Determination of Theophylline and its Metabolites in Human Urine by High-Performance Liquid Chromatography

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High-performance liquid chromatographic method with UV detection was developed for the determination of theophylline and its metabolites in human urine using β-hydroxyethyl theophylline (β-HET) as an internal standard. For extraction of urine sample, quality control sample and xanthine-free blank urine were mixed with decylamine (ion-paring reagent) and β-HET. After saturation with ammonium sulfate, the mixture was then extracted with organic solvent at pH values of 4.0~4.5. All separations were performed with ion-pair chromatography using decylamine as an ion-pairing reagent and 3 mM sodium acetate buffered mobile phase (pH 4.0) containing 1% (v/v) acetonitrile and 0.75 mM decylamine. The detection limits of theophylline, 1,3-DMU, 1-MU, 3-MX and 1-MX in human urine were 0.17, 0.17, 0.39, 0.19 and 0.19 μg/ml, based on a signal-to-noise ratios of 3.0. The mean intraday coefficients of variation (C.V.s) of each compound on nine replicates were lower than 2.0%, while mean interday C.V.s on three days were lower than 1.6%. All separations were finished within 40 minutes.

Key words : Ion-pair chromatography, Theophylline, 1,3-DMU, 1-MU, 3-MX, 1-MX, HPLC

INTRODUCTION

Theophylline is widely used as a bronchodilating agent (Rall, 1980) having optimal plasma therapeutic concentration range of 10~20 μg/ml (Sarrazin et al., 1980). Theophylline is metabolized to 1,3-di-methyluric acid (1,3-DMU), 1-methyluric acid (1-MU) and 3-methylxanthine (3-MX) (Desiraju et al., 1977). Many studies concerning theophylline metabolism reported that only 10% of the drug is excreted in the urine as unchanged parent drug while remainder is metabolized to 3-MX (13~35%), 1-MU (15~19%) and 1,3-DMU (35~40%) (Thompson et al., 1974; Jenne et al., 1976; Jonkman et al., 1981; Tang-Liu et al., 1982; Birkett et al., 1983; Robson et al., 1987). In many pathological situations, metabolic and phar-macokinetic variation of theophylline and its meta-bolites influence its bronchodilating activity. There-fore, many methods have been developed for the analysis of theophylline and its metabolites in bio-logical samples.

Generally, ion-pair chromatography using ion-pair-ing reagent and acidic buffered mobile phase with UV detection is suggested for the analysis of theo-phylline and its metabolites. Muir et al. (1980) re-

ported that xanthine and uric acid were extracted by organic solvent at pH values of 6.0~6.5 and used tetrabutylammonium (TBA) as an ion-pairing reagent for the HPLC analysis of the compounds. Because of acidic nature of xanthine (pK$_a$ of approximately 8) and uric acid (pK$_a$ of approximately 6) (Albert and Ka-tritzky, 1971), partition of these compounds from aqueous to organic phase is largely dependent on pH values of aqueous phase. According to our com-parative experiment, this procedure produced a low assay recovery (48% for 1,3-DMU; 11% for 1-MU; 50.2% for 3-MX; 48% for 1-MX) and resulted poor de-tection limit. Furthermore, a method using TBA as an ion-pairing reagent resulted in extremely short column life (Naline et al., 1987). Consequently, pH values of 4.0~4.5 was found to be adequate for sam-ple preparation because pH values below 4.0 also in-crease interferences extracted from urine blank. The present method also used n-decylamine as an ion-pairing reagent instead of TBA.

Kester et al. (1987) and Naline et al. (1987) pro-posed a method that used Sep-Pak cartridge for ex-traction of urine sample. However, our comparative experiment showed that this method didn't produce reproducible results enough for precise determination of interest compounds.

In the present study, the solvent extraction pro-cedure for purification of urine sample was per-

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formed with organic phase (methylene chloride : ethylacetate : isopropanol=45 : 45 : 10, v/v%) at pH values of 4.0~4.5. Decylamine (ion-paring reagent) and β-HET (internal standard) were used for the analysis of theophylline and its metabolites in urine. Present method had excellent resolution and detection limit of the interest compounds with increased assay recovery, when compared to those of the reported methods previously.

MATERIALS AND METHODS

Materials

Theophylline (1,3-dimethylxanthine; 1,3-DMX), 1,3-dimethyluric acid (1,3-DMU), 1-methyluric acid (1-MU), 3-methylxanthine (3-MX), 1-methylxanthine (1-MX), β-HET (internal standard), n-decylamine were obtained from Sigma (St. Louis, MO, USA). Acetonitrile was obtained from AJAX Chemicals (Auburn, NSW, Australia). Methylene chloride, ethylacetate, isopropanol, acetic acid, ammonium sulfate, and other reagents were all first grade, and used without further purification.

HPLC apparatus

HPLC apparatus consisted of LC-9A pump (Shimadzu, Kyoto, Japan), SCL-6B system controller (Shimadzu) and C-R4AD data processor (Shimadzu). Stainless steel analytical column (Ultrasphere IP (5 μm), 15 cm ×0.4 cm I.D., Beckman Instruments, Berkeley, CA, USA) was used for separation in conjunction with a guard column (packed with μBondapak C_{18}/Corasil 37-50 μm, Waters, Milford, MA, USA). UV detection was carried with SPD-6AV UV/VIS spectrophotometer (Shimadzu). Syringe filter holder for sample filtration and full range litmus paper were obtained from Fisher Scientific (Pittsburgh, PA, USA). Sample condensation was performed with rotary vacuum evaporator (EYELA, Tokyo Rikakikai Instrument, Kyoto, Japan).

Chromatographic condition

3 mM sodium acetate buffer (pH 4.0) containing 1% (v/v) of acetonitrile and 0.75 mM n-decylamine was used as mobile phase. All separations were carried at room temperature (24°C) with flow rate of 1.5 ml/min., and wavelength of UV detection was set at 280 nm with sensitivity of 0.04 AUFS.

Preparation of stock and standard solutions

β-HET, an internal standard, was prepared dissolving in 2 mM NaOH at the concentration of 1 mg/ml. n-Decylamine (i.e., an ion-paring reagent) solution was prepared by dissolving in distilled, deionized water at the concentration of 0.1 M. Xanthine-

free urine was obtained from a subject who did not take any foods and beverages containing xanthine for 48 hours. The stock solutions for calibration curve were made by dissolving each compound in 2 mM NaOH. Standard solution of theophylline (1.37-43.75 μg/ml), 1,3-DMU (1.37-43.75 μg/ml), 1-MU (3.13-100 μg/ml), 3-MX (1.56-50 μg/ml), 1-MX (1.56-50 μg/ml) were prepared by further dilution of stock solutions with distilled, deionized water. Quality control samples of theophylline (17 μg/ml), 1,3-DMU (12 μg/ml), 1-MU (30 μg/ml), 3-MX (10 μg/ml) and 1-MX (10 μg/ml) were prepared by dissolving each compound in 2 mM NaOH.
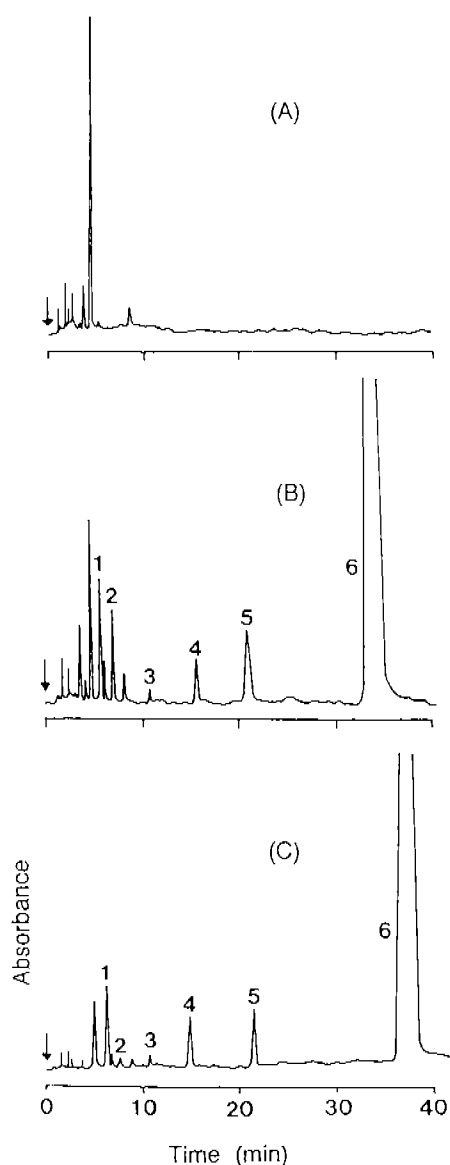
Extraction procedure for urine sample: 0.5 ml of urine sample was mixed with 50 μl of β-HET solution, 0.5 ml of decylamine solution and excess amount of solid ammonium sulfate (over saturation). After vortex-mixing for 30 seconds, pH of the mixture was adjusted to 4.0~4.5 with acetic acid using full range litmus paper. The mixture was then extracted with 10 ml of organic phase (methylene chloride : ethylacetate : isopropanol=45 : 45 : 10, v/v%). The organic phase was collected and evaporated to dryness with rotary vacuum evaporator. The residue was reconstituted with 1 ml of mobile phase. After filtration, 10 μl of clear solution was then injected directly onto the HPLC column. For the preparation of extracted standards for calibration and quality control samples, 1 ml of each standard or quality control sample was mixed with 0.5 ml of xanthine-free urine. The mixture was mixed with 50 μl of β-HET, 0.5 ml of decylamine, and excess amount of ammonium sulfate. The other procedures were the same for preparation of urine sample.

Calculation of recovery

Assay recovery of theophylline and its metabolites were assessed at the concentration of quality control sample. Both quality control sample prepared by above procedure and prepared only in aqueous solution were injected separately. Assay recovery of each compound was calculated using the following equation: % Recovery=(peak area of solvent extraction)/(peak area of direct injection).

RESULTS AND DISCUSSION

Fig. 1 show typical chromatograms of drug-free human urine, drug standards in human urine and human urine from a volunteer collected between 0 and 48 hours after 20 minutes intravenous infusion of aminophylline at 5 mg/kg. No interferences from endogenous substances were observed in any of chromatograms of urine samples. The peaks of theophylline, 1,3-DMU, 1-MU, 3-MX, 1-MX and internal
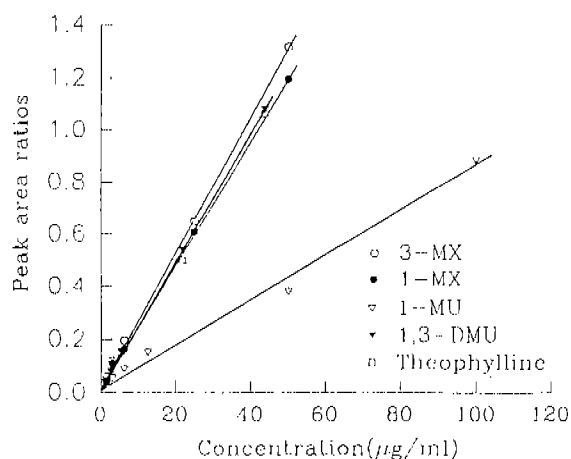
standard were symmetrical, and eluted at approximately 22.1, 16.1, 11.0, 5.9, 7.2, 34.0 minutes, respectively.

The detection limits of theophylline, 1,3-DMU, 1-MU, 3-MX and 1-MX in human urine were 0.17, 0. 17, 0.39, 0.19 and 0.19 µg/ml, based on a signal-to-noise ratios of 3.0 using extraction method (Table I). Mean assay recoveries of spiked theophylline, 1,3-DMU, 1-MU, 3-MX and 1-MX from human urine were 76.4, 74.7, 40.5, 81.7, 74.7 and 74.3%, respectively (Table I).

Calibration curves of theophylline and its metabolites relating the peak area ratios to the corresponding concentration were shown in Fig. 2. Calibration curves of each compound produced good linearity over the range of concentration examined. Correlation coefficients of each curve were 0.9992 for theophylline, 0.9998 for 1,3-DMU, 0.9947 for 1-MU, 0.9999 for 3-MX and 0.9997 for 1-MX.

The mean intraday coefficients of variation (C.V.s) of each compound on nine replicates were lower than 2.0%, while mean interday C.V.s on three days were lower than 1.6% (Table I).

Urinary excretion ratios of theophylline, 1,3-DMU, 1-MU and 3-MX in a human volunteer collected between 0 and 48 hours after 20 minutes intravenous

Fig. 1. Chromatograms of drug-free human urine (A), human urine spiked with 1.4 µg/ml of theophylline and 1,3-DMU, 3. 1 µg/ml of 1-MU, 1.6 µg/ml of 3-MX and 1-MX (B), and urine obtained from a human volunteer collected between 0 and 48 hours after 20 minutes intravenous infusion of aminophylline at 5 mg/kg (C). Peaks: 1=3-MX (5.9 min); 2=1-MX (7.2 min); 3=1-MU (11.0 min); 4=1,3-DMU (16.1 min); 5=theophylline (22.1 min); 6=internal standard (34.0 min). The arrows mark the points of injection. The detector sensitivity was 0.04 AUFS, and signal-to-noise ratios were 3.0.

Fig. 2. Calibration curves of theophylline and its metabolites relating the peak area ratios to the corresponding concentration. Peak area ratio was area of theophylline and its metabolites divided by area of internal standard.

Table I. Extraction recoveries, detection limits, intra and interday coefficients of variation in human urine using solvent extraction method

Compound	Recovery (%)	Detection limit (µg/ml)	Concentration[a] (µg/ml)	Intraday (n=9)		Interday (n=3)	
				mean±S.D. (µg/ml)	C.V. (%)	mean±S.D.(µg/ml)	C.V.(%)
Theophylline	76.4	0.17	17	17.4±0.3	1.8	17.6±0.1	0.6
1,3-DMU	74.7	0.17	12	12.7±0.2	1.4	12.8±0.1	0.9
1-MU	40.5	0.39	30	32.1±0.6	2.0	32.5±0.3	1.0
3-MX	81.7	0.19	10	10.4±0.2	1.6	10.5±0.2	1.6
1-MX	74.3	0.19	10	10.1±0.2	1.7	10.3±0.1	1.2

[a]Concentration=Concentration of quality control samples

infusion of aminophylline at 5 mg/kg showed 15.03, 25.03, 27.98 and 12.08% (w/w), and excretion of 1-MX was little.

Using decylamine as an ion-paring reagent and pH 4.0~4.5 for solvent extraction, this paper presents an increased assay recovery compared to the published methods ranging from 2 times for 3-MX, 1-MX and 1, 3-DMU, 4 times for 1-MU. As a result of increased assay recovery, detection limits were also increased. A method using Sep-Pak cartridge for purification of urine sample showed not enough linearity of calibration curves for precise determination (r^2>0.9912) (Kester et al., 1987).

The present method also could be applied to determine theophylline and its metabolites in human serum with some modification of chromatographic conditions because detection limits of the present method were low enough to determine theophylline with optimal plasma therapeutic concentration range of 10~20 µg/ml (Sarrazin et al., 1980).

In summary, this paper presents an increased sensitivity and good calibration curves through concentration examined with an acceptable intraday and interday variations.

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