0090-9556/07/3502-221–227$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 35, No. 2
12922/3171371
Printed in U.S.A.

DMD 35:221–227, 2007

Triethylenetetramine and Metabolites: Levels in Relation to Copper and Zinc Excretion in Urine of Healthy Volunteers and Type 2 Diabetic Patients

Jun Lu, Yi-Kai Chan, Gregory D. Gamble, Sally D. Poppitt, Asma A. Othman, and Garth J. S. Cooper

School of Biological Sciences, Faculty of Science (J.L., Y.-K.C., A.A.O., G.J.S.C.), Department of Medicine, Faculty of Medical and Health Sciences (G.D.G., G.J.S.C.), Human Nutrition Unit (S.D.P.), and Centre for Molecular Biodiscovery (G.J.S.C.), University of Auckland, Auckland, New Zealand

Received September 14, 2006; accepted November 8, 2006

ABSTRACT:

Triethylenetetramine (TETA), a selective Cu^{II}-chelator used in the treatment of Wilson's disease, is now undergoing clinical trials for the treatment of heart failure in diabetes. Despite decades of clinical use, knowledge of its pharmacology in human subjects remains incomplete. Here, we first used liquid chromatography-mass spectrometry (LC-MS) to detect and identify major metabolites of TETA in human plasma and urine, and then used this method to measure concentrations of TETA and its metabolites in the urine of healthy and diabetic subjects who were administered increasing doses (300, 600, 1200, and 2400 mg) of TETA orally. Twenty-four-hour urine collections were performed before and after dosing participants. Two major metabolites of TETA were detected in human urine, N_1-acetyltriethylenetetramine (MAT) and N_1,N_{10}-diacetyltriethylenetetramine, the latter being novel. Both metabolites were verified with synthetic standards by LC-MS. The proportion of unchanged TETA excreted as a fraction of total urinary drug-derived molecules was significantly higher in healthy than in matched diabetic subjects, consistent with a higher rate of TETA metabolism in the latter. TETA-evoked increases in urinary Cu excretion in nondiabetic subjects were more closely correlated with parent drug concentrations than in diabetic subjects, whereas, by contrast, urinary Cu was more closely associated with the sum of TETA and MAT. These findings are consistent with the hypothesis that MAT could play a significant role in the molecular mechanism by which TETA extracts Cu^{II} from the systemic compartment in diabetic subjects.

Heart disease leads to death in most diabetic patients (Gu et al., 1998; Struthers and Morris, 2002). Recently, we showed that triethylenetetramine (TETA), a Cu^{II}-selective chelator used for the treatment of Wilson's disease (Walshe, 1982), ameliorated left ventricular hypertrophy in diabetic rats and humans (Cooper et al., 2004). We proposed that metabolic changes in diabetes, in particular, those resulting in the accumulation of advanced glycation endproducts in the extracellular matrix, lead to or cause the accumulation of excess extracellular Cu^{II} in the extracellular matrix, and that the resulting tissue-Cu imbalance could cause heart failure (Cooper et al., 2004, 2005). The ability of TETA to remove excess extracellular Cu^{II} thus makes it a candidate pharmacotherapeutic for the cardiovascular complications of diabetes. Consequently, it is now undergoing phase II clinical trials to assess its safety and efficacy as a therapy for heart failure in diabetes (Cooper et al., 2004).

Although TETA administration can elevate urinary Cu excretion (Cooper et al., 2005), the exact mechanism of its action and pharmacology in diabetes is still under investigation. There are relatively few reports on TETA pharmacology in patients with Wilson's disease in the literature, although it has been used as a treatment for this condition for decades (Walshe, 1982; Leggio et al., 2005). Indeed, most relevant human-derived pharmacological data have been derived from studies in healthy volunteers. Most oral TETA is not absorbed, but excreted unchanged in the feces (Gibbs and Walshe, 1986). The 5 to 18% of TETA that is systemically absorbed is said to be extensively metabolized, with the majority being excreted in urine as metabolite(s) (Kodama et al., 1993, 1997), and results from rat studies have been consistent (Kobayashi et al., 1990; Takeda et al., 1995a,b,c). One major metabolite, MAT, has been reported in human urine (Kodama et al., 1997). In those reports, TETA was fluorescence-derivatized and

This study was funded by grants from the Foundation for Research Science and Technology, New Zealand. the Health Research Council of New Zealand, and by Protemix Corporation.

G.G. is a consultant to, J.L., S.P., and A.A.O. are employed by, and G.C. is a Shareholder and Director of Protemix.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.106.012922.

ABBREVIATIONS: TETA, triethylenetetramine; ACS, N_1-acetylspermine; ADME, absorption, distribution, metabolism, and excretion; DAT, N_1,N_{10}-diacetyltriethylenetetramine; HFBA, heptafluorobutyric acid; HPLC, high-pressure liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; MAT, N_1-acetyltriethylenetetramine; NAT, *N*-acetyltransferase; PABA, *p*-aminobenzoic acid; ICPMS, inductively coupled plasma mass spectrometry; HFBA, heptafluorobutyric acid; SIM, selected-ion monitoring; LME, linear mixed-effects model; LLOQ, lower limit of quantification.

detected using HPLC (Nakano et al., 2002). Fluorescence derivatization methods are often associated with problems such as 1) whether the analyte is fully or partially labeled, 2) whether metabolites are labeled, 3) whether metabolites are fully or partially labeled, and 4) whether the analyte-specific signal is separated from those corresponding to other known or unknown metabolites.

Here, we used LC-MS to study TETA metabolism and excretion by analyzing urine samples from drug-treated diabetic subjects and healthy volunteers. We have detected a novel metabolite, N_1,N_{10}-diacetyltriethylenetetramine (DAT) and developed an LC-MS method to measure TETA and both its major metabolites in urine, within one injected sample and without fluorescence labeling. In addition, we concomitantly measured urinary concentrations of TETA and its major metabolites, and analyzed TETA-evoked changes in Cu and Zn excretion in relation to drug and metabolite concentrations.

Materials and Methods

Clinical Data. Six healthy male volunteers and seven diabetic male patients, whose ages ranged between 30 and 70 years, completed this study. One healthy volunteer withdrew from the study during the first dosing period. Diabetic patients had been diagnosed at least 6 months before enrollment, and all control subjects were shown to have normal glucose tolerance. Exclusion criteria included the presence of type 1 diabetes, nephropathy, abnormal hematology or Fe deficiency; a history of significant cardiac disease; previous hepatic, gastrointestinal, or endocrine disease other than diabetes; gangrene or active sepsis; severe retinopathy, nondiabetic renal disease, or renal allograft; malignancy (except cutaneous basal cell carcinoma); or known abnormality of Cu or Fe metabolism; as well as current treatment with diuretics or calcium-channel blockers (Cooper et al., 2005). All protocols received appropriate regulatory approval, and all subjects provided written informed consent.

Authentic Standards. TETA dihydrochloride, MAT trihydrochloride, and DAT dihydrochloride, all of >99.9% purity, were synthesized and supplied by CarboGen AG (Hunzenschwil, Switzerland). N_1-Acetylspermine (ACS) dihydrochloride, of greater than 98% purity, was obtained from Sigma-Aldrich (St. Louis, MO).

Collection of Urine and Plasma Samples. Subjects received increasing doses of TETA dihydrochloride (300, 600, 1200, and 2400 mg/day; Anstead, Essex, UK) in an open-label, dose-escalating trial. Each dose was taken at a single time in the morning (as 300-mg capsules) after an overnight fast, for a period of 7 days, with 6-week washout periods between each dose. Dosing was not witnessed; therefore, the possibility of incomplete compliance was addressed by drug capsule/*p*-aminobenzoic acid (PABA) tablet counts and determination of PABA recovery. For each subject, 24-h urine samples were collected immediately before and on the 7th day of each dosing schedule. Urine samples were collected in precleaned, wide-mouth, trace metal-free plastic receptacles (Nuplex Industries Ltd., Auckland, New Zealand). Gross urine volumes from each 24-h collection were recorded and urine collections were monitored using a PABA test (Cooper et al., 2005). Subjects were given PABA-check tablets (Laboratory for Applied Biology, London, UK) containing 80 mg of the naturally occurring B-vitamin complex to be taken at each of three main meals throughout the day. A recovery of >75% of the oral dose is indicative of complete urine collection over the 24-h period. Low recoveries provide a qualitative indicator of poor urine collection, although failure to adhere to the 80-mg three times per day dosing regimen cannot be excluded when recovery is low, and recovery of <5% is strongly indicative of complete noncompliance. The PABA results were used together with subjects' feedback on drug capsule and PABA tablet intake, completeness of urine collection, urine volume consistency, drug levels in urine, and copper and zinc changes in urine, to determine whether a data point was included in the final statistical analysis. Undiluted urine samples were transferred to 5-ml screw-cap tubes and stored at −80°C until analysis.

In a separate study, 2-ml heparinized blood samples were taken from one healthy male volunteer before and at 10, 20, 40, 60, 90, and 120 min after taking 2400 mg of oral TETA dihydrochloride. Each sample was centrifuged at 10,000*g* and plasma was collected into 1.5-ml tubes. Samples were stored at −80°C until analysis.

Urinary Cu and Zn Analysis. [Cu] and [Zn] were measured in pre- and post-treatment urine samples by inductively coupled plasma mass spectrometry (ICPMS; Elan 6100; PerkinElmerSciex Instruments, Boston, MA) as described previously (Cooper et al., 2005). In brief, diluted samples and matrix-matched standard curve solutions were measured by ICPMS with gallium as the internal standard. Cu and Zn concentrations were calculated from the standard curve. The ICPMS operating parameters were as follows: radiofrequency power, 1500 W; nebulizer gas flow rate, 0.9 l/min; auxiliary gas flow rate, 1.2 l/min; plasma gas flow rate, 15 l/min; reaction gas, NH_3 at 0.3 ml/min; data acquisition mode, peak hopping, three replicates, 20 sweeps per replicate; and sample uptake rate, 1 ml/min.

Metabolite Identification. Urine samples (0.5 ml) were thawed, centrifuged, and transferred into 1.5-ml snap-cap metal-free HPLC vials. One hundred microliters of plasma was added to 50 μl of 10% heptafluorobutyric acid (HFBA; Sigma-Aldrich) and centrifuged to remove precipitated protein. Urine and plasma supernatants were first injected into the mass spectrometer directly. MS conditions were optimized as follows: curved desolvation line temperature, 200°C; heat block, 200°C; nebulizing gas, 1.5 l of N_2/min; interface voltage, 3.5 kV; CDL voltage, 40.0 V; and Q-array voltage, DC 0.0 V, radiofrequency 140 V. The MS positive-ion scan mode was used and ion profiles from pre- and postdrug samples were compared to detect putative major metabolites. Supernatant was then transferred into 1.5-ml HPLC vials, which were loaded onto the temperature-controlled auto-injector of an LC-MS system, comprising an LC-10ADvp pump, a DGU-14AM degasser, a SIL-10ADvp temperature-controlled auto-injector, a CTO-10ASvp column oven, and a 2010A single quadrupole mass spectrometer equipped with an electrical spray ionization interface (Shimadzu Corp., Kyoto, Japan). Fifty-microliter samples were injected into the LC-MS apparatus and chromatographic separation was achieved using a 5-μ Cyano 100 × 4.6 mm column (maintained at 25°C in the column oven) with guard (Phenomenex, Torrance, CA) using 15% (v/v) acetonitrile, 0.1% (v/v) heptafluorobutyric acid in Milli Q water (18 MΩ resistivity; Waters, Milford, MA) as mobile phase. The flow rate was 0.5 ml/min. All samples were analyzed first using MS positive-ion scan mode, with molecular mass range from 50 to 1000 Da, to identify peaks corresponding to TETA and its metabolite(s). Samples were then reanalyzed together with blank urine, to which had been added the authentic standards (TETA, MAT, and DAT), by LC-MS with an additional MS-positive selected-ion monitoring (SIM) signal set at $[M + H]^+ = 147$, 189, and 231 Da, with a micro-scan of 0.5 Da (corresponding to TETA and the metabolites found in previous MS scans), to better identify and characterize the analytes.

Measurement of Urinary Metabolites. Serial dilutions of TETA, MAT, and DAT standards were made in blank urine samples to create standard curves, such that each sample contained one aliquot of each standard. Twenty microliters of HFBA (600 mM) was added into 300 μl of each standard urine sample, and to urine samples from human subjects (with appropriate dilution as necessary). Samples were centrifuged (12,000*g*, 10 min, 4°C), and 200 μl of supernatant was then transferred to a HPLC vial, together with 50 μl of 100 μM ACS as internal standard. Final concentrations of HFBA and ACS were 30 mM and 20 μM, respectively. HFBA (30 mM) stabilized TETA in solution for at least 24 h. ACS appeared to be a suitable internal standard inasmuch as urinary excretion of natural endogenous ACS was undetectable in our experiments. Duplicate 50-μl sample aliquots were analyzed by LC-MS. Separation and detection of TETA and its metabolites were achieved under the same conditions as described above for metabolite identification, with the modification that an extra molecular mass, of 245 Da, was added to the SIM signal to detect the internal standard, ACS. All standards and samples were analyzed within 24 h of preparation.

Data acquisition and MS peak area integration for TETA, MAT, DAT, and ACS peaks were achieved using Shimadzu LCMSsolution software. To construct calibration curves, the area ratios of MS peaks for TETA or its metabolites, relative to that of the internal standard, were plotted against the standard concentrations. Lines of best fit were plotted by nonlinear hyperbolic regression analysis (Prism; GraphPad Software Inc., San Diego, CA). The range of the calibration curves was 1 to 16 μM for each standard ($r^2 = 0.99$ in each case). The relative recoveries were 90 to 120% and coefficient of variation was <10%. The back-calculated values were within 10% of prepared values in each case.

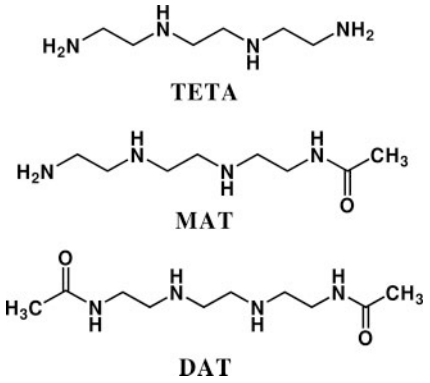

FIG. 1. Molecular structures of TETA, MAT, and DAT.

Statistical Analysis. Descriptive statistics and regression analyses were computed (Prism) and linear mixed-effects models (LMEs) were fitted by restricted maximum likelihood (S-Plus 7.0.2; Insightful Corp., Seattle, WA). Multiple linear regression was performed (SAS 9.1; SAS Institute Inc., Cary, NC) to determine whether TETA, MAT, and DAT were significant independent predictors of Cu or Zn excretion. Once the significant predictors were determined, the models for each significant metabolite alone and in combination were compared for goodness of fit using the Akaike information criterion (SAS 9.1; SAS Institute Inc.).

Results

Identification of Metabolites in Human Urine. Direct injection and mass spectral comparisons of pre- and postdrug urine samples showed that six extra ions were present in postdrug samples. Three of these were of 230, 188, and 146 Da, consistent with DAT, MAT, and TETA, respectively. The other three (of 212, 262, and 338 Da) had less than 1% of the intensity of the preceding three ions in the urine samples of healthy and diabetic subjects, indicating that they are either minor metabolites or other compounds excreted by the body secondary to drug administration. Therefore, their identification was not pursued. Direct injection of plasma samples showed that only three additional ions (of 230, 188, and 146 Da, consistent with DAT, MAT, and TETA) were present in mass spectra of postdrug samples. In LC-MS runs, three extra peaks, with corresponding molecular mass of 230, 188, and 146 Da, were apparent in postdose urine samples compared with predose urine samples, consistent with DAT, MAT and TETA, respectively (see Fig. 1 for molecular structures). Injected standards showed equivalent retention times and molecular masses, consistent with the presence of DAT, MAT, and TETA, respectively (Fig. 2). All three compounds were demonstrated in urine from both healthy and diabetic subjects.

Metabolites in Human Plasma. A TETA peak was present in all post-treatment plasma samples. MAT was detected in plasma samples collected 60 to 120 min after TETA administration, whereas DAT was detected only in the single 120-min plasma samples (Fig. 3).

Comparison of Urinary Excretion of TETA and Its Metabolites between Healthy Volunteers and Diabetic Patients. Drug, including unchanged TETA and metabolites, excreted in the urine during 24 h, was used to estimate total urinary drug excretion. A previous [^{14}C]TETA study showed that 96% of urinary drug excretion occurred within the first 24-h period (Takeda et al., 1995c). Another study measuring 0- to 26-h TETA and MAT in urine of healthy volunteers showed that TETA was not detectable after 10 h, and less than 3% of 0- to 26-h MAT was found in urine between 24 and 26 h (Kodama et al., 1997). Concentrations of TETA and its metabolites in 24-h urine samples were determined by comparison with standard curves (Fig. 4), and 24-h urinary excretion rates were calculated. Total 24-h urinary drug excretion (of all forms of the drug) was calculated as the sum of the amount of TETA and metabolites, and results are summarized in Tables 1 (for healthy volunteers) and 2 (for diabetic patients). Since all incidences of detection under the lower limit of quantification (LLOQ) for TETA and its metabolites occurred in healthy volunteers, the value of LLOQ (1 μM) was used to give a maximum estimate in subsequent statistical analysis. Since some subjects demonstrated apparent noncompliance by the criteria of PABA and/or capsule counts, a combination of PABA results, drug levels, Cu and Zn changes, urine volume consistency, and subjects' feedback of PABA and drug intake were used as inclusion/exclusion criteria to select data points for the final analysis (Tables 1 and 2; excluded data points are italicized). For example, if a subject did not take PABA (i.e., patient Dia5 with a PABA recovery of 2.5% at the 2400-mg dose) but still took the drug and completed urine collection (verified by urine volume consistency), the data point was included. On the other hand, if a subject had an acceptable PABA recovery (i.e., healthy subject HV6 with a PABA recovery of 79.4% at 300 mg dose) but did not take the drug, the data point was excluded.

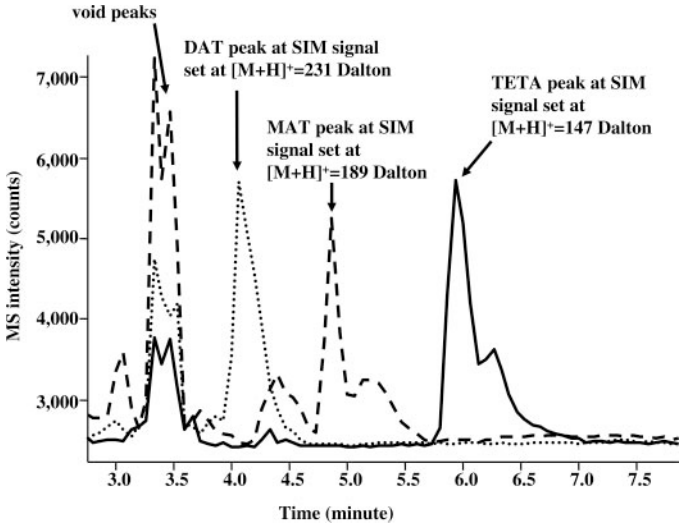

FIG. 2. MS-chromatogram showing separation and detection of TETA, MAT, and DAT in urine sample from a type 2 diabetic patient collected 120 min after oral TETA (2400 mg) administration.

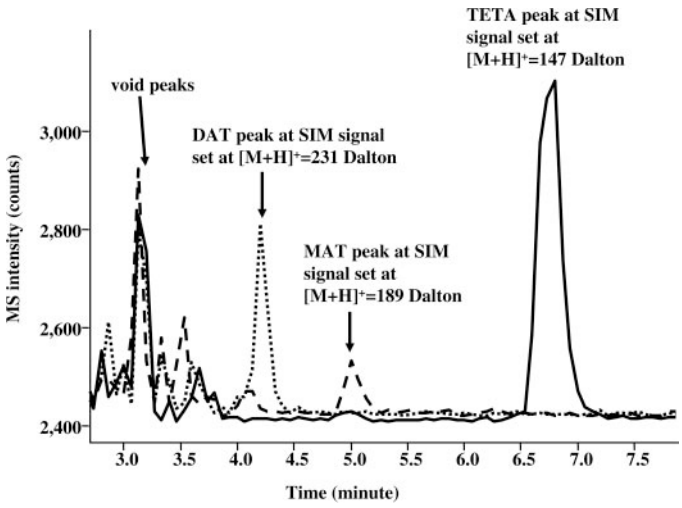

FIG. 3. MS-chromatogram showing separation and detection of TETA, MAT, and DAT in plasma sample of a healthy volunteer collected 120 min after oral TETA (2400 mg) administration.

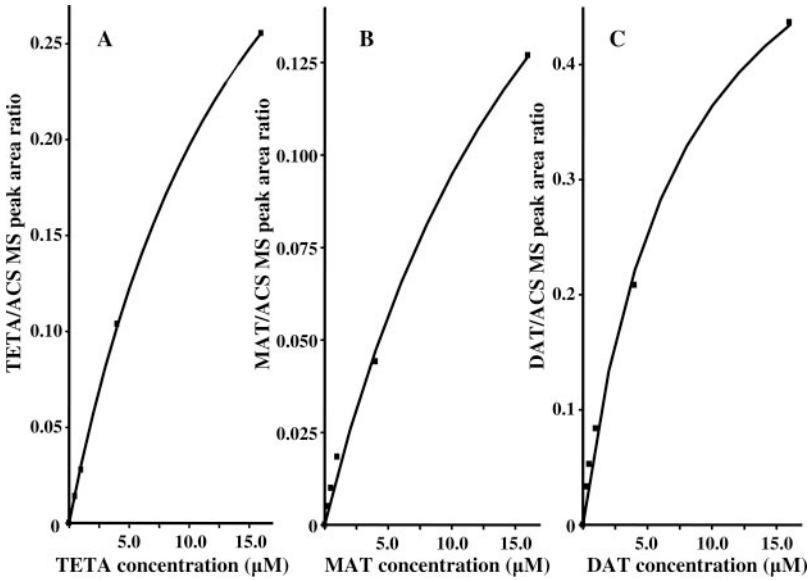

FIG. 4. Standard curves corresponding to TETA (A), MAT (B), and DAT (C) in human urine.

TABLE 1

Twenty-four-hour urinary TETA, MAT, and DAT excretion and corresponding changes in urinary Cu and Zn excretion in six individual healthy human subjects at indicated drug doses

Subject	Dose	PABA Recovery	Urinary Drug Excretion			ΔCu	ΔZn
			TETA	MAT	DAT		
	mg	*%*		*μmol/day*			*μmol/day*
HV1	300	119	3.34	22.4	9.45	1.35	2.19
	600	*33.8*	*0.183*	*6.28*	*14.6*	*0.096*	*−0.834*
	1200	101	11.9	51.7	17.3	1.75	6.88
	2400	85.8	70.6	97.4	63.7	4.24	17.0
HV2	300	123	14.8	54.0	20.5	1.47	2.63
	600	110	11.9	27.9	7.16	1.55	6.20
	1200	100	45.7	52.7	16.1	3.99	22.4
	2400	122	267	1037	165	8.62	59.5
HV3	300	85.9	<LLOQ	<LLOQ	1.78	0.53	0.86
	600[a]	*14.2*	*0.463*	*1.18*		*0.322*	*−0.346*
	1200	1.25	24.3	40.1	15.2	3.17	14.3
	2400	97.6	111	149	65.9	5.29	39.2
HV4	300	80.3	0.288	0.050	0.165	0.455	1.74
	600	143	0.072	0.785	0.066	1.69	2.08
	1200	107	20.2	37.3	24.2	3.40	6.26
	2400	96.7	164	228	93.0	7.66	26.6
HV5	*300*	*−1.56*	*<LLOQ*	*<LLOQ*	*<LLOQ*	*−0.032*	*−1.04*
	600	*33.1*	*<LLOQ*	*<LLOQ*	*<LLOQ*	*0.243*	*1.31*
	1200	60.9	31.9	360	57.6	1.98	9.60
	2400	*59.6*	*<LLOQ*	*<LLOQ*	*<LLOQ*	*0.004*	*0.627*
HV6	*300*	*79.4*	*<LLOQ*	*<LLOQ*	*<LLOQ*	*0.136*	*1.83*
	600	*72.0*	*<LLOQ*	*<LLOQ*	*<LLOQ*	*0.935*	*2.45*
	1200	97.2	2.36	28.6	72.7	1.30	4.12
	2400	*35.1*	*<LLOQ*	*<LLOQ*	*<LLOQ*	*−0.223*	*−8.08*

HV, healthy volunteer.
[a] Italicized numbers represent excluded data points.

As statistical models, LMEs were used to determine the significance of differences using all data points from different doses, because this technique can take multiple factors (dose, disease status, and drug concentration) into consideration simultaneously. There was no significant difference between the amount of unchanged TETA excreted in urine of healthy volunteers and diabetic patients (Fig. 5A; $p = 0.873$, LME, S-Plus). However, diabetic patients excreted significantly more metabolized TETA than did healthy volunteers (Fig. 5B; $p = 0.015$, LME). Total drug (including metabolites) excreted in urine was 0.03 to 13.4% (range) (3.2 ± 1.1%, mean ± S.E.M.) of administered dose in healthy volunteers and 3.7 to 14.6% (9.2 ± 0.4% %) of administered dose in diabetic patients. Thus, diabetic patients excreted significantly higher percentages of administered doses than healthy volunteers (Fig. 6; $p = 0.005$, LME), whereas dose did not affect the percentage of administered drug excreted in urine ($p = 0.121$, LME). In both diabetic and healthy subjects, there was a significant correlation between dose and amount of drug excreted ($p < 0.001$, LME), but there was no combined effect of dose and disease status on drug excretion (both parent and metabolites) ($p > 0.05$, LME). Within urinary drug (parent + metabolites), diabetic subjects demonstrated higher percentages of metabolites (92.9 ± 0.1%) than did healthy volunteers (77.2 ± 0.2%) ($p = 0.007$, LME), but dose elevation did not alter this difference ($p = 0.224$, LME).

TABLE 2

Twenty-four-hour urinary TETA, MAT, and DAT excretion and concomitant changes in Cu and Zn excretion in seven individual type 2 diabetic subjects at indicated drug doses

Subject	Dose	PABA Recovery	Urinary Drug Excretion			Δ Cu	Δ Zn
			TETA	MAT	DAT		
	mg	*%*		*μmol/day*			*μmol/day*
Dia1	*300*[a]	*64.3*	*34.7*	*144*	*30.9*	*1.92*	*−3.42*
	600	*63.2*	*237*	*378*	*49.4*	*4.99*	*15.4*
	1200	*17.6*	*50.0*	*79.9*	*40.0*	*1.83*	*−8.26*
	2400	*35.7*	*767*	*981*	*225*	*13.1*	*59.8*
Dia2	300	83.6	5.46	108	13.6	0.511	−2.18
	600	117	10.2	152	81.1	0.969	9.13
	1200	108	46.2	511	192	2.33	20.0
	2400	92.0	120	659	115	3.66	31.7
Dia3	300	85.1	0.240	60.6	33.0	0.266	5.16
	600	98.8	8.12	156	236	0.879	5.67
	1200	93.5	37.6	389	170	2.38	9.31
	2400	94.6	144	1068	217	6.87	45.9
Dia4	300	66.7	0.122	37.7	29.3	0.367	3.55
	600	7.60	5.34	205	174	1.24	6.48
	1200	53.3	22.0	418	324	1.88	25.2
	2400	94.6	103	1057	320	7.69	60.6
Dia5	300	75.5	2.16	105	56.6	0.213	−5.29
	600	84.9	6.26	245	110	0.639	2.54
	1200	91.6	21.8	394	93.6	1.91	6.74
	2400	2.50	62.8	687	248	1.80	12.0
Dia6	300	81.1	4.38	51.9	20.6	0.947	2.28
	600	93.3	12.7	74.3	20.6	1.11	6.89
	1200	83.7	32.4	152	47.5	1.60	8.32
	2400	79.4	114	372	131	3.62	15.2
Dia7	300	95.8	7.07	48.9	14.7	1.83	5.05
	600	101	17.2	157	25.8	1.48	7.34
	1200	89.7	62.9	350	79.9	3.35	25.8
	2400	85.4	146	601	98.8	6.62	50.2

Dia, diabetic.
[a] Italicized numbers represent excluded data points.

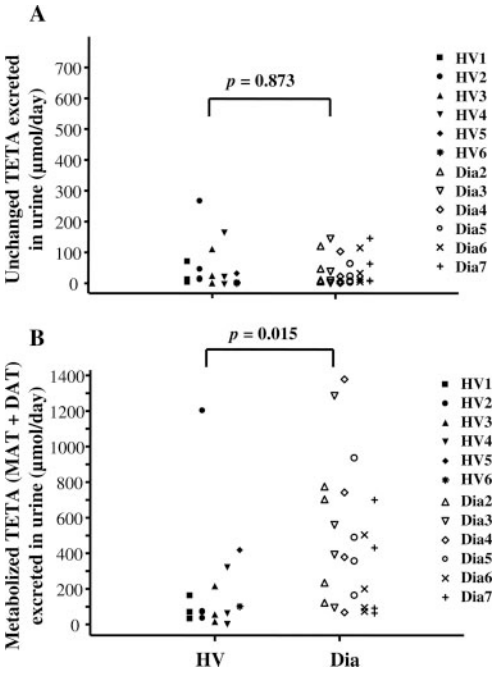

FIG. 5. Comparison of 24-h urinary excretion of unchanged TETA (A) and metabolized TETA (B) between healthy volunteers (HV) and diabetic patients (Dia). Exact *p* values were derived from LME.

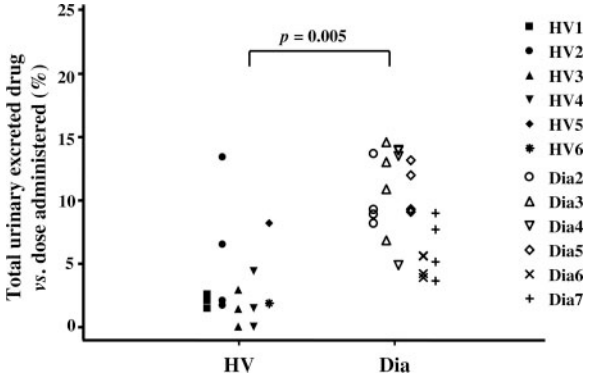

FIG. 6. Comparison of proportions of administered drug excreted in urine between healthy volunteers (HV) and diabetic patients (Dia). Exact *p* values were derived from LME.

Human 24-h Urinary Excretion of TETA and Its Metabolites, and Their Correlation with Changes in Urinary Cu and Zn Excretion. The changes in 24-h Cu and Zn excretion are as shown in Tables 1 and 2. They were calculated as ΔCu or Zn = [Cu or Zn content of 24-h urine after dosing] − [Cu or Zn content in 24-h urine before dosing]. ΔCu and Zn were closely related to dose elevation ($p < 0.001$, LME). Changes in Cu and Zn excretion in healthy volunteers and diabetic patients were then analyzed against amounts of urinary TETA, MAT, DAT, [TETA + MAT], or [TETA plus both metabolites].

TETA and MAT were both found to be significant independent predictors of urinary Cu excretion over drug levels for healthy volunteers and diabetic patients (Table 3). Further refinement of the model shown in Table 4 suggested that TETA alone is the best predictor of change in Cu in response to drug in healthy volunteers, whereas the sum of TETA and MAT is the best predictor for diabetic

TABLE 3

Summary of multiple linear regression models for determination of the significant independent predictors of urinary copper or zinc excretion

Urinary Cu or Zn Changes and Excreted Drug Levels		*p* Values	
		Healthy Volunteers	Diabetic Patients
Urinary Δ Cu-drug	Δ Cu-TETA	**<0.0001***	**0.0057***
	Δ Cu-DAT	0.85	0.60
	Δ Cu-MAT	**0.018***	**0.055***
Urinary Δ Zn-drug	Δ Zn- TETA	**0.001***	**0.035***
	Δ Zn-DAT	0.67	0.67
	Δ Zn-MAT	0.95	0.13

* Bold denotes results considered statistically significant ($p < 0.05$).

TABLE 4

Goodness of fit statistical predictors of copper excretion.

Urinary Cu Changes and Excreted Drug Levels		Adjusted r^2	
		Healthy Volunteers	Diabetic Patients
Urinary Δ Cu-drug	Δ Cu-TETA	**0.89***	0.82
	Δ Cu-MAT	0.51	0.75
	Δ Cu-TETA + MAT	0.52	**0.85***

* Best fit model by Akaike information criterion minimized ($p < 0.05$).

patients. For Zn, TETA alone was the best predictor of change in urinary Zn response to drug in both healthy and diabetic subjects (Table 3).

Discussion

This study identified and characterized the major metabolites of TETA in diabetic patients and healthy human subjects. We found that, apart from TETA itself, there are two major metabolites present in human urine and plasma. One is MAT, which was previously identified (Kodama et al., 1997), whereas, to our knowledge, the existence of the second, DAT, has not previously been reported. Identifying major metabolites of TETA is an important step for further pharmacological investigation, such as absorption, distribution, metabolism and excretion (ADME) studies, in diabetic patients.

Previous studies showed that TETA was poorly absorbed. A study using [^{14}C]TETA showed that only 6 to 18% of orally administered drug was systemically absorbed (Gibbs and Walshe, 1986). Another rat study reported the bioavailability of TETA to be about 13.8% (Tanabe, 1996). In an additional report, the urinary recovery of drug was about 9% of administered dose in healthy humans (Kodama et al., 1997). Our current results showed that urinary recoveries of drug varied between 0.03 and 13.4% of administered dose in healthy volunteers and between 3.7 and 14.6% in diabetic subjects, consistent with these previous studies. Our direct injection studies did not detect any other significant TETA metabolites in urine or plasma. Those findings, together with our recovery levels that were consistent with the previous [^{14}C]TETA study, indicate that no other major metabolites of TETA are likely to be excreted in urine.

Most urinary TETA was present as metabolites, and unchanged TETA only accounted for 22.8 ± 0.6% and 7.1 ± 0.8% of total excreted drug in healthy and diabetic subjects, respectively. In drug excreted via the urine, the percentage of metabolized drug was much higher in diabetic subjects (92.9 ± 0.1%) than in healthy controls (77.2 ± 0.2%). This is in accord with previous findings that more metabolite than parent compound is excreted in urine of humans and rats (Kobayashi et al., 1990; Kodama et al., 1993, 1997; Takeda et al., 1995b). The greater amount of metabolized TETA excreted in diabetic patients is consistent with the hypothesis that TETA might be more extensively metabolized in them than in healthy controls (Fig. 5B). In a recent pharmacokinetic study, we found that MAT and DAT concentrations in the blood of diabetic patients were higher than those in healthy subjects at the same dose (600 mg). The T_{max} values corresponding to TETA, MAT, and DAT in healthy subjects ($n = 24$) were 1.9 ± 0.8, 5.1 ± 0.6, and 5.1 ± 0.7 h, respectively. Serum C_{max} values for TETA, MAT, and DAT in healthy subjects were 5.4 ± 2.3, 4.0 ± 1.6, and 1.1 ± 0.6 μM, respectively (unpublished data). In diabetic subjects, concentrations of TETA, MAT, and DAT were determined in serum sampled 4 to 6 h after dosing, and were 6.3 ± 4.2, 10.5 ± 5.3, and 2.6 ± 1.6 μM, respectively (unpublished data). Although there were no statistically significant differences between TETA concentrations, those of MAT and DAT in diabetic subjects were significantly higher than corresponding values in healthy subjects. This observation further supports our hypothesis that TETA might be metabolized faster in diabetic subjects than in healthy subjects.

One possibility is that diabetic patients may have higher hepatic metabolic rates for TETA. For example, previous rat (Sheweita et al., 2002) and rabbit (Arinc et al., 2005) studies indicated that diabetes increases the activity of certain hepatic enzymes (e.g., those in the cytochrome P450 family). There is also a report that type 2 diabetic patients had a marked increase in hepatic CYP2E1 activity (Wang et al., 2003). However, the nature of the enzymes responsible for TETA metabolism [presumably either *N*-acetyltransferase (NAT) or polyamine acetyltransferase, or both], and whether these enzyme activities are modified by diabetes, are still under investigation. NAT is an enzyme that could mediate TETA metabolism. Polymorphism of NAT could greatly affect NAT expression and, thus, TETA metabolism (Wormhoudt et al., 1999). We have recently studied the plasma pharmacokinetics in two healthy volunteer groups, with fast and slow acetylator phenotypes, and found no difference in any of the pharmacokinetic parameters between the two groups (unpublished data). However, whether diabetic subjects have higher rates of NAT mutation than normal subjects is unknown. We have planned a study to genotype and phenotype NAT in normal and diabetic subjects to determine whether there is any difference. The expression of metabolizing enzymes also changes with age. However, subjects in our study were age-matched; therefore, age was unlikely to influence these results. Another possibility is that, apart from the liver, TETA may be metabolized more extensively by enzyme(s) in other organs such as heart, kidney, or gut, in diabetic than in healthy people. We are currently investigating TETA metabolism in liver, kidney, and heart of healthy and diabetic rats to test these hypotheses.

The evidence that diabetic patients metabolize TETA more completely than healthy people suggests that further ADME studies in diabetic patients may be required. Previous ADME results from healthy volunteers for studies of Wilson's disease may not be applicable in diabetic patients, because a higher metabolic rate of TETA may change its ADME behavior, as well as its pharmacokinetics and pharmacodynamics. Here, a significantly higher proportion of administered drug was excreted by diabetic patients than by healthy subjects (Fig. 6). There is, thus, a correlation between the apparently greater rate of TETA metabolism and its higher intake or bioavailability in diabetic subjects.

The Cu excretion changes in healthy volunteers mainly correlated with urinary [TETA] (Table 4): urinary [Cu] increased in proportion to urinary [TETA], consistent with a prior report (Kodama et al., 1997). Urinary [Zn] showed similar trends in healthy volunteers, increasing with urinary [TETA] (Table 4). This is not surprising, because TETA is a more potent chelator than its *N*-acetyl metabolite in vitro (Kodama et al., 1997).

Interestingly, increases in Cu excretion in diabetic patients showed a different correlation with urinary drug levels, in that their ΔCu values were more closely related to [TETA + MAT] (Table 3) than for control subjects, in whom [TETA] alone was the main correlate. These findings are consistent with MAT acting as a Cu-chelator in vivo. Although MAT reportedly has in vitro Cu-chelating activity (Kodama et al., 1997), we know of no prior reports concerning its in vivo Cu- or Zn-chelating properties.

Our prior studies have shown that the mechanisms by which TETA elevates urinary Cu and Zn are likely to differ between diabetic and healthy people. TETA-mediated increases in urinary Cu and Zn in diabetic subjects resulted largely from their extraction from the systemic compartment whereas, by contrast, elevated urinary Cu and Zn excretion in healthy subjects resulted mainly from drug-mediated increases in metal uptake from the gut (Cooper et al., 2005). The difference between diabetic and control subjects may be due to the up-regulation of metallothionein in small bowel enterocytes in the former group, which could create a defensive barrier to the excessive Cu and Zn uptake that might otherwise result from diabetic hyperphagia.

In summary, we found two major TETA metabolites in human plasma and urine, one of which, DAT, has not previously been reported. Results of urinary drug analyses indicate that diabetic patients metabolize TETA more extensively than do healthy people, which in turn was associated with its higher uptake or bioavailability in those with the disease. These findings may warrant further ADME studies in diabetic patients. Increased urinary Zn excretion was mainly associated with unchanged urinary TETA in both healthy and diabetic subjects. Increased urinary Cu excretion in healthy volunteers showed similar characteristics, being mainly associated with unchanged urinary TETA levels. However, increased urinary Cu excretion in diabetic patients more closely correlated with the urinary [TETA + MAT] than with nonmetabolized urinary TETA alone. MAT may thus be implicated in the mechanism by which TETA extracts excess systemic Cu in diabetes.

Acknowledgments. We thank Tracey Sunderland for critical review of the manuscript.

References

Arinc E, Arslan S, and Adali O (2005) Differential effects of diabetes on CYP2E1 and CYP2B4 proteins and associated drug metabolizing enzyme activities in rabbit liver. *Arch Toxicol* **79:**427–433.

Cooper GJ, Chan YK, Dissanayake AM, Leahy FE, Keogh GF, Frampton CM, Gamble GD, Brunton DH, Baker JR, and Poppitt SD (2005) Demonstration of a hyperglycemia-driven pathogenic abnormality of copper homeostasis in diabetes and its reversibility by selective chelation: quantitative comparisons between the biology of copper and eight other nutritionally essential elements in normal and diabetic individuals. *Diabetes* **54:**1468–1476.

Cooper GJ, Phillips AR, Choong SY, Leonard BL, Crossman DJ, Brunton DH, Saafi L, Dissanayake AM, Cowan BR, Young AA, et al. (2004) Regeneration of the heart in diabetes by selective copper chelation. *Diabetes* **53:**2501–2508.

Gibbs KR and Walshe JM (1986) The metabolism of trientine: animal studies, in *Orphan Diseases and Orphan Drugs* (Scheinberg IH and Walshe JM eds) pp 33–42, Manchester University Press in Association with the Fulbright Commission, Manchester, UK.

Gu K, Cowie CC, and Harris MI (1998) Mortality in adults with and without diabetes in a national cohort of the U.S. population, 1971–1993. *Diabetes Care* **21:**1138–1145.

Kobayashi M, Sugawara M, Saitoh H, Iseki K, and Miyazaki K (1990) [Intestinal absorption and urinary excretion of triethylenetetramine for Wilson's disease in rat]. *Yakugaku Zasshi* **110:**759–763.

Kodama H, Meguro Y, Tsunakawa A, Nakazato Y, Abe T, and Murakita H (1993) Fate of orally administered triethylenetetramine dihydrochloride: a therapeutic drug for Wilson's disease. *Tohoku J Exp Med* **169:**59–66.

Kodama H, Murata Y, Iitsuka T, and Abe T (1997) Metabolism of administered triethylene tetramine dihydrochloride in humans. *Life Sci* **61:**899–907.

Leggio L, Addolorato G, Abenavoli L, and Gasbarrini G (2005) Wilson's disease: clinical, genetic and pharmacological findings. *Int J Immunopathol Pharmacol* **18:**7–14.

Nakano Y, Nohta H, Yoshida H, Saita T, Fujito H, Mori M, and Yamaguchi M (2002) Liquid chromatographic determination of triethylenetetramine in human and rabbit sera based on intramolecular excimer-forming fluorescence derivatization. *J Chromatogr B* **774:**165–172.

Sheweita SA, Newairy AA, Mansour HA, and Yousef MI (2002) Effect of some hypoglycemic herbs on the activity of phase I and II drug-metabolizing enzymes in alloxan-induced diabetic rats. *Toxicology* **174:**131–139.

Struthers AD and Morris AD (2002) Screening for and treating left-ventricular abnormalities in diabetes mellitus: a new way of reducing cardiac deaths. *Lancet* **359:**1430–1432.

Takeda S, Ono E, Matsuzaki Y, Wakui Y, Ono H, Kushida H, Takizawa Y, Kaneko M, Takeda S, Wakamatsu T, et al. (1995a) [Metabolic fate of triethylenetetramine dihydrochloride (trientine hydrochloride, TJA-250) 3. Bioavailability of TJA-250 in rats after single administration]. *Oyo Yakuri* **49:**179–186.

Takeda S, Ono E, Wakui Y, Matsuzaki Y, Ishihara K, Yanagisawa E, Takeda S, and Wakamatsu T (1995b) [Metabolic fate of triethylenetetramine dihydrochloride (trientine hydrochloride, TJA-250) 2. Metabolism study in rats using ^{14}C-TJA-250]. *Oyo Yakuri* **49:**173–178.

Takeda S, Ono E, Wakui Y, Matsuzaki Y, Wakui Y, Mizuhara Y, Asano T, Takeda S, and Wakamatsu T (1995c) [Metabolic fate of triethylenetetramine dihydrochloride (trientine hydrochloride, TJA-250) 1. Absorption, distribution and excretion in rats after single administration of ^{14}C-TJA-250]. *Oyo Yakuri* **49:**163–171.

Tanabe R (1996) Disposition behavior and absorption mechanism of trientine, an orphan drug for Wilson's disease. *Hokkaido J Med Sci* **71:**217–228.

Walshe JM (1982) Treatment of Wilson's disease with trientine (triethylene tetramine) dihydrochloride. *Lancet* **1:**643–647.

Wang Z, Hall SD, Maya JF, Li L, Asghar A, and Gorski JC (2003) Diabetes mellitus increases the in vivo activity of cytochrome P450 2E1 in humans. *Br J Clin Pharmacol* **55:**77–85.

Wormhoudt LW, Commandeur JNM, and Vermeulen NPE (1999) Genetic polymorphisms of human *N*-acetyltransferase, cytochrome P450, glutathione-*S*-Transferase, and epoxide hydrolase enzymes: relevance to xenobiotic metabolism and toxicity. *Crit Rev Toxicol* **29:**59–124.

Address correspondence to: Professor Garth J.S. Cooper, School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand. E-mail: g.cooper@auckland.ac.nz