

Metabolism and Disposition of Tribendimidine and Its Metabolites in Healthy Chinese Volunteers

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Abstract

Background: Tribendimidine is a new anthelmintic agent synthesized by Chinese scientists. It is a broad spectrum agent with high activity against parasites. However, its disposition and metabolism remain unknown.

Objective: To investigate the metabolism, disposition, and metabolites of tribendimidine in healthy human volunteers.

Methods: Twelve healthy Chinese volunteers were chosen after clinical assessment of health status and laboratory tests. They received single oral doses of tribendimidine 400 mg enteric-coated tablets. Blood and urine samples were collected at scheduled timepoints. Samples were qualitatively and quantitatively analyzed by liquid chromatography-mass spectrometric (LC-MS) and high performance liquid chromatography (HPLC) methods, respectively.

Results: Tribendimidine was rapidly and completely broken down to *p*-(1-dimethylamino ethylimino) aniline (dADT) and terephthalaldehyde (TPAL). Furthermore, dADT was partially transformed to acetylated dADT, and TPAL completely transformed to terephthalic acid (TPAC). The main pharmacokinetic parameters (\pm SD) of dADT were as follows: elimination half life ($t_{1/2}$) 4.74 ± 1.80 h; elimination rate constant (K_e) 0.16 ± 0.06 h⁻¹; apparent volume of distribution (Vd/F) 12.23 ± 8.69 L \bullet kg⁻¹; apparent total clearance of the drug from plasma (CL/F) 1.63 ± 0.58 L \bullet h⁻¹ \bullet kg⁻¹; area under the plasma concentration-time curve (AUC) from time 0 to time 24 hours (AUC₂₄) 4.29 ± 1.88 μ g \bullet mL⁻¹ \bullet h; AUC from time zero to infinity (AUC_∞) 4.45 ± 1.81 μ g \bullet mL⁻¹ \bullet h; maximum plasma drug concentration (C_{max}) 0.64 ± 0.27 μ g \bullet mL⁻¹; and time to C_{max} (t_{max}) 4.20 ± 0.71 h. A total of 35.28% dADT and 28.50% TPAC were excreted through the urine within 24 hours after tribendimidine administration.

Conclusion: These results reveal the disposition, metabolism, and main metabolites of tribendimidine in healthy Chinese volunteers.

Introduction

Tribendimidine, N,N'-bis[4'-(1-dimethyl amino ethylidene amino)phenyl]-1,4-phenylene-dimethylidene amine, is a new and effective anthelmintic agent, with broad spectrum activity against helminthes. It is highly active against *Ascaris lumbricoides* and *Necator americanus* without mutagenic and clastogenic effects compared with other anthelmintic agents.^[1] Worldwide, more than 1221 million people are infected with *Ascaris lumbricoides* (the most common intestinal nematode), 795 million people with *Trichuris trichiura*, and 740 million people with hookworms.^[2] Tribendimidine is promising because of its safety profile, broad spectrum, and high levels of activity.

In clinical and pharmacodynamic studies, Yueyi et al.^[3] and Kuyang et al.^[4] showed that tribendimidine was highly active against hookworm and *Ascaris*. The adverse effects of tribendimidine were very mild and transient. Zhongxing and Yixin^[5] compared the therapeutic and adverse effects of single or combination doses of tribendimidine and ivermectin against human nematodiasis. It was indicated that tribendimidine combined with ivermectin had the highest cure rate, suggesting the existence of synergism. Shuhua et al.^[6] assessed the efficacy of artemether, artesunate, praziquantel, and tribendimidine against different stages of *Clonorchis sinensis in vivo*, and found that the artemisinins and tribendimidine possess important clonorchicidal properties.

Few studies have focused on the metabolism and pharmacokinetic profiles of tribendimidine in humans. *In vitro*, tribendimidine is unstable and quickly broken down to *p*-(1-dimethylamino ethylimino) aniline (dADT) and terephthalaldehyde (TPAL) in acidic buffer, human blank plasma, and urine.^[7] dADT was demonstrated to be present in human plasma and urine after oral administration of tribendimidine enteric-coated tablets; however, only 40% of the original dose was recovered, indicating that other metabolites may be produced. TPAL was not detected in human plasma, urine, or feces, but its metabolite, terephthalic acid (TPAC), was present in human urine. dADT and TPAC were detected in human

plasma and urine using liquid chromatography-mass spectrometric (LC-MS) and high performance liquid chromatography (HPLC) methods.^[7,8] The disposition of tribendimidine *in vivo* can be identified by these same methods. In the present study, we aimed to identify the distribution, metabolism, and metabolites of tribendimidine in humans using qualitative and quantitative analyses.

Materials and Methods

Chemicals and Reagents

Tribendimidine, dADT standard (>99%) and tribendimidine enteric-coated tablets (200 mg, No. 0505226) were provided by Xinhua Pharmaceutical Co. Ltd. (Zibo, China). TPAL and TPAC were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Mirtazapine standard (>99%) was provided by Kexinwei Pharmacy Research Company (Jinan, China). Ferulaic acid was provided by the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade acetonitrile and methanol were purchased from J.T.Baker (Phillipsburg, NJ, USA). Glacial acetic acid and triethylamine were purchased from Tedia Company (Fairfield, CT, USA).

Instruments

The LC-MS system equipped with electrospray ionization (ESI) source, HPLC pump, and autosampler (Agilent, Santa Clara, CA, USA) was used for metabolism analysis and dADT detection in human plasma samples. The Agilent HPLC and Waters HPLC systems (Milford, MA, USA) were used to determine dADT and TPAC levels in human urine samples.

Chromatography and Mass Spectra Conditions

The LC-MS method was used to examine dADT levels in human plasma and feces. The separation was performed using a Hypersil C₁₈ column (250 × 4.6 mm inner diameter [ID], 5 μm) with mobile phase containing ammonium acetate 10 mmol/L (containing 0.1% triethylamine): acetonitrile (20:80, v/v) at 25°C. Flow rate was

1.0 mL • min⁻¹. The ESI source was set to positive ion mode with capillary voltage 4000 V, desolvation gas (nitrogen) heated at 350°C (11.0 L • min⁻¹), nebulizer pressure 50 psi, and fragment voltage 90 V. Quantification was performed with selected ion monitoring (SIM) of mass-to-charge ratio (m/z) 178.1 for dADT.

dADT and TPAC in human urine were examined using the HPLC method. dADT was separated through an Extend C₁₈ column (250 × 4.6 mm ID, 5 μm) by isocratic elution using methanol:acetonitrile:water:triethylamine (35:35:30:0.2, v/v/v/v) at 0.7 mL • min⁻¹. Mirtazapine was used as the internal standard and UV detection was at 249 nm. TPAC was separated through an Ultimate XB C₁₈ column (200 × 4.6 mm ID, 5 μm) by isocratic elution with methanol:1% glacial acetic acid (35:65, v/v) at 0.8 mL • min⁻¹. Ferulaic acid was used as the internal standard and UV detection was at 240 nm.

The LC-MS method was used to analyze the metabolite of tribendimidine in urine with an Agilent Extend C₁₈ column (250 mm × 4.6 mm ID, 5 μm) at 25°C. Isocratic elution of the analyte was performed with mobile phase (acetonitrile: methanol:water:triethylamine [35:35:30:0.2, v/v/v/v]) at 1.0 mL • min⁻¹. The analyte was detected and analyzed with ESI ion source in both positive ion mode and full scan mode. The molecular weight ranged from 110 to 600. The detection conditions of capillary voltage, source temperature, drying gas flow, and nebulizer pressure were 4000 V, 350°C, 11.0 L • min⁻¹, and 50 psi, respectively.

Method Validation and Sample Preparation

dADT (1.0 mg • mL⁻¹) stock solution was prepared and diluted in ethanol for the working solutions. Linearity was assessed ranging from 0.5 to 1000 ng • mL⁻¹ in plasma and from 5 to 500 μg • mL⁻¹ in urine. Similarly, TPAC stock solution (2.0 mg • mL⁻¹) was prepared and diluted in 1% sodium hydroxide for the working solutions. Linearity was assessed ranging from 1 to 400 μg • mL⁻¹ in urine.

A calibration curve was constructed on the basis of peak area and this was analyzed using

weighted linear regression. The lower limit of quantification (LLOQ) was defined as the lowest concentration to be produced at least five times with acceptable precision (relative standard deviation [RSD] <20%) and accuracy (80–120% of nominal concentration). Intra- and inter-day precision and accuracy were determined by analyzing spiked samples with low, medium, or high concentrations for 3 days, and five replicates were applied. Three aliquots of the spiked samples at low, medium, or high concentration were stored for the stability studies of dADT and TPAC.

A total of 0.5 mL of plasma, 50 μL of sodium hydroxide (5 mol/L) and 0.3 mL of acetonitrile were added to a 1.5 mL polypropylene centrifuge tube; this was vortex-mixed for 1 minute, and then centrifuged at 11 132 g (revolutions per minute) for 5 minutes. The supernatant was transferred into another 1.5 mL polypropylene centrifuge tube that contained 50–60 mg sodium chloride; this was vortex-mixed for 30 seconds, incubated for 10 minutes at room temperature, and then centrifuged for 5 minutes at 11 132 g. A total of 5 μL of the final supernatant was injected for analysis. Urine samples were diluted and then analyzed without disposition after adding the internal standard (mirtazapine for dADT and ferulaic acid for TPAC).

Study Design

Twelve healthy Chinese volunteers (six men and six women, aged 23–27 years) were screened after clinical assessment of health status and laboratory tests. Informed consents were signed before starting the experiments. The protocol was approved by the Ethic Committee of Qilu Hospital, and was conducted in accordance with the Declaration of Helsinki.

The volunteers fasted for 10 hours. After oral administration of tribendimidine enteric-coated tablets (400 mg), 4 mL of blood was collected before (0 hours), and 0.5, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 12, and 24 hours after administration, and a liquor of urine samples were collected at time zero (blank sample), 0–2, 2–4, 4–6, 6–8, 8–12, and 12–24 hours after administration. Plasma was separated, collected, and stored at -20°C for

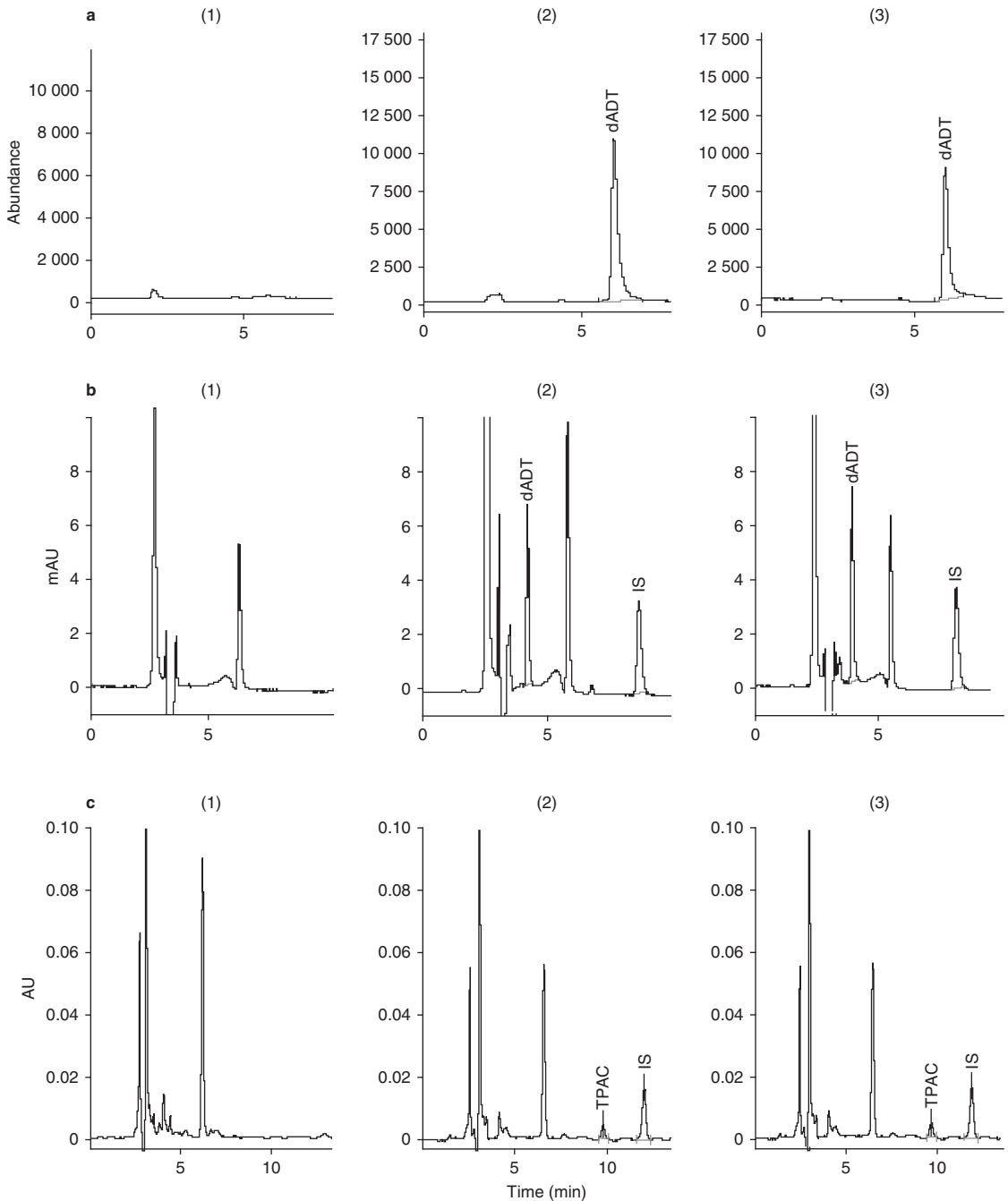


Fig. 1. The typical chromatograms of (a) blank plasma (1), plasma sample spiked with *p*-(1-dimethylamino ethylimino) aniline [dADT] (2), and plasma sample from a volunteer 1 hour after oral administration of tribendimidine (3); (b) blank urine (1), urine sample spiked with dADT and mirtazapine [internal standard; IS] (2), and urine sample from a volunteer 4 hours after oral administration of tribendimidine spiked with IS (3); and (c) blank urine (1), urine sample spiked with terephthalic acid (TPAC) and ferulic acid [IS] (2), and urine sample from a volunteer 4 hours after oral administration of tribendimidine spiked with IS (3). **AU** = absorb unit; **mAU** = mini-absorb unit.

Table I. Precision (relative standard deviation [RSD]) and accuracy for the determination of *p*-(1-dimethylamino ethylimino) aniline (dADT) and terephthalic acid (TPAC) in human plasma and urine

Variable	Concentration	RSD (%)		Accuracy (% of nominal concentration)
		intra-day	inter-day	
dADT				
Plasma	1 ng • mL ⁻¹	2.93	4.88	97.72
	50 ng • mL ⁻¹	1.22	1.74	96.78
	800 ng • mL ⁻¹	1.86	3.22	97.12
Urine	10 µg • mL ⁻¹	2.98	2.66	99.82
	50 µg • mL ⁻¹	0.59	2.52	99.26
	400 µg • mL ⁻¹	1.37	2.79	97.03
TPAC				
Urine	3 µg • mL ⁻¹	5.37	6.24	105.99
	25 µg • mL ⁻¹	4.04	5.62	107.40
	200 µg • mL ⁻¹	3.58	3.89	108.49

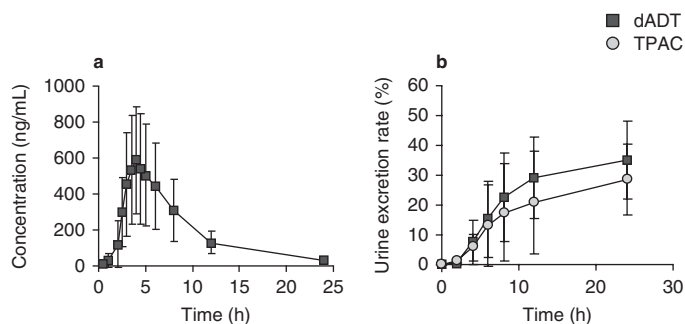
analysis. The Drug and Statistic (DAS, version 2.0)^[9] program was used to fit the compartmental model of dADT and calculate the main pharmacokinetic parameters as follows: elimination half-life ($t_{1/2}$), elimination rate constant (K_e), area under the plasma concentration-time curve from time 0 to 24 hours (AUC_{24}), area under the plasma concentration-time curve from time zero to infinity (AUC_{∞}), apparent total clearance of the drug from plasma (CL/F), and apparent volume of distribution (Vd/F). The maximum plasma drug concentration (C_{max}) and the time to reach C_{max} (t_{max}) were also observed. Accumulated excretion rates of dADT and TPAC in urine were obtained by the following equation: accumulated excretion rate = accumulated excretion amount / time × 100%.

Chromatograms of blank urine samples were compared with those of urine samples collected after oral administration of tribendimidine enteric-coated tablets. The natures of the metabolites were determined by their standard and molecular weight.

Results

Validation of Analytical Methods

The typical chromatograms of plasma and diluted urine are shown in figure 1. No interferences were found in analyzed plasma and urine samples. The retention time of dADT in plasma was approximately 6.08 minutes. The retention times of dADT and its internal standard in urine were 4.10 and 8.76 minutes, respectively. The

**Fig. 2.** (a) The plasma concentration vs time profile of *p*-(1-dimethylamino ethylimino) aniline (dADT) [$n = 12$] and (b) the urine excretion rate vs time curves of dADT and terephthalic acid (TPAC) [$n = 6$] after oral administration of tribendimidine 400 mg enteric-coated tablets.

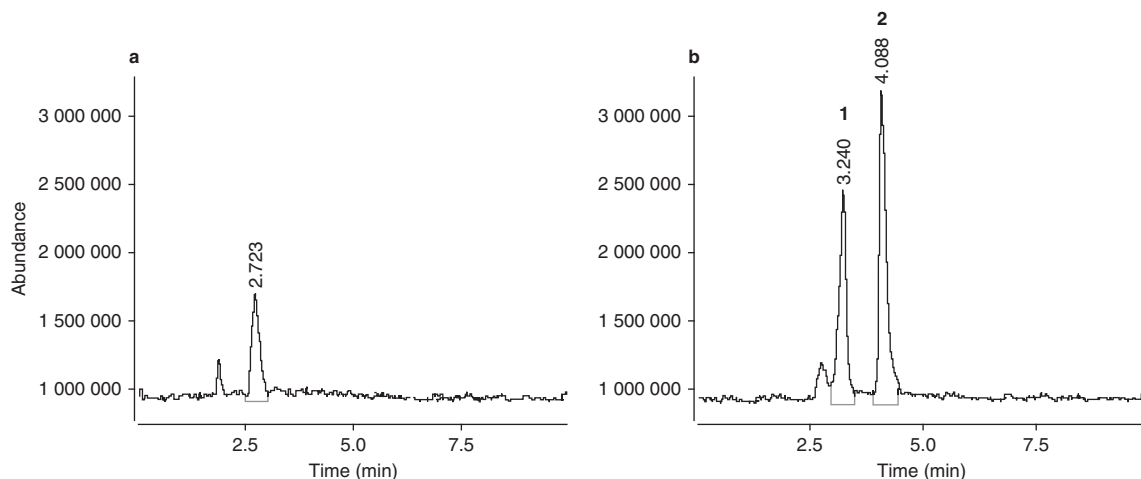


Fig. 3. Chromatograms of (a) blank urine and (b) urine collected 4 hours after administration of tribendimidine. Peaks 1 and 2 in (b) correspond to the peak retention times of acylated *p*-(1-dimethylamino ethylimino) aniline (dADT) and dADT, respectively (see figure 4 for more detailed analysis).

retention times of TPAC and its internal standard in urine were 9.86 and 12.06 minutes, respectively.

The calibration curves of dADT were linear above the concentration ranges in plasma ($n=5$) and urine ($n=5$) with the regression equations (weight = $1/X^2$), which were $Y=14767.7001X+783.85047$ for plasma and $Y=4.283X+0.001$ for urine, and the correlation coefficients were 0.999. The LLOQ of dADT was $0.5 \text{ ng} \cdot \text{mL}^{-1}$ in plasma ($n=5$) and $5 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ in urine ($n=5$).

The calibration curve of TPAC was linear above the concentration range in urine. The regression equation was $Y=0.0268X-0.00147$ with a correlation coefficient of 0.9954. The LLOQ of TPAC was $1 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ in urine ($n=5$).

The intra- and inter-assay precision and accuracy of dADT and TPAC were acceptable for the analysis. All data were obtained from five replicates on 3 consecutive days, and are shown in table I.

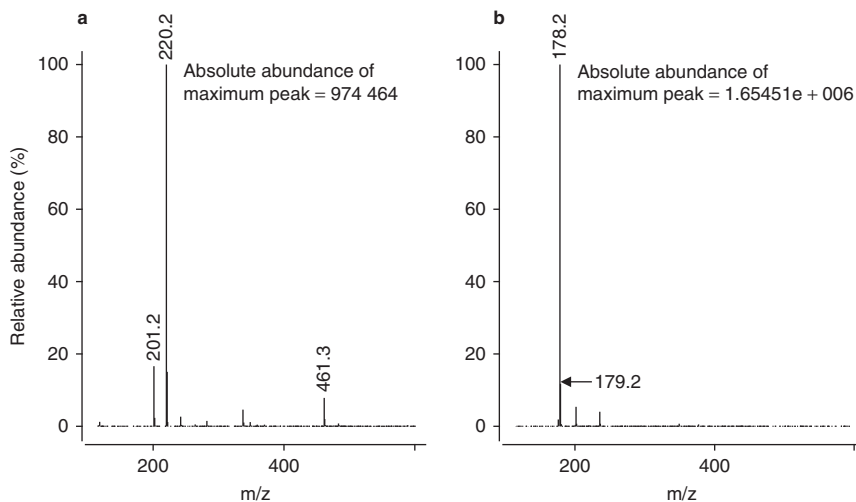


Fig. 4. Mass spectrum of (a) acetylated *p*-(1-dimethylamino ethylimino) aniline (dADT) and (b) dADT. m/z = mass-to-charge ratio.

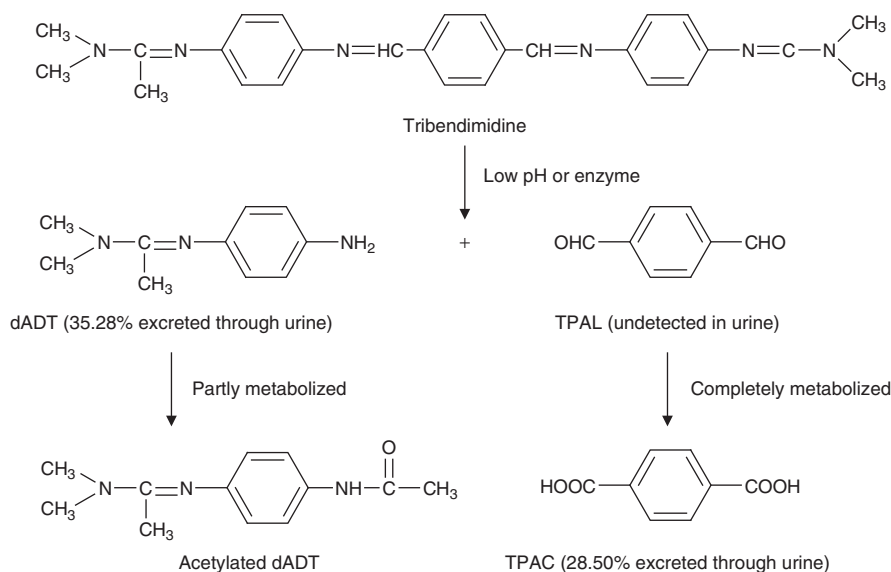


Fig. 5. The breakdown of tribendimidine in the human body. **dADT**=*p*-(1-dimethylamino ethylimino) aniline; **TPAC**=terephthalic acid; **TPAL**=terephthalaldehyde.

The stability of dADT in human plasma and urine, and TPAC in human urine were investigated under different storage and process conditions. The analyte was assayed with three concentrations. dADT was stable after being frozen for 7 days at -20°C (90.00–94.70% of the initial value in plasma and 97.84–99.88% in urine), and stable after two freeze-thaw cycles (-20°C /room temperature) [92.35–100.68% of the initial value in plasma and 98.64–99.78% in urine]. TPAC was stable after being frozen for 7 days at -20°C (95.32–99.70% of the initial value) or two freeze-thaw cycles (-20°C /room temperature) [96.75–102.13% of the initial value].

Metabolism and Disposition of Tribendimidine and Its Metabolites

The plasma concentration and urine excretion rate versus time curves of dADT and TPAC in human plasma and urine are shown in figure 2. The kinetics of dADT fit a one-compartmental model. The main pharmacokinetic parameters were as follows: $t_{1/2}$ 4.74 ± 1.80 h; K_e 0.16 ± 0.06 h $^{-1}$; V_d/F 12.23 ± 8.69 L \bullet kg $^{-1}$; CL/F 1.63 ± 0.58 L \bullet h $^{-1}$ \bullet kg $^{-1}$;

AUC_{24} 4.29 ± 1.88 $\mu\text{g} \bullet \text{mL}^{-1} \bullet \text{h}$; AUC_{∞} 4.45 ± 1.81 $\mu\text{g} \bullet \text{mL}^{-1} \bullet \text{h}$; C_{max} 0.64 ± 0.27 $\mu\text{g} \bullet \text{mL}^{-1}$; and t_{max} 4.20 ± 0.71 h. Accumulated excretion rates of dADT and TPAC were $35.28 \pm 13.00\%$ and $28.50 \pm 11.92\%$ in urine, respectively.

The chromatograms of blank urine samples and urine samples collected after oral administration of tribendimidine are shown in figure 3. Two peaks of retention time, 3.2 minutes and 4.1 minutes, were validated by LC-MS. Peak 1 (protonated molecular ion $[M + H]^+$, 220.2) and peak 2 ($[M + H]^+$, 178.2) are shown in figures 4a and 4b, respectively. Peak 2 is dADT and peak 1 is the acetylated metabolite of dADT based on their molecular weight and standard.

Discussion

In this study, the LC-MS and HPLC methods for determination of dADT and TPAC in human plasma and urine were established. The high sensitivity for dADT was obtained using the electrospray ion source and positive selective ion monitoring mode. The mobile phase for the LC-MS method was optimized by comparing various

percentages of methanol: ammonium acetate 10 mmol/L or acetonitrile: ammonium acetate 10 mmol/L to improve separation and to enhance sensitivity. Use of acetonitrile and triethylamine, instead of methanol, allowed appropriate retention time and sharp peak shape, respectively. No endogenous interferences were found under the optimized LC-MS conditions.

The results indicated that tribendimidine was immediately broken down to dADT and TPAL after oral administration of enteric-coated tablets. dADT in plasma can be used to evaluate the pharmacokinetic behavior of tribendimidine *in vivo* after its oral administration. The metabolite TPAL was also not detected in either urine or plasma, because it was quickly metabolized to a more stable metabolite (TPAC), which was found in urine. dADT was further metabolized to an acetylated form by acetyltransferase, as shown in figure 5.

dADT is the major metabolite of tribendimidine. However, so far, there have been no studies to demonstrate its anti-parasitic activity. The activity of dADT, degree of acetylation, and activity of acetylated dADT are expected to be identified in future studies.

Conclusion

Specific, sensitive, and rapid methods were developed to determine dADT and TPAC, and to investigate the disposition and metabolism of tribendimidine in human plasma and urine. Our data suggest that tribendimidine is rapidly broken down to dADT and TPAL. Furthermore, dADT was partially transformed to acetylated dADT, and TPAL was completely transformed to TPAC. The anti-parasitic activity of dADT and its acetylated metabolite, and the degree of its acetylation need further investigation.

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