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Determination of midazolam and its major metabolite 1′-hydroxymidazolam by high-performance liquid chromatography-electrospray mass spectrometry in plasma from children

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Abstract

We have developed a sensitive, selective and reproducible reversed-phase high-performance liquid chromatography method coupled with electrospray ionization mass spectrometry (HPLC–ESI-MS) for the simultaneous quantification of midazolam (MDZ) and its major metabolite, 1′-hydroxymidazolam (1′-OHM) in a small volume (200 μl) of human plasma. Midazolam, 1′-OHM and 1′-chlordiazepoxide (internal standard) were extracted from alkalinised (pH 9.5) spiked and clinical plasma samples using a single step liquid–liquid extraction with 1-chlorobutane. The chromatographic separation was performed on a reversed-phase HyPURITY^{TM} Elite C18 (5 μm particle size; 100 mm × 2.1 mm i.d.) analytical column using an acidic (pH 2.8) mobile phase (water–acetonitrile; 75:25% (v/v) containing formic acid (0.1%, v/v)) delivered at a flow-rate of 200 μl/min. The mass spectrometer was operated in the positive ion mode at the protonated-molecular ions $[M+l]^+$ of parent drug and metabolite. Calibration curves in spiked plasma were linear ($r^2 \geq 0.99$) from 15 to 600 ng/ml (MDZ) and 5–200 ng/ml (1′-OHM). The limits of detection and quantification were 2 and 5 ng/ml, respectively, for both MDZ and 1′-OHM. The mean relative recoveries at 40 and 600 ng/ml (MDZ) were 79.4 ± 3.1% ($n = 6$) and 84.2 ± 4.7% ($n = 8$), respectively; for 1′-OHM at 30 and 200 ng/ml the values were 89.9 ± 7.2% ($n = 6$) and 86.9 ± 5.6% ($n = 8$), respectively. The intra-assay and inter-assay coefficients of variation (CVs) for MDZ were less than 8%, and for 1′-OHM were less than 13%. There was no interference from other commonly used antimalarials, antipyretic drugs and antibiotics. The method was successfully applied to a pharmacokinetic study of MDZ and 1′-OHM in children with severe malaria and convulsions following administration of MDZ either intravenously (i.v.) or intramuscularly (i.m.).

Keywords: Midazolam; 1′-Hydroxymidazolam; Pharmacokinetics in children

1. Introduction

Benzodiazepines are the drugs of choice for rapid termination of seizures [1]. In resource poor countries, diazepam is routinely used as the standard first line treatment for acute convulsions and status epilepticus, since it is widely available,

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cheap, and rapidly acting. However, diazepam has several disadvantages, such as incomplete and erratic absorption following intramuscular (i.m.) administration [2], and accumulation on repeated administration that may lead to fatal respiratory depression [3,4]. Intravenous (i.v.) administration of diazepam requires trained healthcare personnel and is, therefore, not a practical route for use at most peripheral health facilities in rural Africa. Consequently, there is an urgent need for alternative effective but easy to administer drugs to treat convulsions at such facilities.

Midazolam (MDZ) is a water-soluble benzodiazepine with several pharmacokinetic and pharmacological properties that would make it a suitable anticonvulsant: it is effective in childhood status epilepticus refractory to other anticonvulsants [5,6] and causes less respiratory depression [7]. It can be administered via several routes including i.v. [6], i.m. [8,9], buccal [10] and intranasal [11] routes. The latter routes may be particularly useful in most rural health facilities in resource-poor countries. However, their suitability needs to be formally evaluated in children in these areas.

In humans, MDZ is rapidly and extensively metabolized by the CYP 450 3A isoenzymes in the liver to the major hydroxylated metabolite, 1′-hydroxymidazolam (1′-OHM) and two minor metabolites, 4′-hydroxymidazolam and 1,4′-hydroxymidazolam [12]. 1′-OHM is rapidly deactivated by conjugation with glucuronic acid to form the 1′-OHM glucuronide, which makes up 60–70% of the dose eliminated in urine [13]. Although 1′-OHM is less pharmacologically active than the parent drug and has a shorter half-life (<1 h) than MDZ, it may contribute to the pharmacological effect of MDZ.

The pharmacokinetics of MDZ and its metabolites have not been described in African children, particularly those with falciparum malaria, a common cause of seizures in sub-Saharan Africa. For pharmacokinetic studies in such children, the ideal analytical method for MDZ and its metabolites should be one that is sensitive enough to allow use of small quantities of biological fluid (since large sampling volumes are both not acceptable to parents and impractical), and fully selective to avoid interference from concurrently administered drugs (antimalarials, other anticonvulsants, antibiotics, antipyretics/analgesics). Previously published analytical techniques for the determination of MDZ and its metabolites in biological fluids used high-performance liquid chromatography (HPLC) with UV detection [14–25], gas chromatography (GC) with electron capture detection [26–28], GC–mass spectrometry (MS) [29–31] and HPLC–MS with electrospray ionisation [32,33], atmospheric pressure chemical ionisation (APCI) [34–36], and fast atomic bombardment (FAB) ionization [37]. These techniques have various limitations, including large sample volumes (1–2 ml) [14–16,20,21,23,24,32,35], long retention times [15,17,19], lengthy extraction and derivatisation procedures [30,31], inadequate sensitivity [14–16,20], and use of expensive solid phase extraction cartridges [16,20,37,38].

We report a relatively simple, sensitive and selective liquid chromatography (LC)–mass spectrometry-electrospray ionisation (ESI) method for the simultaneous determination of MDZ and 1′-OHM in volume-limited human plasma samples.

2. Experimental

2.1. Chemicals and reagents

Midazolam base (MDZ; no information available on its purity), 1′-hydroxymidazolam base (1′-OHM; lot # 205-11-2, purity 97.1%) and 4′-hydroxymidazolam base (4-OHM; lot# 137-71-1, purity 99.6%) were purchased from Ultrafine Chemicals Ltd (Manchester, England). 1′-Chlordiazepoxide hydrochloride (internal standard (I.S.)) was kindly provided by Dr Martin S. Lennard (University of Sheffield, Sheffield, UK). Acetonitrile and methanol (both HPLC grade) and formic acid (98/100%; AnalaR® grade) were purchased from BDH Laboratory Supplies (Poole, UK), while 1-chlorobutane (HPLC grade) was obtained from Fisher Scientific UK Ltd (Loughborough, Leicestershire, UK). Sodium bicarbonate (analytical reagent grade) and anhydrous sodium carbonate (SigmaUltra® grade) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Deionized water was prepared using a *Purite Select*™ water purification system (Purite Ltd, Oxford, UK). Blank human plasma for the preparation of calibration curves was obtained from the Merseyside Blood Bank, Royal Liverpool University Hospital, Liverpool, UK.

2.2. Preparation of analytical standards

Stock solutions (1 mg of the base/ml) each of MDZ and 1′-OHM were prepared by dissolving 4.32 mg of MDZ base and 5.0 mg of 1′-OHM base, respectively, in appropriate volumes of methanol. A stock solution (2.43 mg of the base/ml) of the internal standard was prepared by dissolving 11.36 mg of 1′-chlordiazepoxide hydrochloride in methanol (5 ml). Working standard solutions (100, 10 and 1 μg/ml) of MDZ, 1′-OHM and 1-chlordiazepoxide were prepared by appropriate serial dilution of the stock solutions with methanol. All the stock solutions were stored at −20 °C in the dark and used within three months. A 0.5 M solution of sodium carbonate-sodium hydrogen carbonate buffer (pH 9.5) was prepared by mixing equal volumes of 0.5 M aqueous solutions of sodium carbonate and sodium hydrogen carbonate.

2.3. Liquid chromatography–mass spectrometry

The equipment consisted of a Surveyor™ LC pump and a Surveyor™ autosampler coupled to a Finnigan *LCQDECA*™ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). The latter was equipped with an electrospray ionisation source. High purity nitrogen was used

as the sheath and auxiliary gas. The other main parameter settings were: sheath flow gas 44 l/min; auxiliary gas flow 9.5 l/min; ESI capillary temperature 200 °C; ionspray voltage 5 kV and capillary voltage 6.95 V. All spectra were obtained in the positive ion mode, over mass range of *m/z* 280–350, at a scan speed of 0.5 scan/s. The spectra were collected in the form of continuum data. The chromatographic separation was performed on a HyPURITY^{TM} Elite C18 analytical column (5 μm particle size; 100 mm × 2.1 mm i.d.) (Hypersil, Runcorn, Cheshire, England) using an isocratic mobile phase comprising water–acetonitrile (75:25%, v/v) containing formic acid (0.1%, v/v), the pH of the mixture was about 2.8. The flow rate was set at 200 μl/min, generating an operation back-pressure of about 1400 psi.

2.4. Sample preparation

To 200 μl aliquot of plasma (blank, standard or patient sample) in a 15 ml borosilicate glass tube was added 50 μl of a 1 μg/ml solution of the internal standard and 0.5 ml of sodium carbonate–sodium hydrogen carbonate buffer (pH 9.5), followed by vortex-mixing for 10 s. 1-Chlorobutane (5 ml) was then added, and the mixture tumbled for 30 min on a *Heidolph REAX2* rotator drive (Heidolph Instruments, Germany) followed by centrifugation (1942 × *g*; 10 min). The organic phase was transferred to a clean 15 ml borosilicate glass tube and evaporated to dryness in a water bath (37 °C) under a gentle stream of white spot nitrogen gas. The residue was dissolved in mobile phase (100–300 μl) and 50 μl aliquot was injected onto the LC column.

2.5. Preparation of calibration curves

Pooled blank human plasma (50 ml) was spiked with MDZ and 1′-OHM to give final concentrations of 600 and 200 ng/ml, respectively, and stored frozen at −20 °C until used. An aliquot of the spiked plasma stock solution was diluted with pooled drug-free human plasma to give calibration curve nominal concentrations of 0, 15, 30, 60, 90, 120, 180, 300, 450 and 600 ng/ml (MDZ) and 0, 5, 10, 20, 30, 40, 60, 100, 150 and 200 ng/ml (l′-OHM). The samples were extracted and assayed as described above. Peak area ratios (PAR) of MDZ and 1′-OHM to internal standard were plotted against the known concentrations of MDZ and 1′-OHM. Weighted (1/*x*) least-squares linear regression analysis was used to determine the slope, intercept and correlation coefficient (r^2) using *Excalibur*^{TM} software integral with the LC–MS system. Unknown concentrations of MDZ or 1′-OHM were determined (by interpolation) with reference to the calibration lines.

2.6. Analytical recovery

The analytical recovery of the extraction procedure for MDZ and 1′-OHM from human plasma was determined by spiking 200 μl aliquots of drug-free plasma with various amounts of MDZ (40 and 600 ng/ml) and 1′-OHM (30 and 200 ng/ml) and 50 ng of the internal standard. The samples were extracted as described in the extraction procedure above. In another set of tubes, equivalent amounts of MDZ and 1′-OHM and the internal standard (50 ng) were added directly into extracting solvent (5 ml) followed by evaporation of the solvent in a water bath (37 °C) under a gentle stream of white spot nitrogen. The residue was reconstituted in mobile phase and 50 μl aliquots injected onto the LC chromatograph as described above. Recovery was assessed by comparing the chromatographic peak area ratios of MDZ and 1′-OHM to internal standard for the extracted plasma standards to those obtained from equivalent amounts of MDZ and 1′-OHM spiked directly into the organic phase.

2.7. Assay precision and accuracy

Quality control (QC) samples were prepared by spiking drug-free human plasma (5 ml) with various quantities of MDZ and 1′-OHM, corresponding to the low (LQC), medium (MQC) and high (HQC) levels of the calibration curves. The nominal concentrations for the LQC, MQC and HQC levels were 40, 250 and 500 ng/ml (MDZ) and 30, 80 and 180 ng/ml (1′-OHM), respectively. Reproducibility of the assay was assessed by determining the intra- and inter-assay coefficients of variation (% CV) for the QC samples. Intra-assay (within-day) precision and accuracy were determined by analyzing on the same day 200 μl aliquots of each of the QC samples ($n = 7$ for each level). Inter-assay (between-day) precision and accuracy were assessed by analyzing duplicates of each of the QC samples over a period of seven days. The CVs values were used as an index of precision. Accuracy was calculated by comparing the mean experimental concentrations of assayed QC standards with their nominal values, and relative error (RE%) was used as the index.

2.8. Validation study

In a separate study investigating the clinical pharmacology MDZ, children with severe malaria and convulsions were recruited (after obtaining ethical clearance from KEMRI/National Ethical Committee and informed consent from the parents/guardians) and administered MDZ (0.3 mg/kg; *Dormicum*®; 5 mg/ml; Roche, Cyprus), either i.v. (as a slow bolus over 2 min) or a single i.m. injection. Blood samples (0.4 ml) were collected into heparinised tubes pre-dose and at 5, 10, 15, 20, 30, 40, 60 min, and 2, 3, 4, 5 and 6 h after midazolam administration. The blood was centrifuged (1942 × *g*; 10 min), and plasma separated and stored frozen at −20 °C until assayed for MDZ and its metabolite, 1′-OHM. Concentration–time profiles for two of the patients (one from the i.v. MDZ and one from the i.m. MDZ group) are reported.

3. Results and discussion

3.1. Chromatography

Midazolam is a basic compound possessing a tertiary amine in its structure. Therefore, positive-ion mode by ESI-MS is suitable for the determination of MDZ and its metabolites. The production of positive ions proceeded in acidic conditions of the mobile phase used (water–acetonitrile containing 0.1%, v/v formic acid) for HPLC–MS. Mass spectra of MDZ, 1′-OHM and 1′-chlordiazepoxide (I.S.) are shown in Fig. 1, and ions at *m/z* of 326.4, 342.4 and 300.3, respectively, were selected for subsequent quantitative analysis. Under these chromatographic conditions, the retention times for I.S., 1′-OHM and MDZ were approximately 3.5, 5.2 and 6.0 min, respectively (Fig. 2). All the chromatographic peaks were resolved to baseline throughout the calibration curve ranges of 15–600 ng/ml and 5–200 ng/ml for MDZ and 1′-OHM, respectively. Another minor metabolite of MDZ, 4′-OHM, eluted from the column just before the 1′-OHM, with a retention time of about 4.3 min, and thus did not interfere with the assay (Fig. 2A). There was no interference from endogenous compounds or other concomitantly administered antimalarial drugs (chloroquine (500 ng) and its metabolite desethylchloroquine, quinine, sulfadoxine,

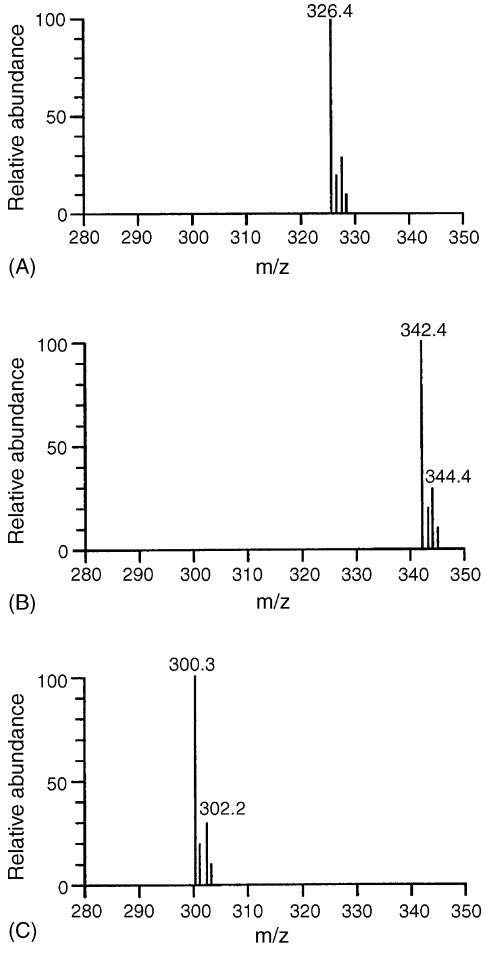

Fig. 1. Mass spectra of (A) midazolam (MDZ; *m/z* = 326.4), (B) 1′-hydroxymidazolam (1′-OHM; *m/z* = 342.4) and (C) 1-chlordiazepoxide (internal standard (I.S.); *m/z* = 300.3).

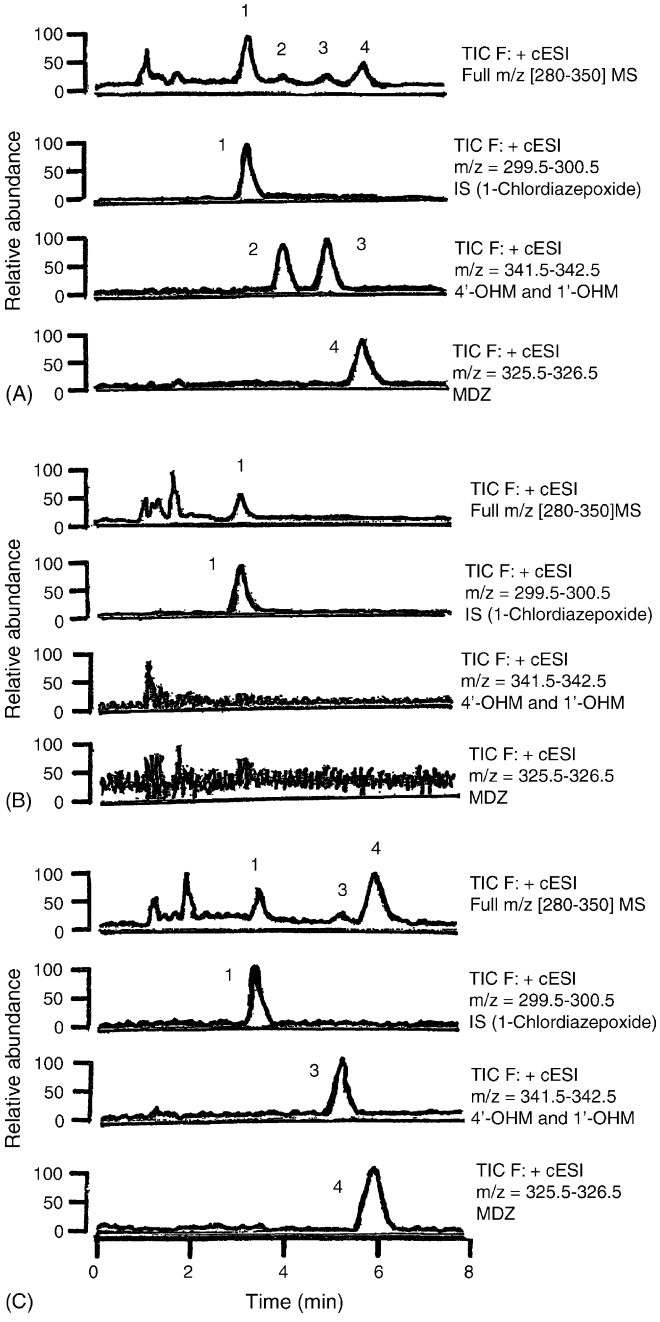

Fig. 2. Reconstructed mass chromatograms following the analysis of 200-μl aliquots of: (A) blank human plasma spiked with 40 ng/ml (MDZ), 30 ng/ml (1′-OHM) and 50 ng of the internal standard (1-chlordiazepoxide); (B) blank plasma from a child spiked with 50 ng of I.S.; and (C) the plasma sample obtained after 40 min following intravenous administration of a single dose of MDZ (0.3 mg/kg) to a child with severe malaria and convulsions. The estimated plasma concentrations of MDZ and 1′-OHM were 266 and 53 ng/ml, respectively. Peaks: 1—1′-chlordiazepoxide (I.S.), *m/z* = 300.3; 2—4′-0HM, *m/z* = 342.4; 3—4′-OHM, *m/z* = 342.4; and 4—MDZ, *m/z* = 326.4.

Table 1
Extraction recoveries of midazolam (MDZ) and 1′-hydroxymidazolam (1′-OHM) in spiked plasma

Compound	Concentration (ng/ml)	No. of replicates (*n*)	Recovery (%) (mean ± SD)	CV (%)
MDZ	40	6	79.4 ± 3.1	3.9
	600	8	84.2 ± 4.7	5.7
1′-OHM	30	6	89.9 ± 7.2	7.9
	200	8	86.9 ± 5.6	6.4
1′-Chlordiazepoxide (I.S.)	50	15	85.5 ± 4.2	4.9

Standard deviation (SD); coefficient of variation (CV); internal standard (I.S.).

pyrimethamine, proguanil, cycloguanil, artesunate), analgesics (acetaminophen, salicylate) or antibiotics (chloramphenicol, benzyl penicillin, ceftrioxone)—data not shown. The limit of detection (LOD) (defined as the lowest concentration yielding a signal-to-noise ratio >3) was 2 ng/ml for both MDZ and 1′-OHM, while the limit of quantification (LOQ; defined as the lowest concentration whose precision and accuracy values were ≤20% ($n = 6$), excluding outliers) was 5 ng/ml.

3.2. Calibration curves and analytical recovery

The calibration curves were constructed by plotting peak area ratios of drug/I.S. against known concentrations of MDZ and its metabolite, 1′-OHM. Calibration curves for MDZ and 1′-OHM in spiked plasma were linear over the concentration ranges studied, i.e. 15–600 ng/ml (MDZ) and 5–200 ng/ml (1′-OHM), respectively. The regression equations for MDZ and 1′-OHM, were $y = 0.012x + 0.135$ ($r^2 = 0.989 \pm 0.005$; $n = 8$), and $y = 0.005x + 0.014$ ($r^2 = 0.992 \pm 0.005$; $n = 8$), respectively. The coefficients of variation of the slopes of eight calibration curves were 0.51% and 7.43% for MDZ and 1′-OHM, respectively. The mean relative recoveries at 40 and 600 ng/ml (MDZ) were 79.4 ± 3.1% ($n = 6$) and 84.2 ± 4.7% ($n = 8$), respectively; for 1′-OHM at 30 and 200 ng/ml the values were 89.9 ± 7.2% ($n = 6$) and 86.9 ± 5.6% ($n = 8$), respectively. The mean relative recovery for the I.S. was 85.5 ± 4.2% ($n = 15$) (Table 1).

3.3. Assay precision and accuracy

Assay precision and accuracy data are shown in Table 2. The intra-assay coefficients of variation (CVs) at 40, 250 and 500 ng/ml of MDZ were 4.4%, 6.5% and 1.3% ($n = 7$ in all cases), respectively; the CV values for 1′-OHM at 30, 80 and 180 ng/ml were 2.4%, 9.7% and 4.9% ($n = 7$ in all cases), respectively. The inter-assay CVs at the above concentrations were 6.9%, 4.1% ($n = 7$ in both cases) and 6.5% ($n = 8$), respectively, for MDZ and 12.8%, 7.5% ($n = 7$ in both cases) and 5.0% ($n = 8$), respectively, for 1′-OHM.

3.4. Validation study

We have successfully used the assay method described here to quantitatively measure the concentrations of MDZ and its major metabolite, 1′-OHM, in plasma samples obtained from pediatric patients who were administered MDZ (0.3 mg/kg) either i.v. or i.m. Fig. 3 shows the semi-logarithmic concentration–time profiles for MDZ and 1′-OHM from two of the patients.

3.5. Discussion and conclusion

Midazolam is commonly used to terminate seizures and sedate patients. Also, it is used as an in vitro and in vivo probe to measure CYP3A4 activity in man. Consequently, many quantitative assays have been developed. Plasma (or

Table 2
Intra- and inter-assay precision (reproducibility) and accuracy of the LC–MS-ESI assay for midazolam (MDZ) and 1′-hydroxymidazolam (1′-OHM) in plasma

	Compound	Nominal concentration (ng/ml)	No. of replicates (*n*)	Estimated concentration (ng/ml); mean ± SD	Precision (CV%)	Accuracy (RE%)
Intra-assay	MDZ	40	7	47.2 ± 2.1	4.4	18.1
		250	7	242.1 ± 15.6	6.5	5.0
		500	7	565.8 ± 7.2	1.3	13.1
	$1' - OHM$	30	7	32.8 ± 0.8	2.4	9.0
		80	7	75.7 ± 7.3	9.7	6.0
		180	7	186.4 ± 9.2	4.9	5.1
Inter-assay	MDZ	40	7	40.9 ± 2.8	6.9	6.2
		250	7	240.6 ± 9.7	4.1	4.7
		500	8	491.3 ± 31.7	6.5	4.9
	$1' - OHM$	30	7	28.6 ± 3.7	12.8	9.9
		80	7	75.6 ± 5.7	7.5	6.8
		180	8	172.6 ± 8.6	5.0	5.3

Standard deviation (SD); coefficient of variation (CV); relative error (RE) [(estimated concentration − nominal concentration)/nominal concentration].

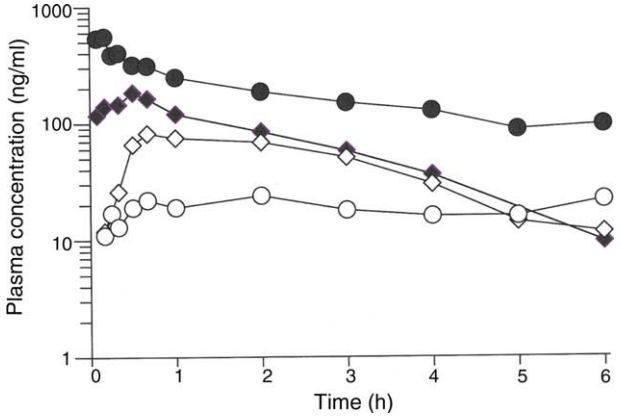

Fig. 3. Semi-logarithmic plasma concentration-time profiles of midazolam (MDZ) and 1′-hydroxymidazolam (1′-OHM) following administration of a single dose (0.3 mg/kg) of midazolam (Dormicum®; 5 mg/ml; Roche, Cyprus) either intravenously (MDZ, *closed circles*; 1′-OHM, *open circles*), or intramuscularly (MDZ, *closed squares*; 1′-OHM, *open squares*), respectively, in two children.

serum) concentrations of MDZ and its major metabolite, 1′-OHM, have been determined by numerous techniques, including HPLC (e.g. refs. [14–25], GC assays with electron capture or nitrogen-phosphorus detection (e.g. refs. [26–28]) and GC–MS (e.g. refs. [29–31]). In general, GC methods are more sensitive than HPLC methods for quantification of MDZ and 1′-OHM in biological fluids, with certain GC methods reporting lower limits of quantification in the sub-nanogram range [30,31]. However, most of these methods involve time-consuming sample preparation and derivatization procedures. HPLC methods have limits of quantification of 5–10 ng/ml for either MDZ or 1′-OHM [14–16,20] from 1 ml of plasma, which is not adequate to characterize the pharmacokinetics of MDZ and its major metabolite using small plasma samples from sick children.

Previously published GC–MS [30,31,38] and LC–MS [29,32,35–37] techniques gave a lower limit of detection than the present technique (< 2 ng/ml). However, our LC–ESI-MS method is considerably more sensitive than most of the HPLC assays [16,18,20,23,29]. Furthermore, most of the reported techniques that had sensitivities lower than that achieved by the present LC–MS method required the use large sample volumes (≥1 ml), which is not suitable for use in young children.

Fig. 3 shows the plasma concentration time profiles following i.v. or i.m. administration of MDZ (0.3 mg/kg). Following i.v. administration of MDZ, maximum plasma concentration of MDZ of 556 ng/ml (MDZ) and 24 ng/ml (1′-OHM) were achieved at 10 min and 2 h, respectively. Maximum plasma concentrations of 183 ng/ml for MDZ and 82 ng/ml for 1′-OHM were achieved at 30 and 40 min, respectively, after i.m. administration. The plasma concentrations of the metabolite declined in parallel with that of parent drug. 1′-OHM was the only metabolite detectable in plasma from these children. These results are consistent with previous studies [39,40]. In pediatric patients receiving 0.15 mg/kg dose of MDZ by i.v. or i.m. administration for sedation, mean (SD) maximum MDZ serum concentrations of 138 ± 46.7 and 93.1 ± 67.9 ng/ml, respectively, were achieved within 15 min [39]. In another study, Rey et al. administered a single 0.2 mg/kg i.v. dose of MDZ to six healthy children, and mean maximum plasma MDZ concentration of 382 ± 59 ng/ml was attained within 2.5 min [40]. In a study involving 12 children aged 4.5 (range: 2–7) years, receiving 0.2 mg/kg MDZ i.v. as an anaesthetic agent, mean (SD) maximum plasma concentration of 1457 ± 1114 ng/ml (range: 400–3805 ng/ml) was achieved within 2 min, but 1′-OHM was not detectable [41].

In conclusion, we have developed a rapid, sensitive and selective LC–ESI-MS method for the simultaneous analysis of MDZ and its major metabolite, 1′-OHM in small volumes of human plasma. It is particularly suitable for pharmacokinetic studies involving young and severely ill children, where sample volume must be kept to a minimum. We have successfully used this assay method to study the pharmacokinetics of MDZ following i.v. and i.m. administration in children with convulsions associated with severe malaria.

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