

EXPERIMENTAL

Chemicals and reagents

All chemicals and reagents were of analytical-reagent grade and used without further purification, unless indicated otherwise.

Two forms of stable isotopically labelled theophylline, 1,3-di(trideuteromethyl)xanthine (TP-d_6· d_6 98%) and [2-^{13}C,1,3-$^{15}N_2$]1,3-dimethylxanthine ([^{13}C,$^{15}N_2$]TP· ^{13}C, 90%, $^{15}N_2$, 99%) were purchased from KOR Isotopes (Cambridge, MA, U S A.) and CEA (Gif-Sur-Yvette, France), respectively. Deuterium-labelled 1,3-dimethyluric acid, 1,3-di(trideuteromethyl)uric acid (1,3-DMU-d_6· d_6 99 4%) was synthesized in this laboratory [4] Unlabelled TP (1,3-dimethylxanthine) and 1,3-DMU were purchased from Sigma (St. Louis, MO, U.S A.). Tetrabutylammonium hydrogensulphate (TBA) was purchased from Aldrich (Milwaukee, WI, U S A.), ethyl iodide and tetra-*n*-butylammonium hydroxide (10% in methanol) from Tokyo Kasei (Tokyo, Japan), N,N-dimethylformamide, butyl iodide, tetraethylammonium hydroxide (25% in methanol), dichloromethane, *n*-hexane and methanol (the last three of HPLC grade) from Kanto Kagaku (Tokyo, Japan)

Stock solutions of the internal standards (TP-d_6 and 1,3-DMU-d_6) and [^{13}C,$^{15}N_2$]TP were prepared in methanol (TP-d_6, 5 136 mg in 50 ml, 1,3-DMU-d_6, 9 958 mg in 100 ml, [^{13}C,$^{15}N_2$]TP, 9 980 mg in 100 ml)

Gas chromatography–mass spectrometry

GC–MS–SIM was performed with a Shimadzu QP1000 gas chromatograph–mass spectrometer equipped with a data processing system. An SPB-1 fused-silica capillary column (30 m×0.32 mm I.D , film thickness 0 25 μm) (Supelco, Japan) was connected directly to the ion source. The mass spectrometer was operated in the electron-impact mode at an energy of 70 eV The trap current was set at 60 μA Helium was used as the carrier gas at 0.5 kg/cm^2

Samples were injected into the chromatograph according to the splitless mode The GC conditions were as follows The injection port temperature was 230°C for TP and 250°C for 1,3-DMU. The splitless injector was used with a septum purge flow-rate of 6 ml/min for TP and 7 5 ml/min for 1,3-DMU and a split flow-rate of 60 ml/min The purge activation time was 0 5 min for TP and 1 min for 1,3-DMU The initial column temperature for TP was set at 120°C and, 1 min after sample injection, was increased at 40°C/min to 230°C. For 1,3-DMU, the column temperature was maintained at 150°C for 1 5 min, then programmed at 40°C/min to 280°C. The ion source temperature was 250°C for TP and 280°C for 1,3-DMU. The multiple ion detector was focused

on the molecular ions of the monoethyl derivatives of TP and of the dibutyl derivatives of 1,3-DMU, i e , at *m/z* 208 for unlabelled TP, *m/z* 211 for [^{13}C,$^{15}N_2$]TP and *m/z* 214 for the internal standard, TP-d_6, and at *m/z* 308 for unlabelled 1,3-DMU and *m/z* 314 for the internal standard, 1,3-DMU-d_6

Extraction and HPLC purification

Theophylline To 0 5–1 ml of serum or urine were added 1 027 μg of TP-d_6 dissolved in 1 ml of methanol and 0 1 ml of 1 *M* KH_2PO_4 solution After vortex mixing for 15 s, the sample was extracted twice with 5 ml of chloroform–isopropanol (90 10, v/v) The organic phase was collected, dried over anhydrous sodium sulphate and evaporated to dryness at 40 °C under reduced pressure The extract was dissolved in 200 μl of mobile phase (solvent A methanol–dichloromethane–*n*-hexane, 4 30 66, v/v/v) and injected into a liquid chromatograph (Hitachi 638-30) equipped with gradient elution facilities and a variable-wavelength UV detector (Hitachi 635M) The HPLC column was normal-phase 50-μm LiChrospher Si-60 (25 cm × 4 mm I D) (Merck, Darmstadt, F R G) and was eluted with solvent A and solvent B (methanol–dichloromethane–*n*-hexane, 13 30 57, v/v/v) at 1 ml/min by the gradient-elution method Following the injection of each sample, the column was eluted with 100% solvent A for 20 min, then a gradient was employed over 10 min until the solvent system became 100% B Elution with 100% solvent B was continued for 20 min and then a reverse gradient was employed until the solvent system became 100% A again The column was then ready for injection of the next sample

The column effluent was monitored at 280 nm The TP fraction corresponding to retention time, $t_R = 14$–20 min was collected and the solvent evaporated at 40 °C under reduced pressure The residue was then ethylated by dissolving it in 100 μl of N,N-dimethylformamide, adding 30 μl of tetraethylammonium hydroxide solution, vortex mixing for 1 min, standing at room temperature for 15 min, adding 20 μl of ethyl iodide and then vortex mixing for 1 min After standing at room temperature for 30 min, the mixture was extracted twice with 2 ml of chloroform Each chloroform extract was washed with 1 ml of saturated sodium chloride solution The combined extracts were dried over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen The residue was dissolved in 50 μl–1 ml of acetonitrile and 0 5 μl of the solution was subjected to GC–MS

1,3-Dimethyluric acid To 1 ml of urine were added 4 979 μg of 1,3-DMU-d_6 dissolved in 1 ml of methanol, 0 5 ml of 0 1 *M* TBA solution and 1 g of ammonium sulphate After vortex mixing for 1 min, 1 ml of 0 1 *M* carbonate buffer (0 1 *M* Na_2CO_3–0 1 *M* $NaHCO_3$, 90 10, v/v), the mixture was extracted twice with 10 ml of chloroform–ethyl acetate–isopropanol (45 45 10, v/v/v) The organic phase was collected, dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure The dried extract was dissolved in

200 μl of mobile phase (solvent A: methanol–dichloromethane–*n*-hexane, 8 30 62, v/v/v) and injected into the chromatograph The liquid chromatograph, UV detector and the column used were the same as above The column was eluted with solvent A and solvent B (methanol–dichloromethane–*n*-hexane, 13 30 57, v/v/v) at 1 ml/min by the gradient-elution method Following the initial elution with 100% solvent A for 25 min, a gradient was employed for 10 min until the solvent system became 100% B Elution with 100% solvent B was continued for 10 min and then a reverse gradient was employed until the solvent system became 100% A again

The column effluent was monitored at 270 nm The 1,3-DMU fraction corresponding to $t_R = 18–22$ min was collected and the solvent evaporated at 40°C under reduced pressure. The residue was then butylated by dissolving it in 100 μl of N,N-dimethylformamide, adding 30 μl of tetraethylammonium hydroxide solution, vortexing for 30 s, adding 20 μl of butyl iodide, heating at 100°C for 1 h and then standing overnight at room temperature The mixture was extracted with chloroform in the same manner as TP described above The dried extract was dissolved in 50 μl–2 ml of acetonitrile and 0 5 μl of the solution was subjected to GC–MS

Preparation of calibration graph

Quantification of TP and [^{13}C,$^{15}N_2$]TP in serum or urine and 1,3-DMU in urine sample was achieved by reference to the respective calibration graphs determined from the analysis of standards (0 05–50 μg TP or [^{13}C,$^{15}N_2$]TP, 0 05–80 μg 1,3-DMU) with the internal standards (TP-d_6, 1 027 μg, 1,3-DMU-d_6, 4 979 μg). For GC–MS analysis, the ratios of the peak areas or heights of the molecular ions of the alkyl derivatives (TP, *m/z* 208; [^{13}C,$^{15}N_2$]TP, *m/z* 211, TP-d_6, *m/z* 214, 1,3-DMU, *m/z* 308; 1,3-DMU-d_6, *m/z* 314) were determined

Determination of accuracy

TP or [^{13}C,$^{15}N_2$]TP in amounts of 0 1, 1 and 10 μg was added to 0 5-ml portions of serum or urine 1,3-DMU in amounts of 0 5, 5 and 50 μg was added to 1-ml portions of urine. After preparation of the sample for GC–MS–SIM as described above, the peak-area or -height ratio was determined

RESULTS AND DISCUSSION

There are several published MS methods with SIM for measuring TP and its metabolites in biological fluids [5–11]. The ideal internal standards for mass spectrometric quantification are the stable isotopically labelled analogues of TP or each metabolite For the selective and accurate quantitation of TP, Floberg et al [6] and Désage et al [10] used TP-d_3 and [^{13}C,$^{15}N_2$]TP, respectively, as internal standards The aim of this work was to design a method

for determining TP, [^{13}C,$^{15}N_2$]TP and 1,3-DMU in biological fluids Vestal et al [8] described solid-probe mass spectral analysis for the simultaneous quantitation of TP and [^{13}C,$^{15}N_2$]TP with TP-d_6 as an internal standard. Their method, however, needs correction for contributions and does not provide the high sensitivity that we require On the other hand, there is no report of the use of stable isotopically labelled forms of TP metabolites as internal standards for GC–MS methods. We then synthesized 1,3-DMU-d_6 [4] and developed a capillary GC–MS method with high selectivity and sensitivity for the simultaneous determination of TP, [^{13}C,$^{15}N_2$]TP and 1,3-DMU in plasma and urine using TP-d_6 and 1,3-DMU-d_6 as internal standards

The isolation of TP and its major metabolite, 1,3-DMU, from serum and urine involved organic solvent extraction and HPLC purification procedures TP was extracted with chloroform–isopropanol (90 10, v/v) as described by Johnson et al [12] and Rovei et al [13] The extraction of 1,3-DMU, which is a highly polar weak acid, on the other hand, was performed by the ion-pair method of Muir et al [14] using the solvent system chloroform–ethyl acetate–isopropanol (45 45 10, v/v/v). The respective extracts had to be further purified by HPLC to provide samples sufficiently clean for analysis by GC–MS The absolute recovery of the isolation procedures was 53% for TP and 47% for 1,3-DMU

For sensitive and selective GC–MS assays, derivatization of the active hydrogens of TP and 1,3-DMU was essential By taking into account both the GC and MS aspects of derivatives, thermally stable alkyl derivatives of TP and 1,3-DMU were considered to be an appropriate choice [5–7, 9–12] Fig 1 shows

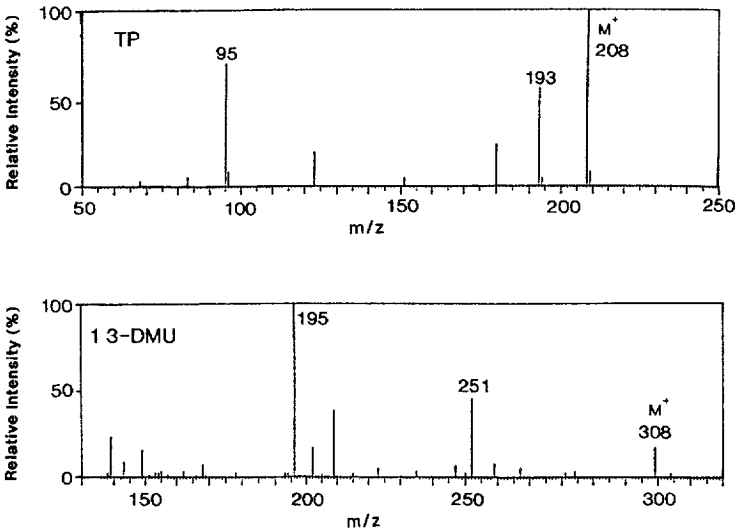

Fig 1 Mass spectra of monoethyl-TP (upper) and dibutyl-1,3-DMU (lower)

the electron-impact mass spectra of the monoethyl derivative of TP and the dibutyl derivative of 1,3-DMU The base peak is the molecular ion at m/z 208 and the analytical procedures described here introduced no interferences on the traces of the molecular ions being monitored for the monoethyl derivatives of TP (m/z 208), [^{13}C,$^{15}N_2$]TP (m/z 211), and TP-d_6 (m/z 214) after processing serum and urine samples (Fig. 2, left).

The ethyl derivative of 1,3-DMU also possessed good GC characteristics and gave a prominent molecular ion on the electron-impact mass spectrum. However, when the molecular ions of the diethyl derivatives of 1,3-DMU (m/z 280) and 1,3-DMU-d_6 (m/z 286) were monitored after processing urine sample, interfering peaks were observed on the SIM chromatograms To overcome the difficulty of removing interfering substances by HPLC purification after the solvent extraction step, 1,3-DMU was derivatized into the N-butyl derivative instead of the N-ethyl derivative for GC–MS analysis

The mass spectrum shown in Fig 1 (lower) indicated that the relative intensity of the molecular ion at m/z 308 was about 25% The SIM chromatograms of the dibutyl derivatives of 1,3-DMU and 1,3-DMU-d_6 showed no substances that caused ion interference at m/z 308 and 314 after processing from the urine sample (Fig 2, right) Specific quantification was then expected for TP, [^{13}C,$^{15}N_2$]TP and 1,3-DMU in biological samples

Calibration graphs for TP and [^{13}C,$^{15}N_2$]TP were prepared without any correction for contributions by using TP-d_6 as an internal standard. Each sample was analysed in triplicate, monitoring the molecular ions at m/z 208 for TP, m/z 211 for [^{13}C,$^{15}N_2$]TP and m/z 214 for TP-d_6 The logarithmic values for the peak-area ratios of the recordings at m/z 208, 211 and 214 were plotted against those for the molar ratios of TP and [^{13}C,$^{15}N_2$]TP to TP-d_6 The graphs

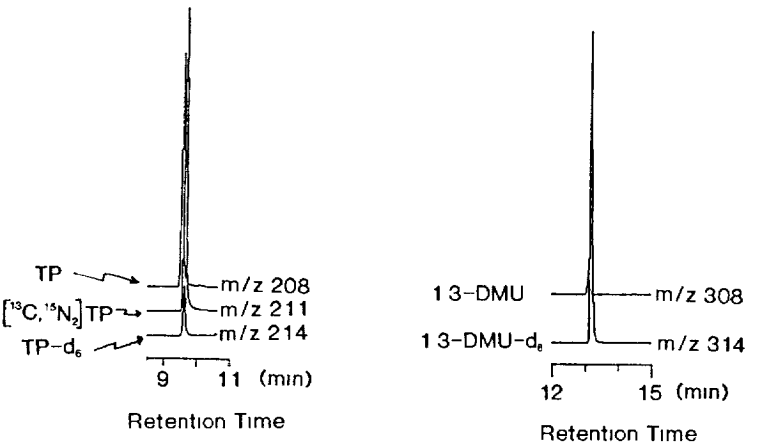

Fig 2 SIM chromatograms of monoethyl derivatives of TP, [^{13}C,$^{15}N_2$]TP and TP-d_6 (left) and dibutyl derivatives of DMU and DMU-d_6 (right)

for TP and [^{13}C,$^{15}N_2$]TP were rectilinear for an examined molar ratio range of 0 05–50 0 Least-squares linear analyses of the logarithmic values of the observed ratio gave regression lines with slopes of 1.003 for TP and 0 997 for [^{13}C,$^{15}N_2$]TP and correlation coefficients of 0 9999 for both TP and [^{13}C,$^{15}N_2$]TP, indicating no contributions or interferences at the monitored masses

A calibration graph for 1,3-DMU was also prepared without any correction for contributions by using 1,3-DMU-d_6 as an internal standard The logarithmic values of the peak-height ratios of the recordings for the molecular ions (*m/z* 308 for 1,3-DMU and *m/z* 314 for 1,3-DMU-d_6) were plotted against those of the molar ratios of unlabelled 1,3-DMU to 1,3-DMU-d_6 The graph was rectilinear for an examined molar ratio range of 0 01–20. There were no significant interferences at the monitored masses as evidenced by the slope of 1 024 and a correlation coefficient of 0 9996.

The accuracy of measurement was determined for TP in serum and urine, [^{13}C,$^{15}N_2$]TP in serum and 1,3-DMU in urine. The results presented in Table I show that the amounts of TP, [^{13}C,$^{15}N_2$]TP or 1,3-DMU added were in good agreement with the amounts of TP, [^{13}C,$^{15}N_2$]TP or 1,3-DMU measured, the relative error being less than 3 6%

TABLE I

ACCURACY OF GC–MS ANALYSIS

Added (μg/ml)	Found (μg/ml)								Coefficient of variation (%)	Relative error (%)
	Individual values[a]							Mean ± S D		
TP in serum										
0 1000	0 1004	0 0998	0 0976	0 1006	0 1006			0 0998 ± 0 0013	1 27	−0 20
1 000	0 985	0 999	0 985	1 001	1 003			0 995 ± 0 0083	0 84	−0 54
10 00	10 10	10 04	10 07	10 01	10 07			10 07 ± 0 0446	0 44	+0 70
TP in urine										
0 1000	0 0978	0 0956	0 0954	0 0982	0 0963			0 0967 ± 0 0012	1 27	−3 30
1 000	0 973	0 965	0 982	1 009	-			0 982 ± 0 0194	1 98	−1 82
10 00	10 48	10 66	10 28	10 19	10 18			10 36 ± 0 2074	2 00	+3 60
[^{13}C,$^{15}N_2$]*TP in serum*										
0 0998	0 0978	0 1001	0 1004	0 1012	0 1021			0 1007 ± 0 0019	1 90	+0 90
0 998	0 961	0 977	0 990	1 058	0 974			0 992 ± 0 0384	3 90	−0 60
9 98	9 95	9 98	10 13	10 01	9 95			9 98 ± 0 0969	0 97	0 00
1,3-*DMU in urine*										
0 4987	0 4629	0 4830	0 4978	0 5168	0 4731	-		0 4867 ± 0 0212	4 36	−2 41
4 987	4 975	4 975	4 817	4 783	4 704	4 898		4 859 ± 0 1104	2 27	−2 57
49 87	49 90	46 91	51 26	53 48	49 66	50 68		50 32 ± 2 155	4 28	+0 90

[a]Each individual value represents the mean of triplicate measurements

The inter-assay coefficients of variation were less than 2 0% for TP in serum and urine and less than 4 4% for [^{13}C,$^{15}N_2$]TP in serum and 1,3-DMU in urine The sensitivity of the GC–MS–SIM assay was 100 pg per injection with signal-to-noise ratios of 18 for TP and 3 5 for 1,3-DMU

In conclusion, the described method provides a sensitive and reliable technique for determining the serum concentrations of TP and [^{13}C,$^{15}N_2$]TP and the urine concentrations of TP and 1,3-DMU. Good accuracy and precision are obtained without complex corrections for contributions by using TP-d_6 and 1,3-DMU-d_6 as internal standards for the GC–MS assay of TP and [^{13}C,$^{15}N_2$]TP and of 1,3-DMU, respectively The method can be applied to a stable isotope coadministration technique for bioavailability and pharmacokinetic studies of TP

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