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Simultaneous analysis of thiamphenicol and its prodrug thiamphenicol glycinate in human plasma and urine by high performance liquid chromatography: Application to pharmacokinetic study

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Abstract

A simple and sensitive method for simultaneous determination of the active compound, thiamphenicol (TAP) and its prodrug, thiamphenicol glycinate (TG) in human plasma and urine is described. The procedure involved extraction of TG and TAP with ethyl acetate (plasma) or 100-fold dilution with the mobile phase (urine) followed by determination by reversed-phase high performance liquid chromatography (HPLC) with UV detection at 224 nm. Separation of the compounds was achieved on a column packed with Hypersil ODS2. The mobile phase consisted of acetonitrile–water containing 0.003 M tetrabutyl ammonium bromide and 0.056 M ammonium acetate (87:13, v/v) with a flow rate of 1.0 ml/min. The chromatograms did not contain interfering peaks due to the suitable extraction procedure and chromatographic conditions. The calibration curves of TG and TAP were linear ranging from 0.78 to 100 μg/ml in plasma and in urine. The intra-day and inter-day relative standard deviations (S.D.) were less than 10%. The recoveries of TG and TAP in plasma and urine were above 80%. TG was not stable in plasma samples and after extraction at ambient temperature or in freeze–thaw cycles, and hence the samples for injection on HPLC column should be stored in refrigerator or under ice cooling prior to analysis, and the plasma samples should not experience the freeze–thaw cycle more than one time. Unlike TAP, TG could not be detected in most urine samples. Application of this method demonstrated that it was feasible for the clinical pharmacokinetic study.

Keywords: Thiamphenicol glycinate; Thiamphenicol; HPLC; Pharmacokinetics; Plasma; Urine

1. Introduction

Thiamphenicol [D(+)-*threo*-2-di-chloroacetamido-1-(4-methylsulphonylphenyl)propane-1,3-diol; (TAP)] is an analogue of chloramphenicol in which the nitro group on the benzene ring is replaced with a methylsulfonic group. To improve its limited solubility in water, thiamphenicol glycinate (TG), obtained by esterification of the primary alcoholic hydroxyl group of thiamphenicol with glycine, is now administered as the prodrug of TAP. The structures of TAP and TG are shown in Fig. 1. In the human organism TG is hydrolyzed by esterase to yield TAP. It was reported that TAP shows particular therapeutic effect in respiratory infections, bacterial prostatitis and venereal diseases [1].

Some papers reported determination methods for TAP in biological samples [2–4]; however, no method was published for the simultaneous determination of TAP and TG. For example, a recently published article reported the serum and lung level results by only determining TAP in experimentally infected guinea pigs with oral administration of TG [4], but this paper presented no data concerning the pharmacokinetics of TG.

It is of vital importance to measure the concentration of the prodrug in plasma and urine samples while studying its pharmacokinetics in clinical trials. The purpose of this work was to develop a simple, sensitive and accurate high performance liquid chromatographic (HPLC) method for measuring TG and TAP in human plasma and urine that can be applied in the clinical pharmacokinetic study.

2. Materials and experiments

2.1. Chemicals and reagents

TG and TAP reference substances and TG hydrochloride were offered by Henan Zhong Shuai Medicine Science and

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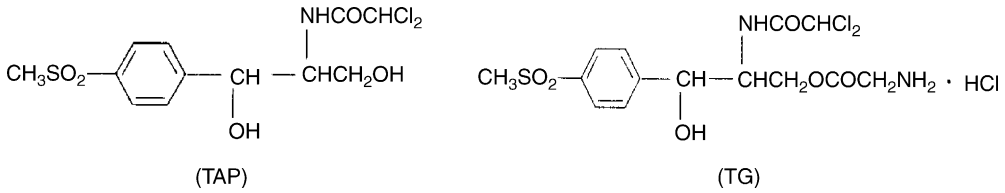

Fig. 1. The structures of TAP and TG.

Technology Development Limited Company, Zhengzhou City, China. HPLC-grade acetonitrile was the product of TEDIA Company, USA. Acetaminophen (*N*-acetyl-*ρ*-aminophenol) was the product of Jingshan Pharmaceutical Factory, Shantou City, China. Other reagents were highest purity available from the market.

2.2. Apparatus

The HPLC system consisted of a Model LC-10AT*vp* solvent delivery system and a Model SPD-10A*vp* multiple wavelength UV detector (Shimadzu, Japan). The apparatus was connected to a personal computer with a HW2000 Chromatography Data System software (Shanghai, China). A XW-80A vortex mixer (Instrumental Factory of Shanghai Medical University, Shanghai, China), a HH-2 thermostat-controlled water bath with digital display (Guohua Electric Apparatus Limited Company, Changzhou, China), a model 0412-1 centrifuge (Shanghai Surgical Instruments Factory, Shanghai, China) and a model RE52CS rotating vaporizer (Yarong Biochemical Instrumental Factory, Shanghai, China) were used for sample preparation.

2.3. Chromatographic conditions

The chromatographic separation was performed with a Hypersil ODS2 analytical column (200 mm × 4.6 mm i.d., 5 μm; Elite Corporation, Dalian City, China). The mobile phase consisted of acetonitrile–water containing 0.003 M tetrabutyl ammonium bromide and 0.056 M ammonium acetate (87:13, v/v). The solution was filtered using 0.45 μm nylon membrane and ultrasonically degassed prior to use. The HPLC system was equilibrated for approximately 30 min at a flow rate of 1.0 ml/min before analysis commenced. The UV detection wavelength was 224 nm, the attenuation was 0.001 AUFS and the injection volume was 20 μl manually with a 100 μl syringe. Chromatography was performed at ambient temperature.

2.4. Plasma and urine samples

Venous blood samples were withdrawn into the heparinized tubes, centrifuged at 4000 rpm for 1 min to separate the plasma. Urine samples were collected in conical tubes. All samples were stored at −70 °C until analysis.

2.5. Standard solutions

Stock solutions of TG and TAP (both of 2 mg/ml) were prepared in pH 3 phosphate buffer. Stock solution of the internal standard (I.S.) of acetaminophen (20 mg/ml) was prepared in methanol. The solutions were stored at 4 °C in refrigerator.

The pH 3 phosphate buffer was the 72.5:27.5 (v/v) mixture of aqueous solutions of phosphoric acid (16.6 ml/1000 ml) and di-sodium hydrogen phosphate (71.63 g/1000 ml).

2.6. Extraction procedures

2.6.1. For plasma

For HPLC determination, 500 μl plasma spiked with 25 μl of 100 μg/ml I.S. working solution was extracted with 2 ml ethyl acetate. Samples were vortexed for 2 min and centrifuged at 4000 rpm for 1 min, then 750 μl of the organic layer was transferred to another tube and evaporated under air stream by a rotating vaporizer. Before analysis, residues were reconstituted in 250 μl of the mobile phase, vortexed and centrifuged again. Twenty microliters of the resulting solution was injected manually into the HPLC system. The internal standard method was used for quantitation.

2.6.2. For urine

For HPLC determination, 20 μl urine in a conical tube was diluted 100-fold with the mobile phase, then 25 μl of 100 μg/ml I.S. was added and mixed. Twenty microliters aliquot was injected manually into the HPLC system for analysis.

3. Results and discussion

3.1. Selectivity

The selectivity was studied by analyzing blank human plasma and urine samples. The chromatogram of blank plasma (Fig. 2A) and urine (Fig. 2D) did not indicate any interfering components extracted from the biological samples. A typical chromatogram of a drug-free human plasma sample spiked with TG and TAP and the I.S. is shown in Fig. 2B and that of a drug-free human urine sample spiked with TAP and the I.S. is shown in Fig. 2E. The chromatogram of the extract of plasma samples after intravenous infusion administration of 1.0 mg TG hydrochloride in human is shown in Fig. 2C and that of the dilution of a urine sample in Fig. 2F. The retention time of TAP and TG was about 5.8 and 11.3 min, respectively, and the retention time of the I.S. (acetaminophen) was about 3.9 min. No interfering peak appears in the chromatograms of real samples at the retention time of the internal standard.

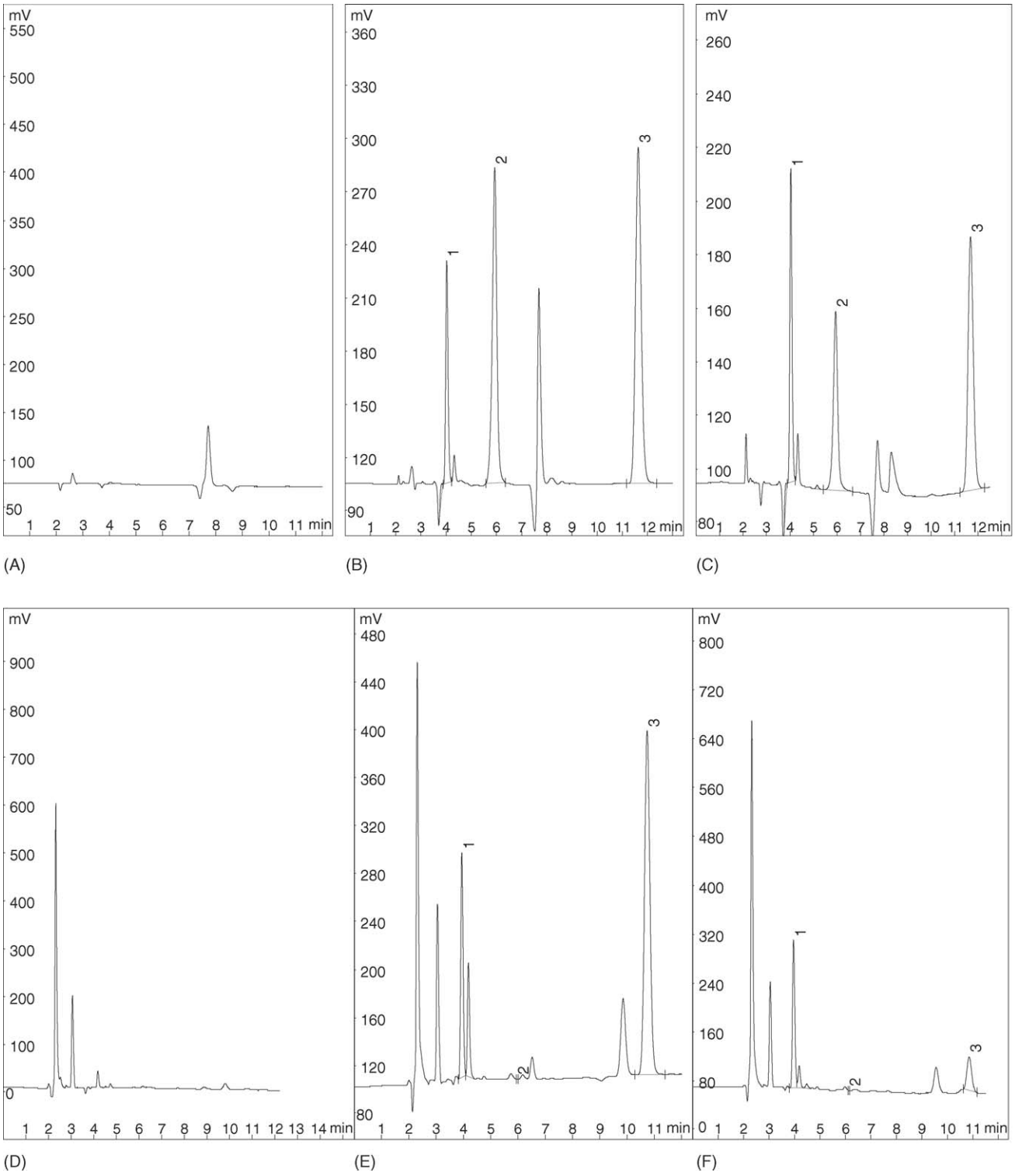

Fig. 2. Chromatograms of blank plasma (A); drug-free plasma sample spiked with TG, TAP and the internal standard (B); plasma sample following intravenous infusion administration of TG hydrochloride (C); 100-fold diluted blank urine (D); drug-free urine sample spiked with TAP and internal standard (E) and urine sample following intravenous infusion administration of TG hydrochloride (F). *Key*: (1) internal standard (acetaminophen), (2) TG and (3) TAP.

3.2. Precision and accuracy

The intra-day and inter-day standard deviations (S.D.) were determined by analyzing plasma samples containing 0.78, 6.25 and 100.0 μg/ml of TG and TAP and urine samples containing the same three concentrations of TAP. The intra-day and inter-day standard deviations were estimated from the results of five replicate assays on 1 day and on 5 continuous days,

Table 1
The intra-day and inter-day precision of the method for TG and TAP in plasma and TAP in urine samples ($n = 5$)

Samples	Concentration (μg/ml)	Intra-day		Inter-day	
		Average ± S.D.	R.S.D. (%)	Average ± S.D.	R.S.D. (%)
Plasma					
TG	0.78	0.81 ± 0.034	4.2	0.86 ± 0.017	2.0
	6.25	6.13 ± 0.28	4.6	6.70 ± 0.39	5.8
	100.0	95.2 ± 7.01	7.4	99.5 ± 4.8	4.8
TAP	0.78	0.83 ± 0.018	2.1	0.83 ± 0.016	1.9
	6.25	6.48 ± 0.29	4.5	6.64 ± 0.19	2.9
	100.0	101.9 ± 1.94	1.9	113.0 ± 5.65	5.0
Urine					
TAP	0.78	0.83 ± 0.047	5.0	0.82 ± 0.039	4.2
	6.25	6.71 ± 0.15	2.2	6.83 ± 0.11	1.6
	100.0	100.8 ± 1.38	1.4	100.2 ± 1.00	1.0

respectively. The results are shown in Table 1. Accuracy was within the range 6.0–13.0% at all concentrations. The results are shown in Table 2.

3.3. Extraction efficiency

The recovery for TG and TAP from plasma and TAP from urine were evaluated at the low, medium and high concentrations of 0.78, 6.25 and 100.0 μg/ml, respectively. The recoveries of TG and TAP from plasma and TAP from urine were calculated by comparing the peak area obtained from extracts of spiked plasma samples or dilutions of urine with the peak area obtained from the direct injection of known amounts of standard solutions of TG or TAP. The overall extraction yields of TG and TAP in plasma and TAP in urine are shown in Table 3.

3.4. Linearity

3.4.1. For plasma

Drug-free plasma was spiked with standard solutions of TG and TAP to get a series of concentrations of 0.781, 1.563, 3.125, 6.25, 12.5, 25.0, 50.0 and 100.0 μg/ml. The calibration curves were constructed by plotting the peak area ratio of TG or TAP to the I.S. against their concentrations. The calibration curves for TG and TAP were linear over the range from 0.78 to 100 μg/ml. The calibration curves were calculated by linear regression method: $Y = aX + b$, where Y is the peak area ratio of TG or TAP to the I.S., X the plasma concentration of TG or TAP (μg/ml), a the slope and b is the y-intercept. Typical values for the regression parameters a, b and r (correlation coefficient) of TAP were 0.0741, 0.0116 and 0.9998, respectively, and those of TG were 0.0628, 0.0129 and 0.9994, respectively.

3.4.2. For urine

Drug-free urine diluted 100-fold was spiked with standard solution of TAP to get a series of solutions with the same concentrations as in Section 3.4.1. The calibration curves were constructed by plotting the peak area ratio of TAP to the I.S. against its concentration. The linearity range and the calculations are also the same as in Section 3.4.1. The regression parameters a, b and r were 0.146, 0.0392 and 0.9996.

3.5. Stability of the biological samples

3.5.1. Stability of processed samples at refrigeration/ambient temperature

3.5.1.1. For plasma. The stability of TG in processed samples was determined at refrigeration temperature at low (0.78 μg/ml), medium (6.25 μg/ml) and high (100 μg/ml) concentrations of its

Table 2
The accuracy of the method for TG and TAP in plasma and TAP in urine samples

Samples	Concentration (μg/ml)	%Bias
Plasma		
TG	0.78	6.0
	6.25	6.3
	100.0	13.0
TAP	0.78	4.1
	6.25	−1.9
	100.0	−4.8
Urine		
TAP	0.78	6.4
	6.25	7.4
	100.0	2.0

Table 3
Extraction efficiency of TG and TAP in human plasma and TAP in urine samples ($n = 5$)

Samples	Concentration (μg/ml)	Recovered (%) ± S.D.	R.S.D. (%)
Plasma			
TG	0.78	104.1 ± 0.04	4.2
	6.25	98.1 ± 0.05	4.6
	100.0	96.1 ± 0.08	8.0
TAP	0.78	106.0 ± 0.02	2.1
	6.25	103.7 ± 0.05	4.5
	100.0	101.9 ± 0.02	1.9
Urine			
TAP	0.78	108.9 ± 0.03	2.9
	6.25	105.7 ± 0.02	2.2
	100.0	100.6 ± 0.01	1.4

calibration standards. Two sets of the samples of the three concentrations were divided into 30 tubes and extracted as described in Section 2.6.1. One set of spiked samples at low, medium and high concentrations was assayed immediately and taken as standard (100%). The remaining set of the evaporated residues in the tubes was stored at refrigeration temperature for 24 h and measured. The stability results were evaluated comparing the subsequent results with the standard and expressed as percent deviation.

For the determination of the stability of TAP at ambient temperature also two sets of samples with the above three concentrations were prepared and divided into 30 tubes. One set of spiked samples was extracted as outlined above, then measured immediately and taken as standard (100%). The remaining set was allowed to stand at ambient temperature for 24 h. Before analysis, the samples were extracted as outlined above, and then measured immediately. The stability results were evaluated as described above.

3.5.1.2. For urine. Urine was diluted 100-fold and the stability of TAP was determined at ambient temperature using the same concentrations of the spiked calibration standards as for plasma. Two sets of the samples of the three concentrations were divided into 30 tubes. One set of spiked samples was assayed immediately as described above and taken as standard (100%). The remaining set was allowed to stand at ambient temperature for 24 h, assayed and the results calculated as outlined above.

3.5.2. Stability during freeze–thaw cycles

To evaluate the impact of freeze–thaw cycles, spiked samples ($n = 2 \times 5$ assays = 10) comprising the earlier described low, medium and high concentrations of TAP or TG in plasma and TAP in 100-fold diluted urine were prepared. The two sets of the samples of the three concentrations of TG and/or TAP were divided into 30 tubes. One set of spiked samples was assayed as outlined above with its plasma/urine calibration standards, without being subjected to the freeze–thaw cycle and the results were referred as 100%. The other set of the samples was frozen at −20 °C for subsequent studies. The second set of samples was allowed to stand at refrigeration/ambient temperature for approximately 30 min until completely thawing, then stored frozen at −20 °C again. This freeze–thaw assay cycles were repeated two more times every 2 days thereafter. After three cycles, the set of samples were assayed with a freshly spiked and processed plasma/urine calibration standard as outlined above. The results of these assays were compared with the standard and expressed as percent deviation.

3.5.3. Stability at −20 °C

Frozen plasma and diluted urine samples spiked with TG and TAP at the above described three concentrations were stored at −20 °C for 1 week. The results of the assay of these samples were compared with standards not being subjected to storage at −20 °C and expressed as percent deviation.

The results of the stability studies are summarized in Table 4.

3.6. Application of the method

The study was designed as a single-dose clinical investigation and carried out on eight adult volunteers with valid medical examination. Of the eight volunteers, four were women and four were men, aged between 20 and 26 years (mean age 24 years, median age 23 years), weighing between 50 and 70 kg (mean 60 kg, median 59 kg) and height between 156 and 180 cm (mean 169 cm, median 170 cm). Written informed consent for participation in the study was signed by all participants and permission from the local Ethics Committee was granted.

The volunteers were hospitalized for at least 24 h prior to investigation and put under standard conditions with respect to diet and physical activity. The day before the investigation any drug treatment was discontinued so that during the study period all volunteers were free of any medication. After a 12-hour overnight fast, on the morning of the study day each participant accepted a single-dose intravenous infusion administration of TG hydrochloride with 100 ml normal saline, the

Table 4
Stability of TG and TAP in plasma and TAP in urine

Sample	Concentration (μg/ml)	Room temperature stability (%) for 24 h	Freeze–thaw stability (%) for three cycles	Frozen stability (%) for 7 days
Plasma				
TG	0.78	96.0 ± 6.8	62.2 ± 8.1	100.5 ± 3.6
	6.25	94.0 ± 4.4	59.8 ± 9.6	94.2 ± 3.1
	100.0	90.7 ± 4.8	48.2 ± 4.8	104.0 ± 1.3
TAP	0.78	103.0 ± 2.7	108.1 ± 5.7	102.4 ± 6.4
	6.25	98.8 ± 2.3	99.2 ± 5.5	101.2 ± 5.4
	100.0	90.7 ± 2.1	102.3 ± 3.8	94.6 ± 3.5
Urine				
TAP	0.78	95.4 ± 3.2	107.4 ± 6.0	108.2 ± 1.8
	6.25	101.9 ± 1.6	108.3 ± 2.1	101.2 ± 4.1
	100.0	102.7 ± 1.8	98.1 ± 4.3	94.6 ± 5.7

The values are shown as recoveries under different storage conditions ($n = 5$). Stabilities of processed samples of TG at refrigeration temperature in plasma and of TAP at ambient temperature in plasma/urine for 24 h were determined. Stabilities of freeze–thaw samples of TG in plasma and TAP in plasma/urine for three cycles were determined. Stabilities of frozen samples (−20 °C) of TG in plasma and TAP in plasma/urine for 7 days were determined.

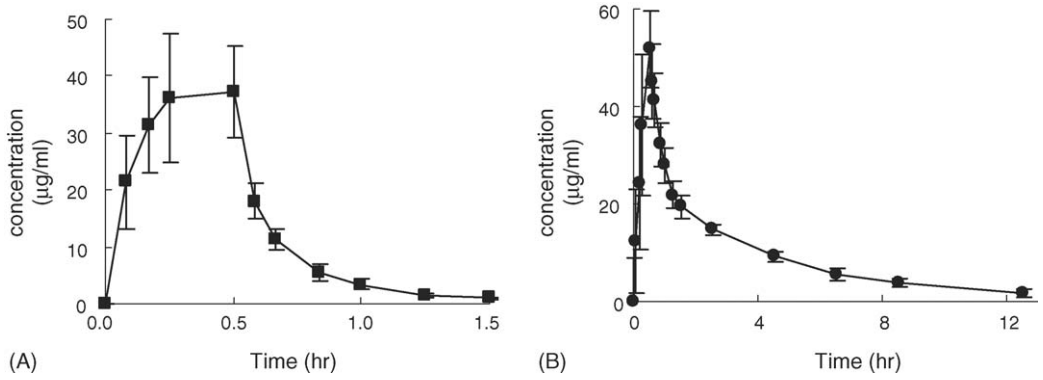

Fig. 3. Plasma concentration–time profiles of TG (A) and TAP (B) in volunteers after intravenous infusion administration of 1.0 g TG hydrochloride ($n = 8$).

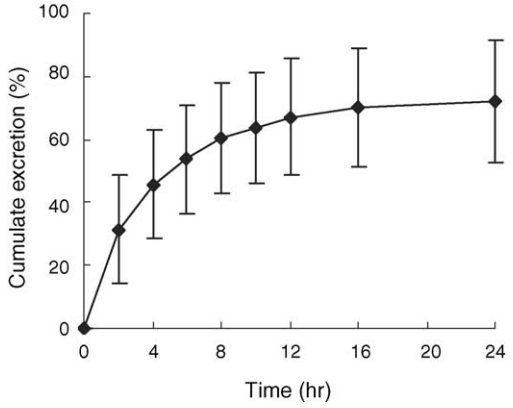

Fig. 4. Cumulate urinary excretion of TAP in volunteers after intravenous infusion administration of 1.0 g TG hydrochloride ($n = 8$).

whole procedure being completed within 30 min, and each participant remained in bed for 2 h.

Approximately 3 ml blood samples were collected into conical tubes before administration, at 5, 10, 15 min during administration, at 0, 5, 10, 20, 30, 45 min and 1, 2, 4, 6, 8, 12, 16 h after administration. The blood samples were centrifuged at 4000 rpm for 1 min and plasma was separated and stored immediately at −70 °C until analysis.

At the same time, urine samples were collected before administration (as blank human urine and time zone) and eight different time points over a 2-hour interval, including 0–2, 2–4, 4–6, 6–8, 8–10, 10–12, 12–16 and 16–24 h. The overall urinary volume of each interval was measured and noted. Approximately 10 ml urine of each participant were collected and stored immediately at −70 °C until analysis (Figs. 3 and 4).

3.7. Discussion

The method described here involves extraction of plasma samples with ethyl acetate or dilution of urine samples with the mobile phase and determination with the use of UV detection at 224 nm.

Extraction was essential to clean the plasma samples. To remove endogenous impurities in plasma to an acceptable level several organic solvents were tried for extraction of the samples. When dichloromethane was used the solvent and I.S. peaks overlapped. If diethyl ether was used as extracting solvent, precise results were difficult to achieve due to its high volatility. Ethyl acetate was finally selected as the optimal extracting solvent which enabled recovery up to 80% to be achieved.

Due to the poor peak symmetry obtained for TG using the chromatographic conditions reported in references [3,5–7] described for TAP appropriate quantities of tetrabutyl ammonium bromide and ammonium acetate were added to the eluent. This improved the peak shape dramatically and enabled complete separation of the analytes and I.S. to be achieved within 13 min. Neither pooled blank plasma from different individuals nor plasma obtained from volunteers receiving TG hydrochloride showed other chromatographic peaks around the retention times of TG, TAP and the I.S. Endogenous peaks (e.g. at 7.8 min in plasma and 9.6 min in urine) did not interfere with the quantification of TG and TAP.

The concentrations of TG in urine samples were so low that they could not be detected in most urine samples. So it was only TAP that was measured in urine samples.

For each analytical run, nine-point plasma and urine standard curves were constructed, and were shown to be linear over the tested range of 0.78–100 μg/ml. Preliminary pharmacokinetic analysis of clinical samples indicated that plasma concentrations of TG and TAP were always lower than 100 μg/ml, and hence the upper level of the curve was set at this concentration. Over the entire concentration range of the standard curve, the mean observed percent deviation was between 5.4 and +6.1%. The lower limit of quantitation (LLOQ) of this HPLC–UV method was 10 ng/ml.

The accuracy and precision of the method are summarized in Tables 1 and 2. Accuracy was assessed by comparing the concentrations of the spiked control samples, estimated from the calibration curve to their true value. An acceptance limit of 15% was applied for validation in the concentration level in the calibration range. Recoveries ranged from 96.1 to 108.9% with the coefficients of variation less than 8% (Table 3).

The in-process stability, freeze–thaw cycles stability study and frozen assay stability study of the compounds at low, medium and high concentration levels in the matrix were assessed. The results were calculated by the analysis of variance (ANOVA) using the STATIS-II program. The results, up to three freeze–thaw assay cycles showed apparent differences

in the calculated spiked concentrations (Table 4) and indicated that TAP was stable under these conditions while TG was not. As a consequence of this instability TG in the control blank plasma samples was extracted as rapidly as possible and the extracted residue was experienced at refrigeration temperature for 24 h. During stability experiments, it was found that TG was relatively stable in methanol or in about pH 3 phosphate buffers, but it was not stable in plasma at ambient temperature and in freeze–thaw cycles. Thus plasma samples obtained from volunteers after intravenous infusion administration of TG hydrochloride in clinical pharmacokinetic trials should be frozen immediately and allowed to stand at $-70\,^{\circ}C$ before analysis. Plasma samples and extracted residues should be stored under ice cooling until chromatography analysis to achieve acceptable results.

4. Preliminary pharmacokinetic study

The validated HPLC–UV method was successfully applied to a preliminary pharmacokinetic study of TG and TAP in clinical trials. The pharmacokinetic parameters for TG and TAP were estimated with the 3p97 computer program of the Chinese Society of Mathematical Pharmacology. The pharmacokinetic data demonstrate that TG is rapidly transformed to TAP in vivo. Plasma TG and TAP concentration–time profiles were fitted to a two-compartment open model and linear first-order elimination from the central compartment.

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