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Article

LC–MS-MS Analysis of Urinary Biomarkers of Imazalil Following Experimental Exposures

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Imazalil (IMZ) is a fungicide used in the cultivation of vegetables, such as cucumbers, in green houses or post-harvest on fruit to avoid spoilage due to fungal growth. Agricultural workers can be occupationally exposed to IMZ and the general public indirectly by the diet. The purpose of this study was to develop and validate an LC–MS-MS method for the analysis of IMZ in human urine. The method used electrospray ionization and selected reaction monitoring in the positive mode. Excellent linearity was observed in the range 0.5–100 ng/mL. The limit of detection of the method was 0.2 ng/mL, and the limit of quantitation 0.8 ng/mL. The method showed good within-run, between-run and between-batch precision, with a coefficient of variation <15%. The method was applied to analyze urine samples obtained from two human volunteers following experimental oral and dermal exposure. The excretion of IMZ seemed to follow a two-compartment model and first-order kinetics. In the oral exposure, the elimination half-life of IMZ in the rapid excretion phase was 2.6 and 1.9 h for the female and the male volunteer, respectively. In the slower excretion phase, it was 7.6 and 13 h, respectively. In the dermal exposure, the excretion seemed to follow a single-compartment model and first-order kinetics. The elimination half-life was 10 and 6.6 h for the female and the male volunteer, respectively. Although the study is limited to two volunteers, some information on basic toxicokinetics and metabolism of IMZ in humans is presented.

Introduction

Modern agriculture is extensively pesticide-dependent, from seed treatment up to post-harvest transport. The 2006 EU monitoring of pesticide residues in plant origin-based products has reported on 769 different pesticide residues (1). Human exposure to pesticides is substantial in the developing countries due to extensive agricultural activities and they may affect human health (2, 3). Populations working in close contact with pesticides are at risk of harmful exposure. Of particular concern is the exposure of women in fertile age during the post-harvest treatment. The general population may also be exposed by intake of contaminated food and the domestic use of pesticides, and may be by living nearby pesticide-treated agricultural fields or common land.

Imazalil (IMZ), 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy) ethyl]-1*H*-imidazole, is a widely used pre- and post-harvest fungicide applied on a variety of vegetable crops, and especially on fruits like citrus to avoid rotting, thus increasing the lifetime of products on the market. IMZ is effective against sporulation and decaying caused by *Penicillium* species and blue-green molds (4), although resistance against IMZ is increasing (5). IMZ is also used in veterinary medicine as a topical antimycotic drug.

In farm workers, dermal exposure has been suggested as the main exposure route to pesticides (6, 7). In closed agricultural set-ups with poor ventilation, inhalation is another important exposure route (8). Workers may be exposed to particulates (dusts), sprays, mist or fog of pesticides depending upon the formulation and the method of application and the general population by the gastrointestinal route. Thus, it is important to evaluate dermal as well as oral exposure in the toxicokinetic studies of pesticides in humans.

In animals, IMZ is reported to cause severe eye irritation (9). Also, the compound may influence the activity of cytochrome P450 isoforms (10), has the potential to affect the endocrine system by interacting with steroidogenesis (11), possesses anti-androgenic activity (12) and is suspected to produce craniofacial malformations in vertebrates (13). *In vitro* studies of IMZ on isolated rat hepatocytes showed depletion of intracellular levels of glutathione, protein thiols and cellular ATP causing cell death (14).

The established acute reference dose (ARfD), based on rabbit fetal toxicity, is 0.05 mg/kg body weight (bw; no-observed-adverse-effect-level, NOAEL 5 mg/kg bw) for pregnant and nursing women, and based on adult (maternal) toxicity it was 0.1 mg/kg bw (NOAEL 10 mg/kg bw) for the general population (9, 15).

In Wistar rats, it has been described that IMZ formed 25 metabolites and 1-(2,4-dichlorophenyl)-2-(1*H*-imidazole-1-yl)-1-ethanol (DCPI) was one of the major metabolites formed by undergoing an oxidative O-dealkylation reaction (16). In a human case study, the half-life of IMZ in serum was reported to be 2 h after intake of a high dose of IMZ as medical treatment (17).

Various methods have been published for the determination of IMZ residues in agricultural and dietary products. However, no method has been described for the determination of IMZ or DCPI in human biological samples, and there is a lack of information on IMZ metabolism in humans. Thus, methods easily applicable to measure the biomarkers of exposure are of interest.

The aim of this study was to develop an LC–MS-MS method for the analysis of IMZ and its metabolite DCPI in human urine. Furthermore, by experiments in humans (although limited to two volunteers), a contribution to a basic understanding of the metabolism of IMZ, including its urinary elimination, was accomplished.

Experimental

Chemicals and materials

IMZ (99% purity) and the internal standard (IS) [$^2H_5$]-Imazalil were obtained from Dr Ehrenstorfer (Augsburg, Germany). The metabolite DCPI was obtained from Toronto Research

Chemicals (North York, ON, Canada). Methanol (LC–MS grade) and hydrochloric acid (HCl) were from Merck (Darmstadt, Germany). Formic acid was from Sigma-Aldrich (St Louis, MO, USA). β-Glucuronidase (*Escherichia coli*) was from Roche Diagnostics (Mannheim, Germany). Water was produced by Milli-Q Integral 5 system, Millipore (Billerica, MA, USA). Solid-phase extraction (SPE) columns, silica-based TELOS C_2 (EC) 100 mg fixed well plates, were obtained from Kinesis (Cambridgeshire, UK).

Instrumentation

The samples were analyzed using a liquid chromatography system (UFLC^{RX}, Shimadzu Corporation, Kyoto, Japan) coupled with a triple quadrupole linear ion trap mass spectrometer, equipped with TurboIonSpray source (QTRAP 5500, AB Sciex, Foster City, CA, USA). Air was used as nebulizer and auxiliary gas, and nitrogen as curtain and collision gas. The temperature of the auxiliary gas was set at 650°C and the ionspray voltage was 5,500 V. The analysis was performed using positive ionization in the selected reaction monitoring (SRM) mode. All the operations were controlled using the Analyst 1.6.1 software from AB Sciex. The LightSight® Software 2.2.1 (AB Sciex) was used for the prediction of metabolites of IMZ in the urine samples. The SPE extraction was performed on 96-channel equipment CEREX 96 (SPEware Corporation, Baldwin Park, CA, USA), which used nitrogen gas to develop a positive pressure to facilitate uniform extraction.

Analysis

Aliquots of 500 μL urine were pipetted into 2 mL 96-well plates and added with 1 M ammonium acetate buffer (6.5 pH), 15 μL of β-glucuronidase and 20 μL of IS. The plates were vortex-mixed and incubated at 37°C for 48 h. Separate tests were performed to optimize the period of sample incubation and suitability of enzyme hydrolysis (Supplementary data). The SPE columns were conditioned with 1 mL of methanol and 1 mL of milli-Q (MQ) water before applying the samples on the columns. Then, the columns were washed with 1 mL of 20% methanol and eluted with 1% HCl (12 M) in methanol. The details of the SPE method are presented in Supplementary data along with the preparation of standards, samples and quality control (QC) samples.

Chromatographic separation was carried out on a Grace Genesis Lightn (USA) C18 (4 μm, ID 2.1 mm, length 50 mm) column. The mobile phase consisted of 0.1% formic acid in MQ water (A) and 0.1% formic acid in methanol (B). The sample injection volume was 5 μL and the flow rate through the column was 0.5 mL/min. The column temperature was maintained at 40°C. The mobile phase gradient started with 5% mobile phase B, progressing linearly to 95% during 5 min and held at 95% for 1 min, including equilibration at 5%. For the dilution correction, density and creatinine were determined in all urine samples. Density was measured using a hand refractometer and creatinine was determined using an enzymatic method (18). The reported precision of the enzymatic method at $n = 50$ was 1.6% coefficient of variation (CV) at 6.4 mmol/L and 1.8% CV at 14.6 mmol/L. The creatinine adjustment was performed by dividing the analyte concentration with the creatinine content of the urine sample. The density adjustment was calculated using the following formula: $C_{(\text{density-adjusted})} = C\ (1 - \rho_{\text{mean}})/(1 - \rho_{\text{sample density}})$, where C = the determined analyte concentration in the sample, ρ_{mean} = the mean of the specific density obtained from samples from the volunteers and $\rho_{\text{sample density}}$ = the specific density of the sample.

Experimental exposure of volunteers

Two volunteers, one female (age 69 and weight 57) and one male (age 44 and weight 75), participated in the study with their written informed consent. The volunteers were nonsmokers and were not under medication during the study period, and they avoided food sources suspected to contain IMZ throughout the study period. Each volunteer received a single oral dose and a single dermal application of IMZ. The oral and dermal experimental exposures were carried out on different occasions with a time gap of 6 months between the exposures. The oral dose of IMZ was administered in an organic orange juice (250 mL) fortified with the appropriate amount of IMZ. The oral dose of IMZ was 0.025 mg/kg bw, which is the established lifelong accepted daily intake (ADI) (9). The dosage of IMZ for dermal exposure corresponded to twice the amount of ADI. The dermal application was administered in 50% (v/v) ethanol as vehicle and applied on an area of 50 cm^2 on the volar side of the forearm. After evaporation of the vehicle, the forearm was covered with aluminum foil to avoid loss of the compound. After 8 h of exposure, the aluminum foil was removed and extracted with 200 mL of methanol. The exposed area was wiped with cotton swabs three times using a few milliliters of the vehicle. The swabs were saved separately and extracted with 30 mL of methanol. Thereafter, tape-stripping was performed on the exposed area. Twelve tapes (Fixomull®, BSN Medical GmbH, Hamburg, Germany) of the same size (10 × 5 cm) as the exposed area were cut out. They were carefully applied on the exposed area and stripped off from the skin one after another, with the help of clean forceps. The tapes were saved separately and extracted in 10 mL of methanol.

A pre-exposure urine sample was collected from each subject prior to the exposures and all voided urine was henceforth collected *ad libitum* up to 100 h for both experiments. The volunteers registered the time of voiding. All collected samples were stored at −20°C until analysis. The total volume of the samples was registered.

Estimation of elimination half-life

The urinary samples from the oral and the dermal experiments were used to calculate the elimination half-life of IMZ and the metabolite DCPI by plotting natural log-linear data of the quantified concentration versus the mid-time points between the two sample collection times. The obtained slope was used for the calculations.

Formation of urinary metabolites

Urine samples from the exposed volunteers showing high concentrations (1–3 h samples) of IMZ were used to study the biotransformation products of IMZ. The samples were analyzed using the LC–MS-MS system without enzyme treatment and data were acquired using the LightSight® software. The samples

were separated, using a Genesis C18 column and scanned for mass ranges of the predicted metabolites according to the phase I and phase II biotransformation. The software creates optimized predicted SRM with information-dependent acquisition methods for the compounds. Acquisition methods with multiple survey scans as well as product ion scans were also performed to identify the conjugated metabolites. The SRMs were extracted from the obtained spectral information and the method was optimized. The log normal values of the peak areas were plotted against time to estimate the elimination half-life of the metabolites.

Results and discussion

Analysis

The analytes DCPI and IMZ separated well on the column. The molecular ions for DCPI $[M+H]^+ = 257.1$ and IMZ $[M+H]^+ = 299.1$ were formed in the positive mode. The quantifier ion selected for DCPI was m/z 257.1/189.0 and the qualifier ion was m/z 257.1/69.1, with a collision energy (CE) of 26 and 28 V, respectively. The quantifier ion selected for IMZ was m/z 299.1/257.0 and the qualifier ion was m/z 297.0/158.9, with a CE of 24 and 30 V, respectively. The fragment m/z 302.1/255.0 was selected as the quantifier ion and m/z 302.1/159.0 was selected as the qualifier ion for [$^2H_5$]-IMZ, with a CE of 26 and 30 V, respectively. The declustering potential, entrance potential and collision cell exit potential for all the ions were 70, 10 and 10 V, respectively.

Method validation

The specificity was tested by analyzing blank urine samples obtained from 10 healthy volunteers. The IS caused no interference in the blank samples and *vice versa*. An example of a chromatogram of the blank sample along with a chromatogram showing IMZ and IS is presented in Figure 1.

Excellent linearity was seen for the calibration standards ranging from 0.5 to100 ng/mL. The correlation coefficient (r^2) observed for both analytes was above 0.995. The mean slope of the regression lines ($n = 9$) was 0.29 ± 0.003 and 0.17 ± 0.02, and an intercept of 0.05 ± 0.04 and 0.11 ± 0.06, for IMZ and DCPI, respectively, at 95% confidence interval.

The limit of detection (LOD) and the limit of quantification (LOQ) were determined by analysis of 10 different blank urine samples collected from healthy volunteers. Since IMZ or DCPI is not ubiquitous among unexposed persons, it was easy to obtain the blank samples. The LOD was calculated as three times the standard deviation of the ratio between the peak area at the analyte retention time and the peak area of IS, divided by the slope of the calibration line. The LOQ was similarly determined as 10 times the standard deviation of the ratios. The determined LOD for both the analytes was 0.2 ng/mL, and the LOQ 0.8 ng/mL.

The within-run precision of the method was determined by replicate analyses of 10 fortified urine samples each at 1, 5 and 20 ng/mL. The CV at the three concentration levels of IMZ and DCPI was below 15%. The values are presented in Table I. The between-run precision was determined by incorporating three QC samples in duplicates in every analytical batch and analyzed over a period of 1 year. The mean quantified values at the three concentration levels were 1.1, 5.5 and 22 ng/mL for DCPI and 0.8, 3.6 and 16 ng/mL for IMZ. The between-batch precision was determined by comparing duplicate analyses of 145 samples from the two orally and dermally exposed volunteers. It was determined at three concentration ranges for IMZ (LOD—10, 10–60 and 60–205 ng/mL) and at two concentration ranges for DCPI (LOD—5 and 5–70 ng/mL) and expressed as the CV, which was calculated as previously described (19). The obtained CVs for IMZ and DCPI for the between-run and the between-batch precision are presented in Table I.

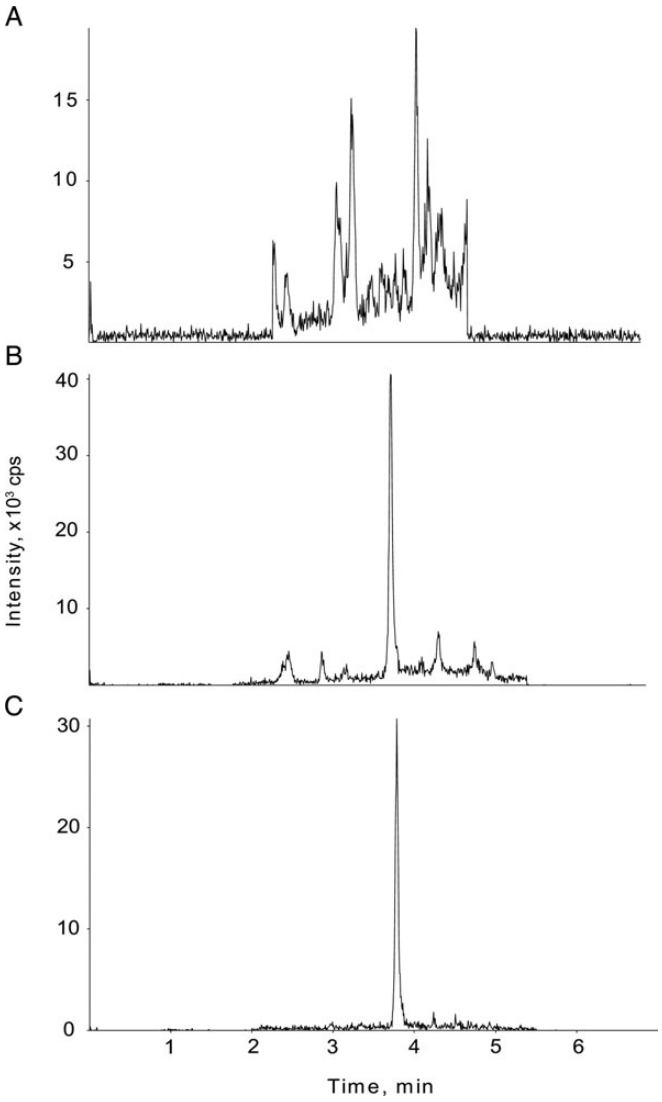

Figure 1. An example of LC–MS-MS chromatogram showing (A) a blank urine sample obtained from a person not exposed to IMZ. (B) An authentic urine sample (28 h after exposure) quantified to 5 ng IMZ/mL urine, transition 299.1/257.0 and (C) IS fortified to 4 ng/mL urine, transition 302.1/255.0. Retention time of IMZ and IS is 3.8 min.

Possible matrix effects of the method were tested by the post-extraction addition approach. The blank urine samples obtained from 10 healthy volunteers were divided into two aliquots each. Both the aliquots were SPE-extracted, and one aliquot was fortified with 1 ng/mL ($n = 10$) and the second with 20 ng/mL ($n = 10$) with both the analytes. The IS was added to all the samples after extraction and the samples were analyzed. The CVs for DCPI at 1 and 20 ng/mL were 12 and 14%, respectively, and for

Table I
The Within-Run Precision, Between-Run Precision, Between-Batch Precision and Stability Determined in Urine Samples at Different Concentrations for DCPI and IMZ

Validation parameters	No. of samples (*n*)	Concentrations of IMZ/DCPI (ng/mL)	Mean (ng/mL)		DCPI CV (%)	IMZ CV (%)
			DCPI	IMZ		
Within-run precision	10	1	1.1	0.8	11	11
	10	5	6.1	3.9	7.1	6.6
	10	20	24	17	5.9	5.3
Between-run precision	35	1	1.1	0.8	15	12
	35	5	5.5	3.6	15	8.8
	35	20	22	16	13	9.6
Between-batch precision[a]	3	LOD—10	–	2.2	–	14
	28	10–60	–	30	–	6.5
	10	60–205	–	114	–	4.7
	48	LOD—5	1.9	–	11	–
	26	5–70	18	–	15	–
Stability	21	25	27	22	12	5.1

[a]The between-batch precision for IMZ was determined at three different concentration ranges, namely from the LOD to 10, 10–60 and 60–205 ng/mL. The between-batch precision for DCPI was determined at two different concentration ranges, namely from the LOD to 5 and 5–70 ng/mL. The precision was determined by comparing the duplicate analyses of the samples obtained from the two orally and dermally exposed volunteers.

IMZ at 1 and 20 ng/mL were 9.2 and 3.4%, respectively. Thus, there was a low matrix effect in this experiment. In addition, all the samples were fortified with the labeled IS, which compensated the effect of the different matrices.

The recovery of the method was evaluated at the concentration levels of 1 and 20 ng/mL. A set of blank urine samples obtained from 10 healthy volunteers was fortified with standards before the SPE extraction and a set of samples was fortified after the SPE extraction. The latter was considered as 100% recovery. The peak area ratios between the analyte and the IS of both sets were compared. The average recovery ($n = 10$) of DCPI at concentration levels of 1 and 20 ng/mL was 104 and 99%, respectively, and the average recovery ($n = 10$) of IMZ at 1 and 20 ng/mL was 89 and 91%, respectively.

Both the analytes stored at −20°C were found to be stable in the standard solutions (in methanol) for a period of 1 year. The QC samples were stable at least for a year under storage and the estimated variability was under a CV of 15%. The degradation of analytes during enzyme hydrolysis was tested by continuous incubation of samples at 37°C for up to 144 h with six time points of 24 h. The stability varied with a CV of 12% and is presented in Table I.

The method validation is comprehensively described in Supplementary data.

Experimental oral exposure

After the oral dose of IMZ, the maximum concentration of IMZ in the female volunteer was 218 nmol/mmol creatinine (84 ng/mL) and DCPI was 104 nmol/mmol creatinine (35 ng/mL) excreted in the 3 h sample. And, the maximum concentration of IMZ in the male volunteer was 140 nmol/mmol creatinine (158 ng/mL) and DCPI was 88 nmol/mmol creatinine (86 ng/mL) excreted in the 2 h sample. The difference may be due to the differences in the metabolic rate of the volunteers. Within the first 24 h, 9.8% of the dose in the female volunteer and 9.9% in the male volunteer was excreted as IMZ and DCPI. The excretion of the compounds showed an exponential decrease exhibiting first-order kinetics and seemed to follow a two-compartment model. Figure 2A and B shows the elimination half-life curves (creatinine-adjusted) of IMZ and DCPI, respectively. For the density-adjusted half-life curves, see Supplementary Figure 1A and B, and for the unadjusted half-life curves, see Supplementary Figure 1C and D. The raw elimination kinetics data (creatinine-adjusted) for both the compounds are presented in Supplementary Figure 7A and B. The estimated elimination half-lives of IMZ and DCPI for both volunteers are presented in Table II. After 100 h, in total, 10% of the dose was recovered as IMZ and DCPI together, in both volunteers. Out of the 10%, 6.7% was found as IMZ and 3.3% as DCPI in the female volunteer; and 6.3 and 3.7% in the male volunteer, respectively. No free IMZ and DCPI could be found in the urine samples, and thus a large fraction is probably conjugated. The data suggest that the compounds are mainly glucuronidated.

Experimental dermal exposure

After the dermal application, the maximum concentrations of IMZ and DCPI were achieved after 10 h in both volunteers. In the female volunteer, the maximum concentration of IMZ excreted was 25 nmol/mmol creatinine (41 ng/mL) and DCPI was 8 nmol/mmol creatinine (13 ng/mL) in the 11-h sample. In the male volunteer, the maximum concentration of IMZ was 52 nmol/mmol creatinine (77 ng/mL) and DCPI was 15 nmol/mmol creatinine (19 ng/mL) in the 10-h sample. Within the first 24 h, 1.4% of the dose in the female volunteer and 4.2% of the dose in the male volunteer was excreted as IMZ and DCPI, respectively. The excretion seemed to follow first-order kinetics and a single-compartment model. The excretion curves and the urinary elimination half-life (creatinine-adjusted) for IMZ and DCPI are shown in Figure 2C and D, respectively. For the density-adjusted half-life curves, see Supplementary Figure 2A and B, and for the unadjusted half-life curves, see Supplementary Figure 2C and D. Of the applied dose, 1.8% in the female and 4.6% in the male was recovered in the urine as IMZ and DCPI, respectively. Fifty-two percentage of IMZ in the female and 22% in the male were recovered from the swabs, tapes and covering foil. This can be assumed as loss of the dose. Hence, the total recovered dose in the urine as well as in the swabs, tapes and covering foil accounted for 54% in the female and 27% in the male volunteer.

The tape-stripping is used to study the localization and the distribution of substances in the stratum corneum. It has been suggested that the occupational dermal exposures to some chemicals can be monitored by a tape-stripping method (20). Analysis of the tape extracts showed a serial decrease in the concentrations of IMZ (Supplementary Figure 3). In both volunteers, the extracts from the 12th tape contained 3 μg/mL of IMZ, which indicates the penetration of IMZ into the skin. The higher concentrations of IMZ were found in the urine of the male volunteer compared with the female. This may explain the higher concentrations of IMZ in the tape of the female volunteer. Our data suggest that a tape-stripping method can be applied for the dermal exposure assessment of IMZ, but it has to be interpreted together with other exposure measurements.

Adjustment of dilution

The toxicokinetic data in both the oral and dermal exposure experiments indicate that good correlation coefficients were

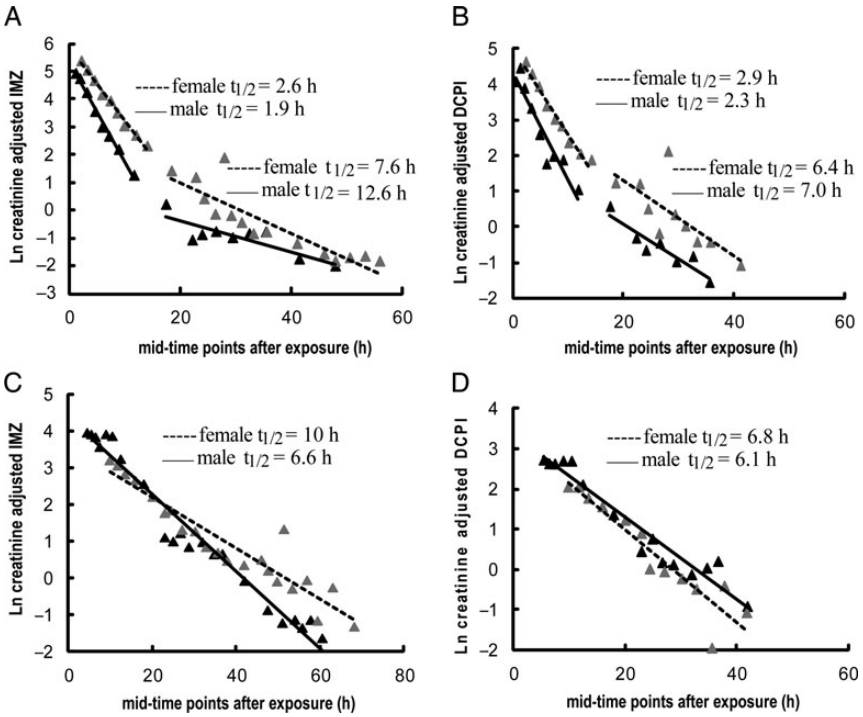

Figure 2. The mid-time points are plotted against natural log-linear data of the quantified IMZ and DCPI in the samples from both the volunteers. The obtained slope of the curve was used for the calculation of elimination half-life ($t_{1/2}$) of the compounds. (A and B) Urinary elimination half-life of IMZ and DCPI (creatinine-adjusted) after the oral exposure. (C and D) Urinary elimination half-life of IMZ and DCPI (creatinine-adjusted) after the dermal exposure.

Table II
Estimation of the Elimination Half-life of IMZ and DCPI in Urine Following the Oral and Dermal Exposure in the Two Volunteers

Volunteer	$t_{1/2}$ creatinine-adjusted Urinary concentrations (h)	*r*	$t_{1/2}$ density-adjusted Urinary concentrations (h)	*r*	$t_{1/2}$ unadjusted Urinary concentrations (h)	*r*
IMZ (oral exposure)						
Female						
Rapid phase	2.6	0.99	2.9	0.97	3.0	0.76
Slower phase	7.6	0.78	7.4	0.84	8.6	0.84
Male						
Rapid phase	1.9	0.99	2.1	0.97	2.1	0.88
Slower phase	13	0.71	11	0.72	7.6	0.85
DCPI (oral exposure)						
Female						
Rapid phase	2.9	0.97	3.2	0.95	3.3	0.76
Slower phase	6.4	0.55	7.0	0.60	9.0	0.57
Male						
Rapid phase	2.3	0.90	2.5	0.86	2.4	0.72
Slower phase	7.0	0.87	5.6	0.85	3.8	0.98
IMZ (dermal exposure)						
Female						
Single phase	10	0.89	10	0.89	10	0.90
Male						
Single phase	6.6	0.97	7.2	0.94	7.4	0.84
DCPI (dermal exposure)						
Female						
Single phase	6.1	0.88	7.5	0.75	7.8	0.76
Male						
Single phase	6.8	0.94	7.0	0.87	5.9	0.88

The half-life ($t_{1/2}$) is estimated by plotting natural log-transformed values of quantified concentration versus calculated mid-time points and *r* is the correlation coefficient of the obtained curve.

obtained for the excretion curves using both the creatinine and the density-adjusted data when compared with the unadjusted ones. The data indicate that an adjustment for the urinary dilution is to be recommended, but both creatinine concentrations and urinary density may be applied. However, creatinine concentrations in urine may be affected by several factors such as gender, age, muscularity and consumption of meat. Also, the density can be affected by similar factors, but it has been shown that the urinary density may be preferred in the studies of populations with mixed genders and age groups (21).

Moreover, this study was limited to two individuals, and thus one should be cautious on a definite conclusion on which adjustment method for urinary dilution to choose.

Formation of urinary metabolites

The analysis of the crude diluted urine samples without the enzyme treatment was performed to study the metabolites of IMZ. In rats, DCPI is suggested as the major metabolite after exposure to IMZ (16). In the present study, the formation of DCPI (at m/z 257) was confirmed and it further conjugated with glucuronide (at m/z 433). In rats, IMZ also undergoes epoxidation to form a reactive compound, which can be hydrolyzed by the enzyme epoxide hydrolase (16). In our study, the epoxidation was indicated by the formation of 1-[2-(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy) ethyl]-1*H*-imidazole or dihydroxy-IMZ (at m/z 331). This metabolite is found either in free form in the unhydrolyzed urine (Supplementary Figure 6) or it may further form conjugates with glucuronide (at m/z 507) before excretion (Supplementary Figure 4D). It was also found that IMZ forms conjugates with glucuronide (at $m/z = 475$; Supplementary Figure 4B). Furthermore, we also observed single oxidation of IMZ followed by glucuronide conjugation (at m/z 491) and trioxidation followed by glucuronide conjugation of IMZ (at m/z 523; Supplementary Figure 4C and E). The natural log-linear plots of peak areas of the glucuronide–IMZ conjugate and the three oxidated forms of the glucuronide–IMZ conjugates, against the mid-time points, were used to verify the presence of the metabolites, as a result of IMZ exposure. It also gave an estimate of the half-lives of these metabolites (Supplementary Figure 5). These were in the same range as IMZ and DCPI. In the enzyme suitability test, the β-glucuronidase was found more efficient compared with the β-glucuronidase/arylsulfatase, suggesting that IMZ and DCPI may not form conjugates with sulfates *in vivo*.

In both the oral and the dermal exposure experiments, IMZ undergoes biotransformation to form urinary metabolites. According to the published animal studies, DCPI was assumed as the major metabolite and was commercially available. Thus, only IMZ and DCPI were monitored in the current study. In further investigations, it would be relevant to develop methods for monitoring the dihydroxy-IMZ in humans, since it indicates a potentially toxic reactive epoxide.

Conclusions

A method for the quantification of IMZ and one of its metabolites, DCPI, in human urine was developed using LC–MS-MS. The method has a good precision and a low LOD was achieved due to the sample clean-up by SPE and analysis using LC–MS-MS. The method was applied for the urine samples obtained from two experimentally exposed volunteers. In the case of the oral exposure, the urinary excretion seemed to follow first-order kinetics and a two-compartment model. The elimination half-life of IMZ (creatinine-adjusted) following the oral exposure was 2.6 and 1.9 h for the rapid excretion phase, for the female and male volunteer, respectively. In the slower excretion phase, it was 7.6 and 13 h, respectively. For the dermal exposure, the excretion seemed to follow a single-compartment model and first-order kinetics. The elimination half-life (creatinine-adjusted) was 10 and 6.6 h for the female and the male volunteer, respectively. IMZ is suggested as a urinary biomarker to monitor the exposure to IMZ. The study complements to the understanding of the metabolism of IMZ in humans. However, the study involved only one female and one male volunteer of different ages, and the presented data should be interpreted cautiously, taking into consideration that the results may vary in larger studies.

Ethics

The investigations involving experimental exposure of volunteers to IMZ were ethically approved by the Regional Ethical Review Board at Lund University in Lund, Sweden (Dnr 463/2005; Dnr 2010/41; Dnr 2010/465 and Dnr 2013/6). The volunteers participating in the study had given their written informed consent.

Supplementary data

Supplementary data is available at *Journal of Analytical Toxicology* online.

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