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Determination of midazolam and 1-hydroxymidazolam from plasma by gas chromatography coupled to methane negative chemical ionization mass spectrometry after sublingual administration of midazolam

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ABSTRACT

A sensitive and selective gas chromatographic mass spectrometric method for the determination of midazolam and its biologically active metabolite, 1-hydroxymidazolam, in rabbit plasma has been developed and validated. Sample preparation includes mixed-mode solid-phase extraction and derivatization with silylating reagents. Midazolam-d4 was used as an internal standard for the determination of parent drug and its active metabolite. The instrumentation consisted of a capillary column gas chromatography and a single quadrupole mass spectrometer with a negative chemical ionization. The method was found to be valid in terms of selectivity, linearity, precision, accuracy, and recovery over the concentration range of 2–200 ng/ml and 1–100 ng/ml for midazolam and 1-hydroxymidazolam, respectively. For both analytes, the lower limit of quantification was 2 ng/ml. Midazolam was stable in stock solutions stored three months at −20 °C and in human plasma stored for three months at −80 °C. In addition, no degradation of midazolam was found after three freeze–thaw cycles, in short-term stability at room temperature for 24 h, or in post-preparative stability in the autosampler. The validity of the method was further tested by performing a pharmacokinetic study of sublingual administration of midazolam in rabbits. The method will be used in studies related to a formulation development of novel midazolam formulations for use in paediatric anaesthesia.

1. Introduction

Midazolam is a short-acting 1,4-imidazole benzodiazepine with anxiolytic, sedative, anticonvulsant and muscle relaxant properties. It has been used orally in sleep disorder either intravenously or intramuscularly for preoperative sedation or induction of anaesthesia. In therapeutic use, commercially available injectable and oral midazolam formulations have limitations especially when used in paediatric patients. Intramuscular injections are not well tolerated by children and are therefore usually avoided. Oral administration of midazolam is preferred and this has become one of the most commonly used preanaesthetics in children. However, the bioavailability of midazolam after oral administration is only 15–27% as a result of its extensive first-pass metabolism in liver and intestine [1]. Intraoral (buccal or sublingual) or intranasal administration of midazolam can also be used. These administration routes achieve a more rapid onset of action and higher midazolam plasma levels with bioavailabilities up to 75% due to the avoidance of first-pass metabolism [2–4]. Sublingual administration has been reported to be effective in anaesthesia premedication [5,6] or in the treatment of seizures [7,8] in children. Unfortunately, a suitable midazolam formulation for intraoral administration is not freely available and the injectable solution of midazolam is commonly used for this purpose. However, the unpleasant taste of midazolam hinders the use those injectable formulations in children medication [9]. The European Medicines Agency has listed midazolam as an important drug which requires new formulations for paediatric use [10].

The metabolism of midazolam occurs by oxidation of the imidazole ring by cytochrome P450-3A (CYP3A) isoforms to its main and pharmacologically active metabolite 1-hydroxymidazolam (75%)

Abbreviations: BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; CRS, certified reference substance; CZE, capillary zone electrophoresis; IS, internal standard; MECC, micellar electrokinetic capillary chromatography; NCI, negative chemical ionization; P450, cytochrome P450 (also termed heme-thiolate P450); TMCS, trimethylchlorosilane.

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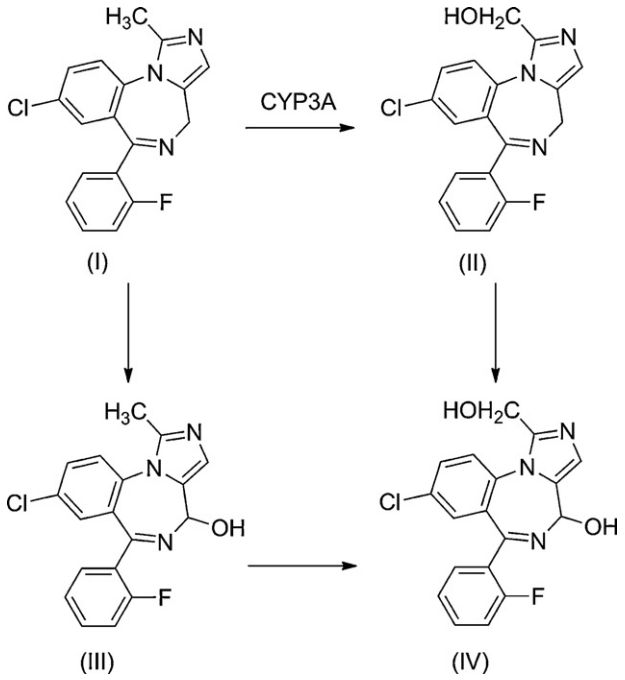

Fig. 1. The molecular structures of midazolam (I) and its phase I metabolites (II-IV). Phase I metabolism in humans is mediated by CYP3A isoforms. The phase one metabolites are 1-hydroxymidazolam (II), 4-hydroxymidazolam (III), and 1,4-dihydroxymidazolam (IV).

(Fig. 1). This metabolite has about 10% of the biological activity of midazolam [11]. In addition, small amounts of inactive metabolites, 4-hydroxymidazolam (3%) and 1,4-dihydroxymidazolam (1%) are formed [11,12]. These phase I metabolites are further conjugated with glucuronic acid to form glucuronides as phase II metabolites [13].

A number of non-chromatographic methods such as immunoassays have been used for the determination of midazolam and other benzodiazepines in biological samples [14]. Immunoassays are versatile for drug screening and they are widely used in clinical laboratories. However, due to their lack of selectivity, immunoassays provide only preliminary results and therefore further confirmation of positive findings is required by more selective method i.e. chromatographic methods. Several chromatographic methods have been described for the simultaneous determination of midazolam and 1-hydroxymidazolam including reversed-phase or normal phase high performance liquid chromatography (HPLC) coupled with single wavelength or photodiode-array ultraviolet detectors [15–18]. Midazolam has been measured from human urine and plasma by gas chromatography with a nitrogen phosphorus detector (GC-NPD) and/or an electron capture detector (GC-ECD) [19,20].

The majority of current analytical methods for benzodiazepines and their metabolites make use of selective and sensitive mass spectrometric detection rather than other detection systems. Midazolam and its metabolites have been measured by gas chromatographic mass spectrometry (GC–MS) mainly as their silylated derivatives with a splitless injection technique, a low polarity stationary phase coated capillary column, and electron ionization (EI) with quantification by selected ion monitoring (SIM) [13,21]. In addition, a highly sensitive negative chemical ionization (NCI) in combination with GC–MS has been used [22,23]. Midazolam and its metabolites have been analysed without derivatization with liquid chromatography mass spectrometry (LC–MS) with positive mode electospray ionization (ESI) and a single quadrupole [24–26] or a triple quadrupole instrument [27–30]. In addition to chromatographic techniques micellar electrokinetic capillary chromatography (MECC) has been used for the confirmation of benzodiazepine compounds in human urine which tested positive by immunoassay screening [31]. It has also been reported that capillary zone electrophoresis (CZE) can provide both different selectivity and greater sensitivity than micro-HPLC for the detection of midazolam and its phase I and II metabolites [32].

Midazolam has also been analysed from plasma without any sample preparation or chromatographic step before tandem mass spectrometric detection with a new ion source, Direct Analysis in Real Time (DART) [33]. This novel technique has been mainly used for qualitative analysis, but it also offers reasonable sensitivity and accuracy in a quantitative analysis in a fraction of the time required for LC–MS techniques [33]. Another very rapid determination of midazolam from human plasma has been attributed to the application of automated chip-based infusion connected to nanoelectrospray ionization triple quadrupole MS [34].

For chromatographic techniques analytes of interest are usually isolated from the specimen by different procedures before their injection into the instrument. Midazolam and its phase I metabolites have been analysed from plasma and liver microsomal incubations after simple protein precipitation with acetonitrile [30]. Dostalek et al. [30] used a highly selective triple quadrupole mass spectrometer (MS/MS) for the analysis and reported the method to be selective and free from any matrix effect. In ESI-LC/MS instruments in combination with the SIM mode, biological samples require an additional purification step to remove interfering components in order to obtain cleaner sample extracts and matrix effect free spray in the ion source [35,36]. Midazolam and its metabolites have been commonly extracted from the biological specimen by liquid–liquid extraction (LLE) after pH adjustment. Extraction solvents with non-polar, medium polarity, or a mixture of several solvents e.g. n-butyl acetate, toluene, or ethylacetate-hexane (75:25, v/v) have been reported [16–18,22,23,27,32]. More selective extraction techniques, e.g. solid-phase extraction (SPE), have also been widely used with reversed-phase sorbents [20,25,29,32] and also with a strong cation-exchange ion exchanger sorbent [26]. In addition, a mixed-mode SPE sorbent, combining nonpolar and cation-exchange packing materials has been used for isolation of midazolam and its phase I and II metabolites from the specimen. The mixed-mode SPE method has been used for the determination of midazolam and its metabolites in oral fluids [21], plasma [15,37], serum [24], and urine [15,26,28,31]. Midazolam has also been assayed from human plasma by direct immersion solid-phase microextraction (SPME) using a polyacrylate fiber after plasma deproteinization step [13] and from human urine by headspace-SPME using a polydiemethylsiloxane fiber [19].

The aim of this study was to develop a highly selective and sensitive GC–MS method for the determination of midazolam and its pharmacologically active metabolite in plasma samples obtained from pharmacokinetic studies in rabbits. This study is related to our project aiming to develop a novel sublingual midazolam formulation for use in children. As far as we are aware, this is a first report to describe the quantitative determination of midazolam and 1-hydroxymidazolam from plasma by mixed-mode SPE and GC–MS with NCI. The method presented in this report was validated in terms of selectivity, linearity, precision, accuracy, recovery, and stability [38,39]. The developed GC–MS method is highly selective and sensitive, and the suitability of the method was evaluated for measuring plasma levels after sublingual administration of a commercial midazolam injection solution in rabbits.

2. Experimental

2.1. Chemicals and materials

Midazolam (8-chloro-6-(2-fluoro-phenyl)-1-methyl-4H-imidazo-[1,5-a][1,4]-benzodiazepine) (CRS) was purchased

from European Pharmacopoeia (Strasbourg, France). 1-Hydroxymidazolam (99%) and deuterated midazolam (internal standard (IS), midazolam-d4, 98%) in methanol (100 μg/ml) were purchased from Cerilliant (TX, USA). N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA with 1% TMCS) and pyridine were of GC-grade obtained from Sigma–Aldrich (Steinheim, Germany). All other chemicals and solvents were of analytical grade. Drug-free human blank plasma from five different individuals was purchased from the Finnish Red Cross (Helsinki, Finland). Sodium citrate was used as anticoagulant in human plasma. Strata-X-C Mixed-Mode polymeric strong cation 30 mg/3 ml SPE cartridges were purchased from Phenomenex (Torrance, CA, USA).

Three male New Zealand White (NZW) rabbits were obtained from HB Lidköpings Kaninfarm (Lidköping, Sweden). Midazolam solution (Dormicum) for animal tests was purchased from Roche (Espoo, Finland), ketamine (Ketalar) from Pfizer (Helsinki, Finland), medetomidine (Domitor) from Orion Pharma (Espoo, Finland) and fentanyl citrate–fluanisone (Hypnorm) from Janssen Pharmaceutica (Beerse, Belgium).

2.2. Preparation of calibration and quality control samples

A stock solution of midazolam was prepared at a concentration of 100 μg/ml in methanol. 1-Hydroxymidazolam and IS stock solutions were commercially available solutions (100 μg/ml) prepared in methanol, which were diluted at a concentration of 1 μg/ml. Separate stock solutions of midazolam and 1-hydroxymidazolam were prepared for calibration and quality control (QC) samples. Stock solutions were protected from light, stored at −20 °C and used within three months. Working solutions of midazolam and 1-hydroxymidazolam were diluted in methanol at concentrations of 10–1000 ng/ml and 5–500 ng/ml, respectively The working solution of IS was prepared at 200 ng/ml.

Calibration and QC samples were prepared by adding 100 μl of the working solution of midazolam, 1-hydroxymidazolam, and IS to the test tubes. Solutions were evaporated under a stream of nitrogen (40 °C), after which 0.5 ml human blank plasma was added in the tubes to make final plasma concentrations for midazolam and 1-hydroxymidazolam of 2–200 ng/ml and 1–100 ng/ml, respectively. Sample preparation of calibration and QC samples was conducted according to Section 2.3. In order to prepare reference standards, 100 μl of midazolam, 1-hydroxymidazolam, and IS working solutions were added to a screw capped Kimax® borosilicate glass test tubes, evaporated to dryness under nitrogen and derivatized with 50 μl of BSTFA and 50 μl of pyridine.

2.3. Sample extraction

Rabbit plasma samples were allowed to thaw at room temperature. The working solution of IS (100 μl) was added to the test tubes and solution was evaporated under a stream of nitrogen (40 °C). After evaporation, 0.5 ml of plasma was added to the tube and the sample was vortex-mixed for 5 s. One ml of 50 mM phosphate buffer (pH 6) and 50 μl of 2 M HCl were added to the sample, which was then vortex-mixed (5 s).

Strata-X-C Mixed-Mode strong cation exchange 30 mg/3 ml SPE cartridges were used for sample extraction. Cartridges were conditioned with 2 ml of methanol and equilibrated with 2 ml of 50 mM phosphate buffer (pH 6). After equilibration, the sample was loaded into a cartridge under vacuum and washed with 2 ml water and 2 ml methanol. Cartridges were dried under vacuum and elution solvent was allowed to soak into the sorbent for 1 min. The sample was eluted with 2 ml of 5% ammonia in methanol. After elution, the sample was evaporated to dryness under a stream of nitrogen at 40 °C and 50 μl of BSTFA and 50 μl of pyridine was added in a screw capped Pyrex® borosilicate glass test tube. After vortex-mixing (5 s), tubes were heated for 30 min at 50 °C, transferred to vials and analysed with GC–MS.

2.4. GC–MS instrumentation

The analyses were performed with Agilent Technologies GC–MS system combined with a gas chromatograph 6890N, injector/autosampler 7683 and a mass detector 5973 (Palo Alto, CA, USA). The column was a cross-linked 5% phenyl methyl siloxane capillary column (HP-5MS 30 m × 0.25 mm, 0.25 μm film thickness; Agilent Technologies). A hand-packaged, deactivated splitless single-taper liner with glass wool (part number 5188-6567) from Agilent Technologies was used. The initial temperature of the analytical column was 110 °C for 1 min and was then increased to 300 °C at a rate of 30 °C per min. The temperature was held at 300 °C for 5 min resulting in a total runtime of 12.33 min. Helium was used as the carrier gas at a constant flow of 1.0 ml/min after pulsed injection in the splitless mode (0.2 μl) with an injection pressure of 30 psi for 1.5 min and inlet temperature of 250 °C. The split vent was opened 1.5 min after the injection.

In NCI, methane was used as a reagent gas at 40% of the maximum methane flow. The temperatures of the transfer line, the ion source and the quadrupole were set at 290 °C, 150 °C and 150 °C, respectively. Quantitative analyses were performed in the SIM mode. One SIM ion time window was used: 5.00–12.33 min for midazolam, 1-hydroxymidazolam, and IS (5.80 cps). The dwell time was set at 50 ms per ion and the solvent delay at 5.00 min. The followed ions were *m/z* 325.1, 413.3, and 329.3 for midazolam, 1-hydroxymidazolam, and IS, respectively. In all scanning mode experiments, a mass range of 100–500 amu (3.58 cps) was applied. The EI mode was using an ionization energy of 70 eV.

2.5. Validation

This method was validated in terms of selectivity, linearity, precision, accuracy, recovery, and stability [38,39].

2.5.1. Selectivity

The selectivity of the method was assessed by analyzing reference standards, authentic sample matrix with and without analytes, buffers, reagents, and solvents for interfering peaks at the retention times of analytes. In addition, human analyte-free blank plasma samples obtained from five different individuals were analysed for possible interference peaks at the retention time of midazolam and 1-hydroxymidazolam. In addition, analyte-free plasma samples from five rabbits collected after administration of anaesthesia medication (ketamine, metedomidine, fentanyl citrate and fluanisone) were analysed.

2.5.2. Calibration

A calibration curve included a blank sample (analyte-free plasma used in calibration, processed without internal standard), a zero sample (analyte-free plasma used in calibration, processed with internal standard) and seven non-zero samples. Seven calibration concentrations with three replicate samples at each level were used for the linearity experiments (for midazolam 2, 5, 10, 20, 60, 100, 200 ng/ml and for 1-hydroxymidazolam 1, 2, 5, 10, 20, 50, 100 ng/ml). Peak area ratios of midazolam and 1-hydroxymidazolam to IS were plotted against the known concentrations, after which the calibration was performed by using least-squares linear-regression. Correlation coefficient (R^2) was used for evaluating the linearity of the regression lines. Furthermore, the deviation of standards from the nominal concentration was calculated. Calibration curve fulfilled the following conditions:

less than 20% deviation in the lower limit of quantification (LLOQ) and less than 15% deviation of the other standards.

LLOQ was determined by using five replicate plasma samples independent of the standards at a concentration of 2 ng/ml for both midazolam and 1-hydroxymidazolam. It is recommended that the response of the analyte signal should be at least 5 times the response compared to blank response and it should be reproducible with a precision of 20% (relative standard deviation, RSD) and accuracy of 80–120%.

2.5.3. Precision, accuracy and recovery

The intra-batch precision of the assay was assessed by calculating the relative standard deviation (RSD) for the analysis at four different QC sample concentration levels in five replicates, and inter-batch precision was determined by the analysis of QC samples on three consecutive days. The QC samples were prepared at the concentrations of 2, 5, 60 and 160 ng/ml for midazolam and 2, 5, 40 and 80 ng/ml for 1-hydroxymidazolam. It is recommended that the precision determined at each concentration level should not exceed 15% of the RSD except at the LLOQ, where it should not exceed 20% of the RSD.

Accuracy was calculated by comparing the mean experimental concentrations of assayed QC samples with their nominal values. Accuracy is expressed as the ratio between the experimental and the nominal values observed. The recommendation is that the mean value of accuracy should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%.

The recovery of midazolam and 1-hydroxymidazolam in an assay was measured as the detector response, obtained from an amount of the analyte added to analyte-free plasma and extracted from these samples, then compared to the detector response obtained for the true concentration of the pure unextracted authentic reference standard that represents 100% recovery. Recovery experiments were performed at four concentration levels, which were the same as the QC standards.

2.5.4. Stability

The stability of midazolam and 1-hydroxymidazolam in plasma was studied with triplicate samples for the low and the high concentrations. Stability samples were prepared from freshly prepared standard solutions, which were spiked in the drug-free human blank plasma giving final concentrations of 5 ng/ml and 160 ng/ml for midazolam and 5 ng/ml and 80 ng/ml for 1-hydroxymidazolam. The stabilities of midazolam and 1-hydroxymidazolam in plasma were determined after 24 h storage at room temperature (+24 °C), after 3 months at −80 °C and after three freeze (−80 °C) and thaw (+24 °C) cycles. In addition, the stability of stock solutions and post-preparative stability in instruments autosampler after 24 h was studied. Stability is expressed as a percentage and was calculated by dividing the sample concentration, at each study point, by the sample concentration at the outset of the study and multiplying the resulting value by 100. The predetermined limits for stability were set at 20% for variation and mean deviation.

2.6. In vivo study

The animal study was approved by the Animal Ethics Committee of Finland. Three male NZW rabbits were used for in vivo tests. The animals weighed 3.5 kg at the beginning of the study. Ketamine (15 mg/kg), medetomidine (0.25 mg/kg) and fentanyl citrate (0.05 mg/rabbit)–fluanisone (1.5 mg/rabbit) solution were used for sedation. Rabbits received 1 mg dose of commercially available midazolam solution (Dormicum 5 mg/ml, Roche Oy) by the sublingual route. Blood samples (2 ml) were collected to the EDTA plasma tubes at the time points between 0 and 240 min from the central artery (90%) or the marginal vein (10%) of the ear. The blood was centrifuged 10 min at 20 °C ($3700 \times g$) to obtain plasma and stored at −80 °C prior to analysis. The samples were analysed within four weeks. The area under curve between 0 and 240 min ($AUC_{0-240\,min}$) was calculated by the linear trapezoidal method. The elimination rate constants (k_{el}) were calculated from the individual concentrations versus time data sets based on a noncompartment model using the WinNonlin Professional v5.0.1 software (Pharsight Corporation, Mountain View, CA, USA). $AUC_{240-\infty}$ was determined by using the following equation: $AUC_{240-\infty} = C_{(240\,min)}/k_{el}$. In equation, $C_{(240\,min)}$ presents the concentration of midazolam or 1-hydroxymidazolam at 240 min. $AUC_{0-\infty}$ is a sum of AUC_{0-240} and $AUC_{240-\infty}$.

3. Results and discussion

3.1. Method development

Analytical methods for determination of midazolam and its metabolites from biological samples are mainly based on mass spectrometric (MS) detection in combination with either gas chromatography (GC) or liquid chromatography (LC). In our laboratory, the limited instrument time on LC/MS/MS and availability of a very sensitive GC–MS instrument with a negative chemical ionization (NCI) encouraged us to develop and validate a quantitative method for use with GC–MS instrumentation. Benzodiazepines have electronegative moieties, GC–MS with NCI can improve the responses to these compounds by a factor of several thousand when compared to GC–MS methods with either positive chemical ionization (PCI) or electron ionization (EI) techniques [22,23,40]. The high sensitivity and selectivity of NCI in GC–MS confer significant benefits in terms of increased signal-to-noise ratios and decreased background interface, and the technique also allow a reliable analysis from microvolumes of sample. This is a great benefit especially when planning sampling protocols from paediatric patients. In addition, GC–MS with NCI possesses advantages over LC–MS based techniques in terms of higher separation efficiency and the absence of matrix-dependent ion suppression. A relatively strong matrix effect in plasma and urine samples has been reported for midazolam with LC/MS/MS instrumentation after reversed-phase SPE and electrospray ionization [29].

Some benzodiazepines have been reported to have carry-over effects derived from the vaporized injection solvent and analytes coming into contact with the gold plate and/or the metal surface of injector [23]. A similar effect was observed in our laboratory during the method development with deactivated, tapered borosilicate glass wool liners. In these GC–EI–MS studies, the injection volume was 1 μl and plasma concentrations as low as 1 ng/ml could be quantified (data not shown). Unfortunately, the glass raw material of the above mentioned commercial liners changed, which caused midazolam peak tailing after only a few injections with the new glass material incorporated in the liners. To overcome this problem, a hand-packaged deactivated splitless single-taper liner with glass wool was obtained from a supplier. In addition, when the ionization was changed from EI to NCI, a smaller injection volume could be used and as a consequence both liner and column lifetime were considerably increased and the need for maintenance of split/spitless injector and MS ionization source was decreased. The capillary column exhibited no degradation or other problems during the validation and in the study sample analysis. In our laboratory the septum is changed every two to three hundred samples and the liner every five hundred to one thousand samples on a

routine basis. This depends on how "dirty" samples have been analysed. In the case of NCI, a change of septum is necessary to keep the instrument free from oxygen contamination, which could greatly decrease the intensity of ionization.

All the samples were silylated by BSTFA with 1% TMCS, which is the most commonly used derivatization reagent for benzodiazepines in biological samples [14]. Midazolam has no active group to bind to the silylating reagent and thus does not form a derivative, but its phase I metabolites form stable and volatile TMS derivatives. This derivatization step is necessary to improve the chromatographic characteristics and the stability of capillary column efficacy by forming less polar derivatives which are more compatible with the non-polar capillary column stationary phase. In addition, derivatization decreases the vaporization temperature by lowering the compound's capability to form hydrogen bonds and/or introducing electronegative moieties to increase the peak intensity in NCI mode.

3.2. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample [38]. Fig. 2 shows the mass spectra of analytes and IS obtained by NCI and EI. Capillary GC–MS with EI is one of the most reliable techniques for drug identification due to its excellent chromatographic resolution power, high reproducibility of chromatographic peak retention times, and the availability of library-searchable spectral information using EI spectra. The base peak in EI spectra of midazolam, the TMS derivative of 1-hydroxymidazolam, and IS were m/z 310, 310, and 314, respectively. The molecular ion of midazolam, TMS derivative of 1-hydroxymidazolam, and IS were also present at m/z 325, 413, and 329, respectively. The fragmentation of analytes in the NCI spectra is much smaller than in the EI spectra (Fig. 2). In the NCI spectra the molecular ions of midazolam, the TMS derivative of 1-hydroxymidazolam, and IS were m/z 325, 413, and 329, respectively. The specific response of NCI to the analytes and the virtually nonexistent background interference in MS spectra further assists in the reliable identification of midazolam, the TMS derivative of 1-hydroxymidazolam, and IS in the plasma samples. For quantitative analysis, ions for SIM of each analyte were carefully selected on the basis of the selectivity experiments made in both the EI and NCI modes. The candidates for SIM ions were chosen from the full-scan mass spectra (Fig. 2) of each analyte and the robustness of the ion was ensured by dynamic mass range calibration (data not shown).

Five human and five rabbit plasma samples from different donors were analysed for possible selectivity problems in the chromatographic run. No interference peaks were detected in human blank plasma, in human blank plasma with IS or in rabbit plasma with anaesthesia medications having the same retention times as midazolam, the TMS derivative of 1-hydroxymidazolam, or IS. Representative selected ion monitoring NCI chromatograms of a quality control sample at LLOQ level and human drug-free blank plasma are shown in Fig. 3.

3.3. Calibration

The seven point calibration curves for midazolam and 1-hydroxymidazolam were highly linear over the range of the method. The simplest model to describe the concentration–response relationship was achieved by using a non-weighted least squares linear regression. The slope and intercept of the calibration curves with the 95% confidence interval and correlation coefficients (R^2) are summarized in Table 1. Deviation of the calibration standards from their nominal concentrations was invariably less than 10%.

The LLOQs, defined as the lowest concentration analysed from five replicate samples, were independent from calibration samples and determined with a precision less than 20% and an accuracy of 80–120%. The LLOQ for both analytes was 2 ng/ml corresponding 0.4 pg injection to the instrument (Table 2). Similar LLOQ values have been reported with GC–MS with NCI [22] and chip-based infusion nanoESI tandem mass spectrometry [34]. Lausecker et al. [32] reported CZE/MS/MS to be superior to that of micro-LC/MS/MS with regard to detection limits which were in the low ng/ml range, even with only nanolitre injection volumes typical in CZE techniques.

3.4. Accuracy, precision and recovery

The intra-day precision and accuracy of all QC samples were within the predetermined acceptance criteria (Table 2). The average recoveries from four QC levels of midazolam and 1-hydroxymidazolam were $99 \pm 2\%$ and $104 \pm 10\%$ (mean $\pm$ SD), respectively (Table 2). Similar results have been reported with mixed-phase SPE methods [28]. Slightly lower extraction recovery values were found for midazolam and its metabolites by a reversed phase SPE [29,32] and LLE with a mixture of ethylacetate and hexane (75:25, v/v) [27]. A recovery of over 95% with a strong cation exchanger has been reported, but recoveries of midazolam metabolites were at the same time less than 90% [26]. The SPE cartridges used in our method contained mixed-mode polymers with reversed phase and strong cation exchange properties. The styrene divinyl benzene polymer achieves good retention for polar and aromatic compounds, and strong cation exchange groups for basic compounds. Midazolam is a weak base with pKa of 6.1. Acidification of plasma with 2 M HCl ionizes the basic nitrogen of midazolam which is then retained on the cartridge through an ion exchange mechanism. This enables washing with pure organic solvents and as a result, a very pure and interference free extract for the silylation and instrumentation steps is obtained. In addition, drying of the stationary phase or extreme pH values are not limiting factors when using polymer based stationary phases, and this improves the robustness of the sample preparation step in the present method. Mixed-mode SPE has been reported to be suitable for extraction of midazolam phase II metabolites from urine [15]. Conjugation with glucuronic acid is a common metabolic pathway for many drugs and the formed phase II metabolites are often amphoteric and therefore more water-soluble than the parent drug. This leads to a poor extraction efficacy of these metabolites with conventional extraction methods such as LLE or reversed-phase SPE [15].

3.5. Stability

There was no significant degradation of midazolam or 1-hydroxymidazolam after three freeze–thaw cycles, compared to the analyses conducted at the outset. The short-term stability at room temperature for 24 h showed no degradation of analytes, and measured stability values were between 91 and 102% for midazolam and 96 and 101% for 1-hydroxymidazolam. Plasma samples were stable during storage at $-80\,^{\circ}C$ for 3 months with stability values of 92–105% and 90–115% for midazolam and 1-hydroxymidazolam, respectively. Similar stability results have been reported previously [29]. Post-preparative stability was found to be constant when the samples were stored for 24 h at $+24\,^{\circ}C$ in the autosampler. In contrast, low post-preparative stability in autosampler has been reported and the midazolam level was observed to decline to 69% over 15 h [28]. However this could partly be due to reversible ring-opening in the HPLC mobile phase (pH 3), which would change the chromatographic properties of midazolam [12,28].

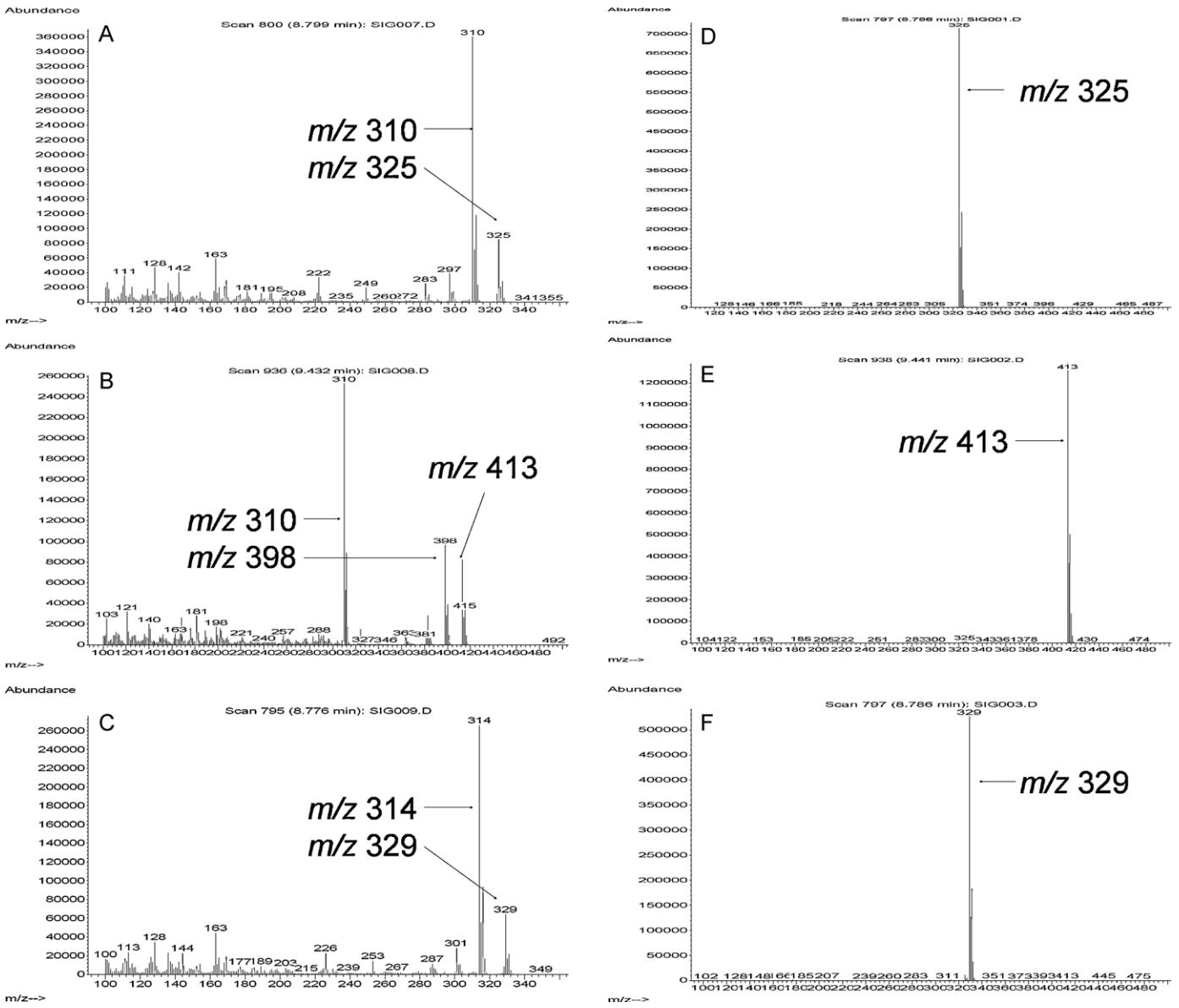

Fig. 2. Electron ionization mass spectra of (A) midazolam, (B) TMS derivative of 1-hydroxymidazolam, and (C) midazolam-d4 (IS). Negative ionization mass spectra of (D) midazolam, (E) TMS derivative of 1-hydroxymidazolam, and (F) IS. Instrumentation parameters have been described in Section 2.4.

3.6. In vivo study

The suitability of GC–MS method for monitoring midazolam levels was evaluated after administration of a commercial midazolam injection solution sublingually in rabbits. Midazolam was absorbed rapidly from this route of administration. All three rabbits displayed plasma concentrations (4.6–6.4 ng/ml), which were above LLOQ of the method already at the first sampling point (2 min). Maximum plasma concentration (C_{max}) of midazolam was 69.6 ± 10.6 ng/ml and it was achieved at 30 min (t_{max}). After drug administration, 1-hydroxymidazolam has been formed in 30 min at concentrations above LLOQ of the method. The C_{max} of 1-hydroxymidazolam was 7.1 ± 2.0 ng/ml, this being achieved at 60 min (t_{max}). The area under the plasma concentration–time curves from 0 min to 240 min ($AUC_{0-240\,min}$) were 8500 ± 1300 ng/ml × min (mean ± SD) for midazolam and 1300 ± 500 ng/ml × min for 1-hydroxymidazolam. The curves of plasma concentration versus time and representative SIM chromatograms of rabbit sample collected after sublingual administration for midazolam and 1-hydroxymidazolam are presented in Fig. 4.

Table 1
The linear range, correlation coefficient (R^2), and calibration curve parameters with 95% confidence intervals of midazolam and 1-hydroxymidazolam ($n = 3$). The linear curve fit with ignored origin was used for non-weighted least squares linear regression. LLOQ was determined by calculating precision and accuracy for five LLOQ samples that were independent of the calibration curve. All the samples were prepared according to Sections 2.2 and 2.3.

Compound	Linear range (ng/ml)	Correlation coefficient (R^2)	Calibration curve parameters		LLOQ (ng/ml)
			Slope ± 95% CI	Intercept ± 95% CI	
Midazolam	2–200	0.999	0.0277 ± 0.0009	0.0427 ± 0.0752	2
1-Hydroxymidazolam	1–100	0.997	0.0223 ± 0.0009	0.0102 ± 0.0401	2

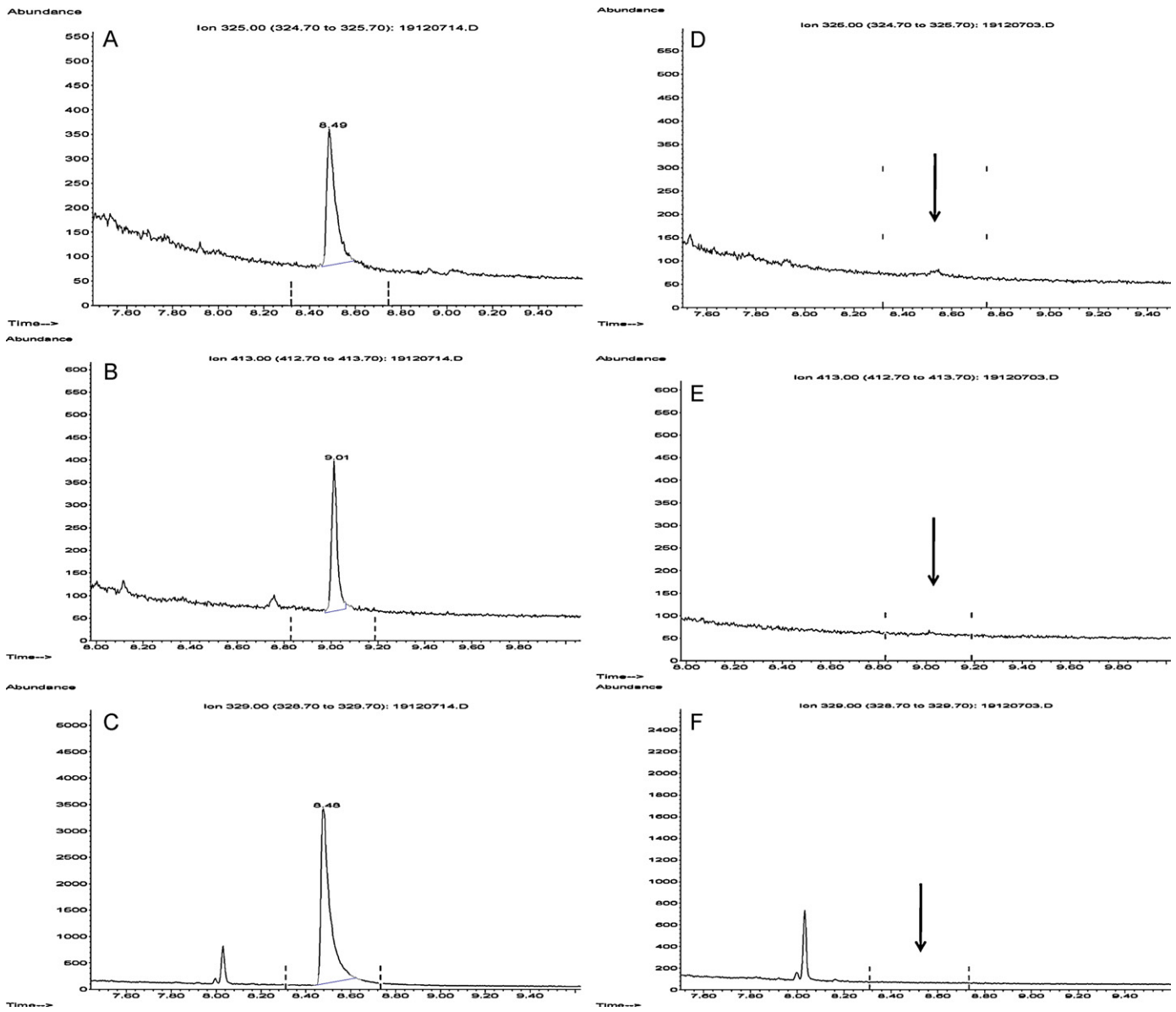

Fig. 3. Representative selected ion monitoring (SIM) NCI chromatograms at a LLOQ level of (A) midazolam (2 ng/ml, retention time (RT) 8.49 min, and RMS $S/N = 127$), (B) 1-hydroxymidazolam (2 ng/ml, RT 9.01 min, and RMS $S/N = 162$), and (C) midazolam-d4 (IS, 40 ng/ml, RT 8.48 min, and RMS $S/N = 1000$). In analyte-free plasma, no disturbing peaks were found at the retention times of (D) midazolam (not detected), (E) 1-hydroxymidazolam (not detected), or (F) IS (not detected). All the samples were prepared according to Sections 2.2 and 2.3. Instrumentation parameters have been described in Section 2.4. The arrows are used to indicate the retention time of analytes and IS in chromatograms.

Table 2
Intra-day and inter-day precision, accuracy, and recovery for midazolam and 1-hydroxymidazolam, and their nominal values at four QC levels. All samples were prepared according to Sections 2.2 and 2.3.

Compound	Nominal conc. (ng/ml)	Intra-day precision, accuracy, and recovery				Inter-day precision	
		Mean conc. ($n = 5$) (ng/ml)	RSD (%)	Accuracy ($n = 5$) (%)	Recovery (%) (RSD %)	Mean conc. ($n = 3$ days) (ng/ml)	RSD (%)
Midazolam	2	2.0	11	100	101 (8.5)	2.2	15
	5	4.9	5.9	99	97 (3.3)	4.5	5.4
	60	55	1.2	92	99 (1.6)	58	6.8
	160	150	1.3	94	99 (4.5)	155	3.8
1-Hydroxymidazolam	2	2.2	10	112	108 (19)	2.0	13
	5	5.0	2.8	99	109 (6.9)	5.3	5.7
	40	40	3.5	99	109 (7.2)	40	3.4
	80	75	2.9	94	88 (16)	76	3.3

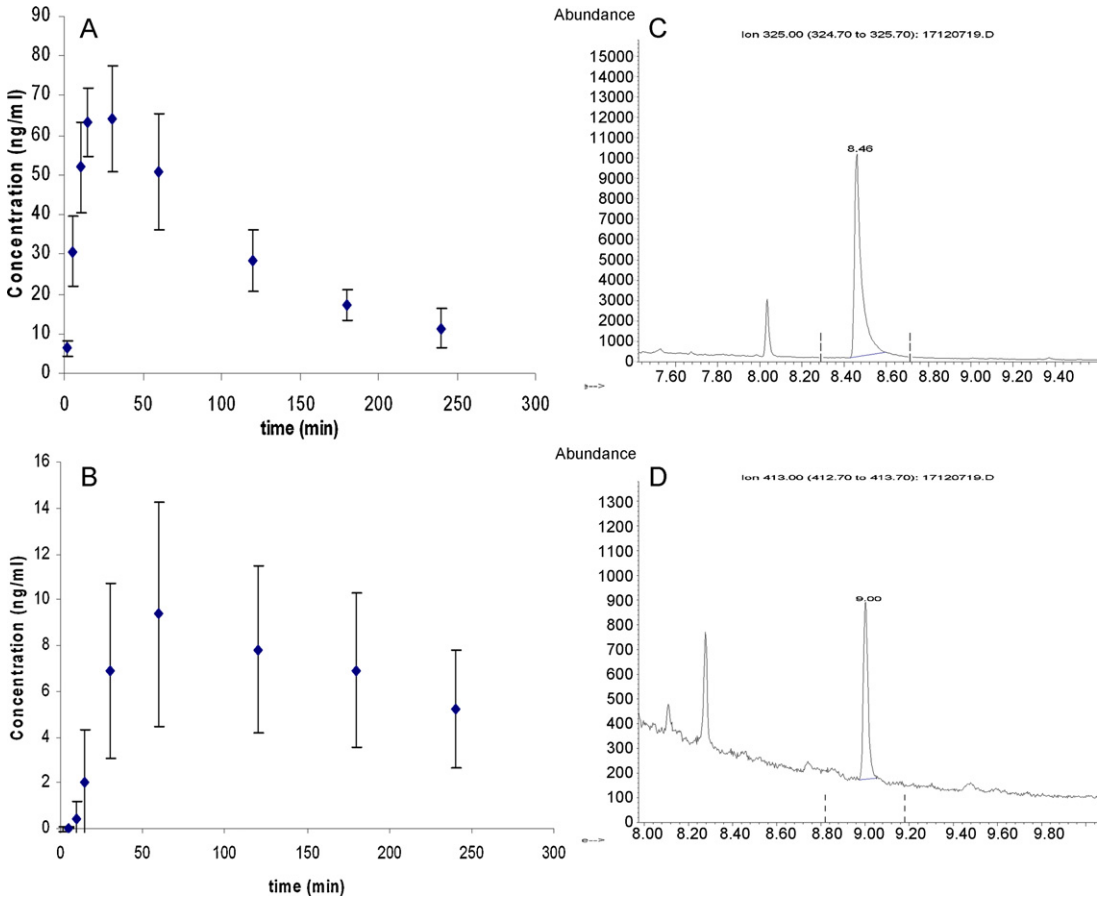

Fig. 4. Plasma concentration versus time profiles of midazolam (A) and 1-hydroxymidazolam (B) after sublingual administration of midazolam solution (1 mg). Values are averages from three rabbits ($n = 3$), and the variation is presented as standard deviation (SD). Representative SIM chromatograms of (C) midazolam (78 ng/ml; RT 8.46 min) and (D) 1-hydroxymidazolam (5.0 ng/ml; RT 9.00 min) extracted from rabbit plasma 30 min after sublingual administration of midazolam.

The main metabolite of midazolam, 1-hydroxymidazolam, is pharmacologically active and therefore it should be analysed together with midazolam. Several analytical methods have been developed with high sensitivity at very low LLOQ concentrations, but they may require extensive work and considerable expense and do not necessarily confer any benefits to the outcome of pharmacokinetic study. The LLOQ in the present method was 2 ng/ml, and although it could easily be lowered to 1 ng/ml or even lower, as this was not systematically investigated. All the measured midazolam concentrations in this study were over 4.6 ng/ml and hence clearly above the LLOQ of the method. According to the European Medicines Agency's (EMA) guideline for bioavailability and bioequivalence [39], sampling points should cover the plasma concentration versus time curve for at least 80% of the AUC from time zero extrapolated to infinity ($AUC_{0-\infty}$). For midazolam, this criterion was fulfilled i.e. the calculated $AUC_{0-240\,min}$ was $84.3 \pm 2.2\%$ (mean ± SD) of the extrapolated $AUC_{0-\infty}$. In the case of 1-hydroxymidazolam, $AUC_{0-240\,min}/AUC_{0-\infty}$ ratio was lower, $48.2 \pm 28.9\%$. For more reliable estimates, the sampling time should have been lengthened and the sensitivity of the method would need to be improved e.g. by increasing the injection volume or introducing electronegative moieties into the metabolite by derivatization. However, this was not considered to be necessary in this study. The plasma concentration of 1-hydroxymidazolam was 12% of the midazolam concentration in the present study, and since 1-hydroxymidazolam has only about 10% of the biological activity of midazolam [11], a concentration of 1-hydroxymidazolam below LLOQ of the method (2 ng/ml) was considered to have minimal therapeutic importance. In addition, from the ethical point of view, it was not justified to continue sampling from the rabbits simply to monitor this phase I metabolite of midazolam.

4. Conclusion

In this report, a highly sensitive and selective GC–MS method for the determination of midazolam and 1-hydroxymidazolam from rabbit plasma is presented. This method was developed and validated for use in pharmacokinetic studies of a novel sublingual midazolam formulation for children. The plasma samples were purified with the mixed-mode strong cation exchange solid-phase extraction cartridges. The extract was injected after silylation into the GC–MS instrument with sensitive negative chemical ionization. The method was selective, linear, accuracy, precise, and sample preparation resulted in high recovery values. Plasma samples were stable during sample handling, storage and after three freeze and thaw cycles. The method was successfully used in pharmacokinetic studies of midazolam administrated sublingually in rabbits.

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