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Determination of the immunostimulatory drug - Glucosoaminyl-Muramyl-Dipeptide – in human plasma using high performance liquid chromatography-tandem mass spectrometry and its application to a pharmacokinetic study

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Short running head: Pharmacokinetics of Glucosaminyl-muramyl-dipeptide (GMDP)

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ABSTRACT

Introduction: GMDP (glucosaminyl-muramyl-dipeptide), a synthetic analog of the peptidoglycan fragment of the bacterial cell wall, is an active component of the immunomodulatory drug Licopid®. But, the pharmacokinetic parameters of GMDP in humans after oral administration have not been investigated yet. A present study aimed to develop and validate a sensitive liquid chromatography-tandem mass spectrometry method for the analysis of GMDP in human plasma. **Methods:** Sample preparation was performed by solid-phase extraction using Strata-X 33um Polymeric Reversed Phase 60 mg/3ml cartridges. The separation of analytes was performed using an Acquity UPLC BEN C18 column 1.7 μm 2.1 x 100 mm (Waters, USA). GMDP and internal standard GHRP-2 (pralmorelin) were ionized in positive electrospray ionization mode and detected in multiple reaction monitoring mode. **Results:** The developed method was validated within a linear range of 50 - 3000 pg/mL for GMDP. Accuracy for all analytes, given as deviation between nominal and measured concentration and assay variability (CV) ranged from 1.61 % to 3.02 % and from 0.89 % to 1.79 % respectively both for within- and between-run variabilities. **Conclusions:** The developed and validated HPLC-MS/MS method was successfully used to obtain the plasma pharmacokinetic profiles of GMDP distribution in human plasma.

Keywords: Immunostimulatory drugs; GMDP; Glucosaminyl-muramyl-dipeptide (*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-d-isoglutamine); muramyl peptides; pharmacokinetics; LC-MS/MS.

Introduction

N-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-d-isoglutamine (GMDP) is an *N*-acetylglucosamine analog of muramyl dipeptide (MDP), which is a component of the cell walls of almost all known bacteria (Adam and Lederer, 1984; Andronova and Ivanov, 1991; Ellouz et al. 1974; Kotani et al. 1975; Kusumoto et al. 1976; Merser et al. 1975). Muramylpeptides (MP) are part of cell wall biosynthesis and bacterial communication, and are also reported to have a possibility to activate an immune response in eukaryotic cells (Girardin et al. 2003a; Girardin et al. 2003b; Girardin et al. 2003c; Boubreau et al. 2012; Woodhams et al. 2013; Dworkin, 2014; Irazoki et al. 2019). MDP and MDP analogs were reported as having potential for clinical use as vaccine adjuvants, immunostimulators, anti-cancer agents and anti-inflammatory agents (Ellouz et al. 1974; Adam and Lederer, 1984; Andronova and Ivanov, 1991; Traub et al. 2006; Rostovtseva et al. 1981; Ogawa et al. 2011; Leclerc et al. 1979). While MDP is highly pyrogenic in humans, GMDP retains the favorable activity of MDP but is less pyrogenic (Dinarello et al. 1978; Lyons et al. 2000). MP administration to a body leads to the stimulation of signal pathways that induce the expression of a big variety of moieties, which can induce proinflammatory and antimicrobial responses (Akira et al. 2006; Nesmeyanov et al. 1992).

The biological activity of the MP is realized via binding to the intracellular receptor protein NOD2, which is expressed in the peripheral blood leukocytes (Molinaro et al. 2019; Bourhis et al. 2007; Laman et al. 2016). The detection of MP by NOD2 protein outcomes with the activation of intracellular reactions that trigger the synthesis of nuclear factors, that initiate inflammatory responses and antimicrobial activity (Fritz et al. 2006; Meylan et al. 2006; Franchi et al 2009).

In mammals, plants, and some insects, muramyl peptides can be recognized by the innate immune system and help to protect the host from bacterial infections (Boneca, 2005; Royet et al. 2011; Gust, 2015; Capo et al. 2016; Pashenkov et al. 2018; Wolf and Underhill, 2018). The intestinal microbiota is a source of muramyl peptides that can travel from the intestines to the bloodstream (Clarke et al. 2010). The presence of muramyl peptides in the body has been studied in numerous works, which provide sufficient evidence of the presence of muramyl peptides in the system circulation and animal tissues (Irazoki et al. 2019; Fosset et al. 2003; Clarke et al. 2010; Krueger, 1985).

Despite a huge number of MP-containing microbiota and numerous studies reporting MP as immunostimulatory signal molecules, little is known about the systemic concentration of MP (Irazoki et al. 2019; Boneca, 2005

The synthetic analog of MDP, Licopid®, enhances the activity of phagocytes, presentation of antigens, the proliferation of lymphocytes, increases the synthesis of specific antibodies, and helps to normalize the balance of Th1/Th2 lymphocytes in the direction of the prevalence of Th1 (Girardin et al. 2003a; Girardin et al. 2003b; Girardin et al. 2003c; Shimizu et al. 1992). Positive effects of GMDP based pharmaceutical, Licopid®, was shown at the treatment of children with bronchial asthma and atopic dermatitis (Guryanova et al. 2010).

The pharmacokinetic parameters of GMDP in humans after oral administration have not been investigated. GMDP pharmacokinetic studies have been conducted mainly in animals using radioisotope labeled compounds ([^{14}C] GMDP) by liquid scintillation counting (Lyons et al. 2000).

The development of peptides and proteins as oral formulations has however been limited due to their low bioavailability when administered via this route. In this regard, initial studies examining the oral bioavailability of [^{14}C] GMDP in beagle dogs have indicated that the absolute bioavailability of GMDP is low (6.7%) (Lyons et al. 2000). The bioavailability of

radio-labeled GMDP was also low when administered orally as a solution to male Sprague–Dawley rats because of degradation in the intestine (8.3 - 4.4%) (Lyons et al. 2000).

Tandem mass spectrometry (MS/MS) coupled with HPLC (high-pressure liquid chromatography) is the analytical technique of choice for most assays used during new drug discovery (Moskaleva et al. 2017; Shpak et al. 2005; Mesonzhnik et al. 2018; Zherdev et al. 2009; Appolonova et al. 2008). Pharmacokinetics parameters of MEPACT® (the liposomal-encapsulated formulation of Muramyl tripeptide phosphatidyl-ethanolamine, mifamurtide (MTP-PE), Takeda Pharmaceutical Company Ltd) in healthy adult volunteers and with hepatic impairment were studied (Venkatakrishnan et al. 2012; Venkatakrishnan et al. 2014). Total MTP-PE concentrations were measured using liquid-liquid extraction of total MTP-PE from human serum samples, followed by quantitative analysis using liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Venkatakrishnan et al. 2012; Venkatakrishnan et al. 2014).

The purpose of this study was to develop a method for determining the concentration of GMDP in human plasma samples using the HPLC-MS/MS technique, which can be used for pharmacokinetic studies after the oral administration of GMDP (Licopid®). The method was validated and successfully applied for real samples analysis from a Licopid® pharmacokinetics study.

Materials and methods

Chemicals and materials

GMDP (99% HPLC) was provided by the “Peptek” company. GHRP-2 (Pralmorelin, ≥97% purity by HPLC) (Fig. 1) was used as an internal standard (IS) and was supplied from Sigma-Aldrich. Cartridges with Strata -X 33um Polymeric Reversed Phase 60 mg/3ml were

purchased from Phenomenex (USA, California). High-grade water (18 MOmwas obtained using a Milli-Q water purification system (Merck KGaA, Darmstadt, Germany). HPLC grade methanol was from Thermo Fisher Scientific Inc. (Madrid, Spain). Extra pure formic acid and H_3PO_4 were purchased from Fluka (Steinheim, Germany). The plasma samples were obtained from healthy volunteers that had previously signed a consent form. Drug-free human plasma was supplied from the blood bank of I.M. Sechenov First Moscow State Medical University, Moscow, Russia. Blood plasma was stored at -70ºC prior to use.

Calibration and QC samples

Stock solutions of GMDP and the internal standard GHRP-2 were prepared in 10% methanol at a concentration of 1.0 mg/ml and were kept at -70ºC. Working solutions were made by dilution of stock solution with 10% methanol in water and were used for the calibration curve and quality control (QC) samples preparation. For calibration standards and QC samples preparation human plasma was preliminarily analyzed and six lots without GMDP peaks (GMDP concentration less than the limit of detection) were selected. To obtain six calibration samples appropriate amount of working solutions were added to the previously screened human plasma samples and the concentrations were 50, 600, 1200, 1800, 2400, and 3000 pg/ml. Quality control samples were prepared at three concentration levels: 150, 1500, and 2700 pg/ml using working standard solutions. GHRP-2 (internal standard) concentration was 3000 pg/ml.

Application to the pharmacokinetic study

The method was used to determine the plasma concentrations of GMDP from a clinical trial study in which each of the 40 healthy male volunteers aged 18 to 45 years received a single oral dose of 2 mg of Licopid® after an overnight fast. The study protocol was approved by the Local Ethical Committee at the I.M. Sechenov First Moscow State Medical University,

Moscow, Russia, and informed consent was obtained from all volunteers. Venous blood samples were obtained prior to dosing (0) and at 0.3 h (20 min), 0.6 h (40 min), 1 h, 1h 15 min, 1 h 30 min, 1 h 45 min, 2 h, 2 h 30 min, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h, and 24 h post-dose. Blood samples were transferred into tubes with K_2EDTA (anticoagulant), centrifuged for 15 min and the plasma was kept frozen at −70 °C until analysis. Pharmacokinetic parameters were calculated by a noncompartmental model using the Equiv Test PK software (Statistical Solutions Ltd., Ireland).

Sample preparation

A 1000 µL aliquot of human plasma sample, calibration or quality control samples were mixed with 100 µL of 30 ng\ml solution of GHRP-2 (IS) and 1000 µL of 4% H_3PO_4 in water. Analytes were extracted using Strata -X 33um Polymeric Reversed Phase 60 mg/3ml Cartridges. SPE cartridges were washed with 1 mL methanol and then equilibrated with 1 mL water. After loading the samples, the cartridges were washed with 1 mL H_2O, and analytes were eluted with 1 mL of methanol. Before analysis, the eluate was dried under vacuum and reconstituted using 100 µL of water: methanol mixture (90:10, v/v), and after 150 min in autosampler 10 µL of the sample was analyzed.

HPLC-MS/MS analysis

The liquid chromatography separation was conducted using a Waters Acquity UPLC system equipped with ACQUITY UPLC BEN C8 column 1.7um 2.1x50mm (Waters, USA). The mobile phase consisted of the H2O + 0.1% formic acid (A) and methanol + 0.1 % formic acid (B). The column temperature was 40ºC, and the injection volume of the samples was 10 µl. The linear gradient was formed as follows: 0 min (2% B), 2 min (20% B), 4 min (90% B), 6 min (2% B) at flow rate 0.3 ml/min.

MS analysis was performed by multiple reaction monitoring (MRM) in positive mode using a Waters TQ-S micro triple quadrupole mass spectrometer with an ESI source. The operating parameters: the capillary voltage was set at 1.5 kV, cone voltage was 20 V, source temperature 150 °C, desolvation gas temperature 500 °C, desolvation gas flow at 900 L/h, and cone gas flow at 50 L/h. Precursor and product ions, dwell time, cone voltage and collision energy were optimized for each analyte (Table 1). Peak detection, integration, and quantification were made using the Masslynx and TargetLynx software.

Method validation

GMDP is a universal minimal component of the peptidoglycan of the cell walls of almost all known bacteria and released from the bacterial cell wall by the action of lysozyme and amidases (EMA, 2011; USFDA, 2019; Irazoki et al. 2019). In healthy humans, the normal intestinal microflora can serve as a source of GMPD and the authors of a number of articles note the possible presence of GMDP in the blood plasma of a healthy person (Fosset et al. 2003; Irazoki et al. 2019), but data about blood GMDP concentrations have not been published yet. During a typical bioanalytical validation procedure, calibration and QC samples are prepared by spiking known amounts of analytes into the same biological matrix as the study sample. The possible presence of endogenous GMDP in the control matrix presents another analytical challenge (Thakare et al. 2019). Currently, the preliminary version of the ICH guide to the bioanalytical validation method highlights a section reflecting the problems of validation of the endogenous compound analysis, which outlines various strategies for quantitative analysis (EMA, 2019; USFDA, 2018). But according to the ICH guide, the biological matrix with an endogenous level of target compound sufficiently lower than LLOQ (< 20% of the LLOQ) can be used as a blank matrix (EMA, 2019). After preliminary studies, six pools of human plasma without GMDP peaks presented on the

chromatograms (the concentration is lower than the detection limit and < 20% of the LLOQ), were selected as blank samples and used for the preparation of the calibration samples and QC.

Calibration curves were obtained by plotting peak area ratios of the GMDP and GHRP-2 (IS) against known analyte concentrations using the Masslynx and TargetLynx software (Waters Inc.). The data were calculated using a linear regression model with 1/x weighting.

Quality control samples (QC) at three concentration levels of GMDP were analyzed within one batch for within-day accuracy and precision and on three days for between-day accuracy and precision evaluation. Accuracy was calculated as the percent relative error (%RE) and precision as the relative standard deviation (RSD).

The extraction recovery, matrix effect, and stability were investigated using six samples at two concentration levels corresponding to LQC and HQC. The extraction recovery of analytes was determined by comparing peak areas from blank plasma samples spiked with GMDP and GHRP-2 before extraction with those from plasma samples spiked with the same analytes post-extraction.

The matrix effect was studied by comparing the peaks of GMDP added to the prepared blank plasma samples after extraction with equivalent aqueous samples. Matrix related ion suppression or enhancement was evaluated by calculating the IS normalized matrix factor.

The stability of the working solutions of GMDP was evaluated at room temperature (20ºC) for 6 h. Plasma analyte stability was tested on LQC and HQC spiked plasma samples. The processed (extracted) sample stability was evaluated after keeping in the autosampler at 10ºC for 24 h. The freeze-thaw stability was conducted after three freeze-thaw cycles. Long-term stability was evaluated after storage at -70 ºC during the 30 days.

Results and discussion

Method development

During the development of the method, the conditions of the chromato-mass-spectrometric determination were optimized. GMDP is a hydrophilic compound and methanol was used as an organic component of the mobile phase to increase retention time. The gradient is also optimized to acquire precise compound retention time.

Electrospray ionization (ESI) showed to give a better response of analyte in positive ion mode. Following the ionization in ESI source, the GMDP and GHRP-2 (internal standard) formed ions with m/z 696.3 for GMDP ($[M+H]^+$) and 410.0 for GHRP-2 ($[M+2H]^{2+}$). During the GMPD fragmentation processes in collision cell characteristic fragments with m/z 475 and 458 are formed due to the loss of the n-Acetylglucosamine moiety and water. The spectrum is shown in Fig. 2 and the fragmentation pattern is shown in Fig. 3. MRM transitions, fragmentor voltage, collision energy (CE) applied to the analyte and internal standard are summarized in Table 1.

GMDP is a hydrophilic compound. In the solution GMDP is presented in a dynamic equilibrium of anomeric α- and β-isomers (Halls et al. 1980; Meshcheryakova et al. 2015), with an axial orientation of the C_1 muramic hydroxyl in the α-isomer and equatorial orientation in the β-isomer (Fig. 4). During reversed-phase chromatographic separation, α- and β-isomers form two peaks showed in Fig. 5. After the sample was dissolved in the diluent (water-methanol 90:10), it was kept for 150 min in an autosampler before analysis until a dynamic equilibrium between the anomers was reached. According to the research of Meshcheryakova et al. the equilibrium relationship between the forms depends mainly on the composition of the final diluent (Meshcheryakova et al. 2015). Validation of the method and quantitative determination was carried out by the sum of the peaks of the anomers.

The similarity of peak intensity ratio in calibration and test samples was proved by one-way analysis of variance (ANOVA) (Table 2). The results show the comparability of the ratios of the intensities of the peaks of the anomers in the calibration and test samples, which make it possible to estimate the total concentration of GMDP by the sum of the peaks.

Despite the great interest in muramyl peptides as biologically active endogenous compounds and using of GMDP as a medicine, the concentrations of the compound in the blood are unknown. The main goal of this work was to develop and validate a bioanalytical method for the quantification of GMDP in human plasma that can be applied to pharmacokinetic and bioequivalence studies. In animals, GMDP pharmacokinetics studies were performed using radioisotope-labeled compounds ([14C] GMDP) by liquid scintillation counting (Lyons et al. 2000). The detection limit was 1 ng / ml and the range of the calibration curve was 1-200 ng/ml. LC-MS method (single quadrupole analyzer) was used for the determination of plasma MDP concentration in rats after gavage and intravenous injection of MDP (Fosset et al. 2003). The limit of detection was 4 ng/ml and the limit of quantification was 13.3 ng/ml (Fosset et al. 2003). The use of tandem mass spectrometric detector and solid-phase extraction for sample preparation and concentration allowed reaching the limit of quantitative determination of 50 pg/ml, which is sufficient for studying the pharmacokinetics of GMDP.

Assay validation

Selectivity was checked by comparing the chromatograms of blank plasma with the chromatograms from the same plasma samples spiked with GMDP and GHRP-2. Fig 5. demonstrates the selectivity of the method, with chromatograms of blank human plasma, LLOQ sample, and plasma sample.

The linearity of the calibration curve was determined by plotting the peak-area ratio (y) of GMDP to GHRP-2 versus the ratio of their nominal concentrations (x). The calibration curve

was linear with the coefficient of correlation (r2) values more than 0.99. The R2 values, slopes, and intercepts were calculated using weighted (1 / x) linear regression analysis. The back-calculated concentrations for calibration standards were within 15 % of their nominal values. Calibration curve parameters for GMPD were: linear range – 50-3000 pg/ml, slope – 1.923 ± 0.027, intercept – 0.016 ± 0.012, r^2 – 0.9982 ± 0.0009 (mean ± SD).

The LLOQ for GMDP for this method was 50 pg/ml, which was sufficient for clinical pharmacokinetic study in humans following oral administration of a 2 mg of Licopid®. The responses for all analytes at the LLOQ were at least 5 times bigger than the response of that in the blank sample and the peaks of analytes were identifiable, reproducible, and discrete with an accuracy of 80-120 % and precision of 20 %.

The precision and accuracy of the GMDP quantification were determined by the analysis of several replicates of QC samples (n=6) at three concentrations on the same day and the three consecutive days. The data from these QC samples were calculated by ANOVA. Within and between-run variability data is summarized in Table 3. Accuracy for GMPD (% RE), given as deviation between nominal and measured concentration and assay variability (CV) ranged from 1.61 % to 3.02 % and from 0.89 % to 1.79 % respectively for both within- and between-runs. Results demonstrated that the values were within the acceptable range and the method was accurate and precise and was in line with the FDA guidance requirements.

GMDP is very hydrophilic and the concentration of GMPD in plasma is very low, so it is difficult to select the extraction conditions. For solid-phase extraction, we tried to use Oasis WCX and Oasis MAX (3cc 60 mg 60um, Waters, USA) cartridges, but we managed to achieve the maximum extraction recovery by Strata -X 33um Polymeric Reversed-Phase SPE cartridge after addition of phosphoric acid. The SPE extraction significantly improves the sensitivity of the method. Extraction recovery and ion suppression were determined

simultaneously and were calculated after normalization by IS. The results are shown in Table 4. Mean extraction recoveries for GMDP at 150 and 2700 pg/mL were 93.85 and 95.58 % (n=6). All recoveries had an RSD better than 9.8 %. Estimated percent nominal concentrations were within the acceptable limits (98.0–101.0%) after evaluating six different lots of plasma. Overall recovery was about 95.1 % for GMDP. Acceptable values of extraction recovery and matrix effect confirm the possibilities to use the GHRP-2 as an internal standard in this method. Observed effects were consistent and reproducible. The method showed good consistency throughout the entire concentration range.

Stability experiments demonstrated that no significant degradation of GMDP has occurred during chromatography, extraction, and storage processes for plasma samples. The results showed that GMDP was stable in working solutions for 6 h at room temperature at about 20ºC and in processed samples for 24 h in the autosampler at 10ºC. Repeated freeze and thawing (three cycles) and storage at –70 ºC during 30 days of plasma samples spiked with GMDP did not affect the concentration of GMDP in plasma samples. Stability data for GMDP are summarized in Table 5.

Application to a pharmacokinetic study

The validated HPLC-MS/MS method was used to obtain the pharmacokinetic profiles of analyte in human plasma after taking two tablets of Licopid® containing 1 mg of GMDP each. This dose is recommended for a single dose and will allow for accurate and reliable determination in the blood plasma of volunteers of GMDP concentrations necessary for assessing the complete pharmacokinetic profile of drugs. The dose chosen for research does not exceed the therapeutic dose. The pharmacokinetic data of GMDP have not yet been investigated, therefore, when determining the optimal number of volunteers and time points, they were guided by the parameters of similar drugs. Conducted pharmacokinetic studies in

healthy volunteers suggest the moderate variability of the drugs of the MP group. So, when studying the comparative pharmacokinetics of the drug Mepact (INN mifamurtide) in patients with impaired liver function and healthy volunteers, valid data were obtained on a sample of 37 volunteers at 24 hours after dosing (Venkatakrishnan et al. 2014). The pharmacokinetic parameters for muroctasine are described in a study of data from 35 healthy male volunteers after a single administration of 400 μg, Cmax was 6.93 ± 1.32 ng/ml after 1.32 ± 0.26 h after administration and $T_{1/2}$ was 27 ±0.8 h (Ichihara et al., 1988). In this study, 40 volunteers participated to obtain reliable pharmacokinetic parameters. The pharmacokinetic curve was constructed based on the changes in the concentration of GMDP at 17 points within 24 hours after administration.

The mean plasma concentration-time profile for GMDP is presented in Fig.6. The concentration of GMPD reaches a maximum of 1276.96± 375.56 pg/ml after 1.61 ± 0.48 hours after administration. $T_{1/2}$ is 11.10 ± 2.98 hours and $AUC_{0\rightarrow t}$ is 9969.21± 2940.73 pg×h/ml. The main pharmacokinetic parameters are shown in Table 6.

Low plasma concentrations of GMDP are consistent with data on the low bioavailability of GMDP in dogs and rats (Lyons et al. 2000; Fosset et al. 2003).

In healthy humans, the normal intestinal microflora can serve as a source of GMPD and the authors of a number of articles note the possible presence of GMDP in the blood plasma of a healthy person (Irazoki et al. 2019; Fosset et al. 2003). According to the results of the analysis of samples before taking the drug, we can conclude that the endogenous content of GMDP could be estimated as less than 50 pg/ml and has great interindividual variability. The concentration of GMDP after taking Licopid® significantly exceeds the endogenous level.

The limit of quantification using this technique is 50 pg/ml, which is sufficient to assess the pharmacokinetic parameters of the drug. If it is necessary to determine the endogenous levels,

the limit of quantification can be improved by using a larger amount of plasma or more sensitive detection.

Conclusions

A specific, rapid, and sensitive HPLC-MS/MS method for the determination of GMDP in human plasma has been developed. Simple sample preparation and short (8.0 min) sample analysis allow applying the proposed method for pharmacokinetic and bioequivalence studies. In our case, more than 100 samples were analyzed in one day. The method was successfully applied to determine GMDP plasma concentrations in a pharmacokinetic study involving healthy volunteers. After a single dose, 2 mg of GMDP concentration reaches a maximum of 1276.96± 375.56 pg/ml after 1.61 ± 0.48 hours after administration. $T_{1/2}$ is 11.10 ± 2.98 hours and $AUC_{0\rightarrow t}$ is 9969.21± 2940.73 pg×h/ml.

Author contributions

The author responsibilities-NEM participated in method development, validation, interpreted biological information, and wrote the manuscript. PAM and RMK performed sample LC-MS/MS analysis, performed statistical pharmacokinetics analyses, interpreted information. TMA participated in sample LC-MS/MS analysis, method development, and result interpretation. SAA conceived the main study and supervised the study. SAA has final responsibility for all parts of this research.

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Conflict of interest

TMA is affiliated with the “Peptek” company, the developer of the Licopid®. None of the other authors have a conflict of interest to declare.

Ethical approval

This research was approved by the Ethics Committee at the I.M Sechenov First Moscow State Medical University, Moscow, Russia. Written signed informed consent was obtained from each volunteer before entry into the study. The study was performed according to the ethical principles stated in the Declaration of Helsinki for medical research involving humans.

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Table 1. HPLC and MS parameters of GMDP

Analyte	Retention time (min)	Precursor (m/z)	Product (m/z)	Cone (V)	CE (V)
GMDP	1.87	696.3	475.0	20	20
		696.3	458.0	20	20
GHPR-2	3.58	410.0	170	30	30

Table 2. One-way ANOVA comparison of the anomeric peaks intensity ratio

Group	Mean α:β anomers intensity ratio	SD withing group	RSD (%)	SD Between group	F, (f test; 0.95; 60 <1.53)	t, (t test; 0.95; 60 <2.00)
Calibration standard	0.31	0.03	9.33	0.03	0.95	0.60
Samples	0.32	0.03	8.96			

Table 3. Within- and between-run precision and accuracy of GMDP quantification in human plasma

	Within-run analysis (n=6)			
Analyte	Spiked concentration (pg/mL)	Calculated concentration (Mean ± SD)	RSD (%)	Relative error (%)
GMDP	150	154.5 ± 3.43	2.22	3.02
	1500	1524 ± 20	1.34	1.61
	2700	2754 ± 13	0.46	2.02
	Between-run analysis (n=18)			
Analyte	Spiked concentration (pg/mL)	Calculated concentration (Mean ± SD)	RSD (%)	Relative error (%)
GMDP	150	152.7 ± 5.88	3.85	1.79
	1500	1513 ± 24	1.29	0.89
	2700	2738 ± 51.55	1.88	1.04

Table 4. Matrix effects and recovery for GMDP determination in human plasma (n=6)

Analyte	Concentration (pg/mL)	Ion suppression (%)		Extraction recovery (%)		Overall recovery (%)	
		Mean	CV	Mean	CV	Mean	CV
GMDP	150	98.0	3.41	93.8	3.09	101.2	9.8
	2700	101.0	1.03	95.6	1.61	103.1	2.5

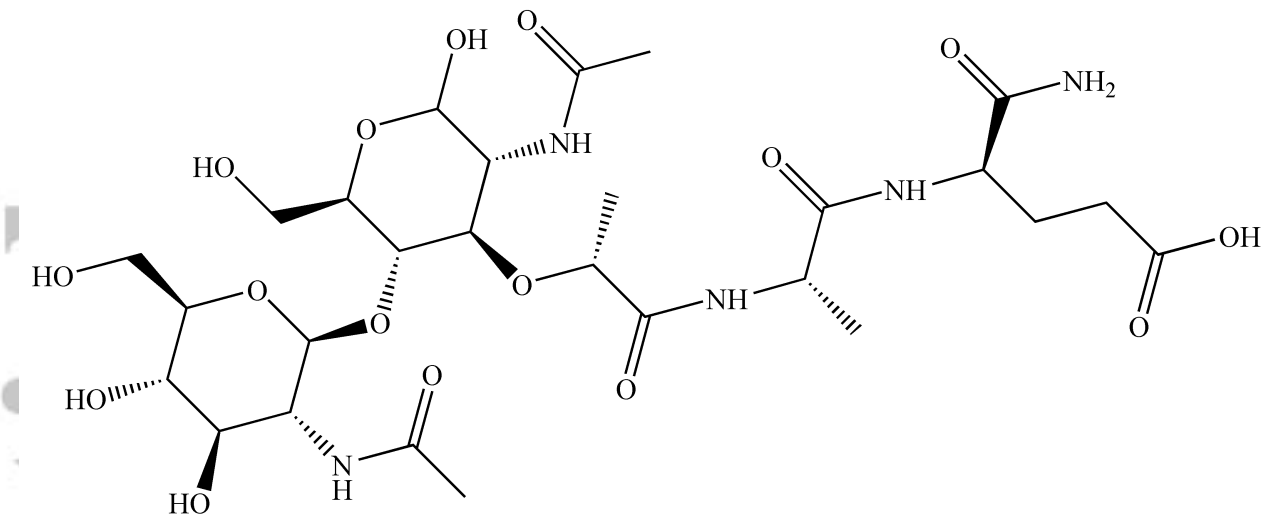
Table 5. Stability of GMDP in working solutions and human plasma

Working solution storage at room temperature for 6 h (n=6)						
Analyte	***Conc. (pg/mL)***	***Bias[a] (%)***	***CV (%)***	***Conc. (pg/mL)***	***Bias (%)***	***CV (%)***
GMDP	*150*	*10.69*	*1.73*	*2700*	*3.57*	*0.75*
Three freeze–thaw cycles (n=6), human plasma samples						
QC	***Conc. (pg/mL)***	***Bias[a] (%)***	***CV (%)***	***Conc. (pg/mL)***	***Bias (%)***	***CV (%)***
GMDP	*150*	*2.1*	*4.07*	*2700*	*1.42*	*1.14*
Long-term stability 30 days at -70 °C (n=6), human plasma samples						
QC	***Conc. (pg/mL)***	***Bias[a] (%)***	***CV (%)***	***Conc. (pg/mL)***	***Bias (%)***	***CV (%)***
GMDP	*150*	*5.4*	*7.21*	*2700*	*1.3*	*2.3*
Extracted samples for 24 h at 10 °C in the Autosampler (repeated analysis, n=6)						
Conc. (pg/mL)	***Conc. (pg/mL)***	***Conc. (pg/mL)***	***Conc. (pg/mL)***	***Conc. (pg/mL)***	***Bias (%)***	***CV (%)***
150	*150*	*10.28*	*1.48*	*2700*	*11.43*	*0.72*

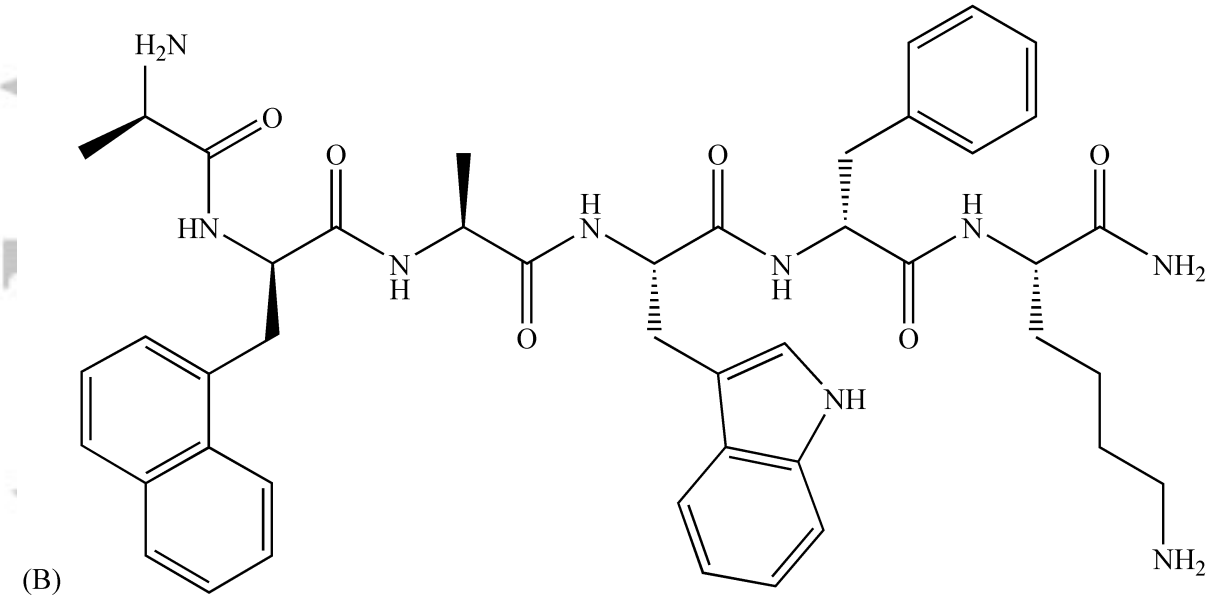
[a] *Bias means deviation of the mean from the mean of non-treated samples*

Table 6. Pharmacokinetic parameters of GMDP (data are Mean ± SD, n=40).

Parameter	***GMDP***
C_{max} *(pg/ml)*	**1276.96 ± 375.56**
T_{max} *(h)*	**1.61 ± 0.48**
$AUC_{0\rightarrow t}$ *(pg×h/ml)*	**9969.21 ± 2940.73**
$AUC_{0\rightarrow\infty}$ *(pg×h/ml)*	**12324.47 ± 3969.02**
K_{el} (h^{-1})	**0.0673 ± 0.0194**
MRT (h)	**13.86 ± 2.93**
$T_{1/2}$	**11.10 ± 2.98**
$C_{max}/AUC_{0\rightarrow t}$ (h^{-1})	**0.1286 ± 0.0118**
$C_{max}/AUC_{0\rightarrow\infty}$ (h^{-1})	**0.1050 ± 0.0122**



(A)



(B)

Figure 1. *Chemical structures of GMDP (A) and GHRP-2 (B).*

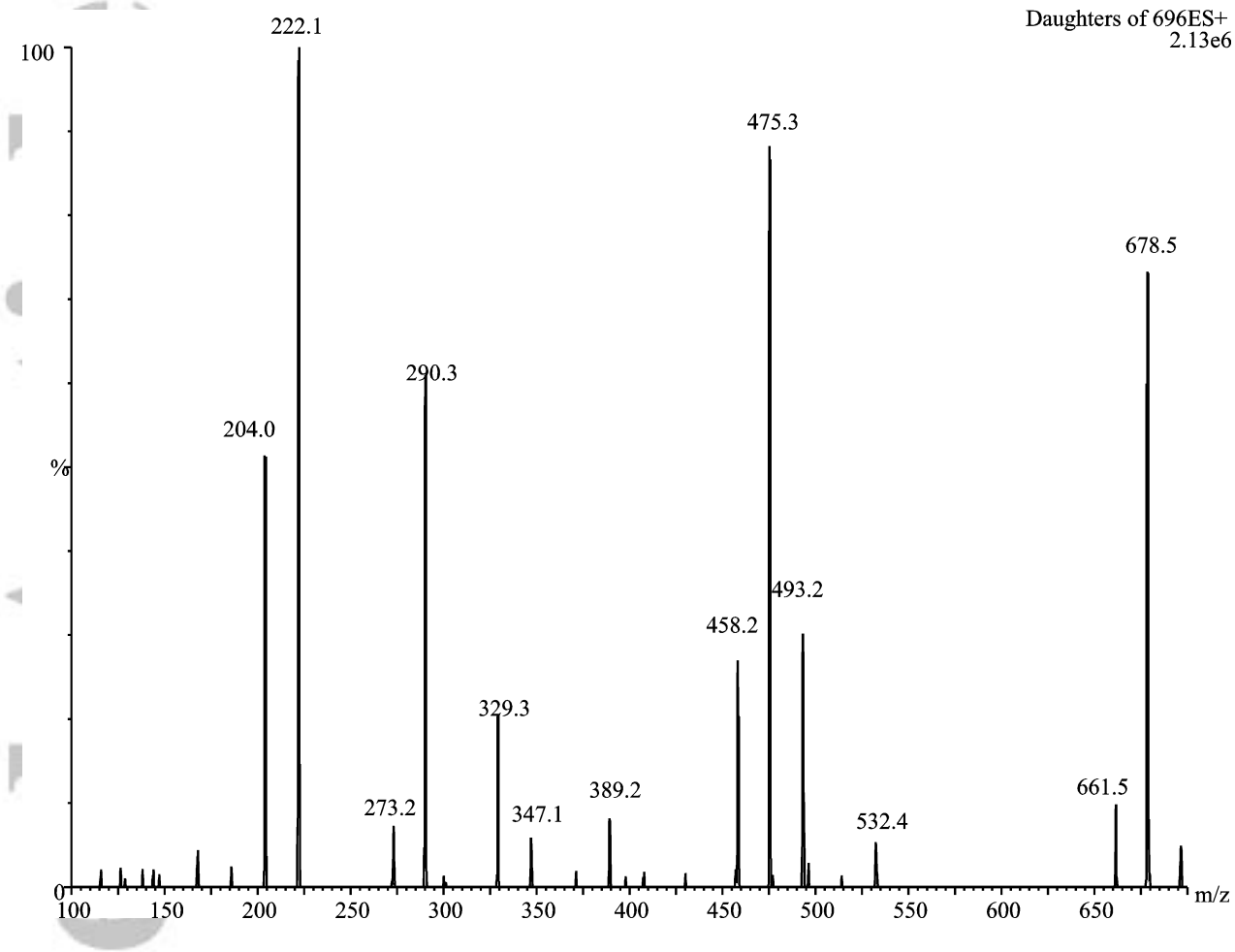


Figure 2. *Mass spectra of GMDP.*

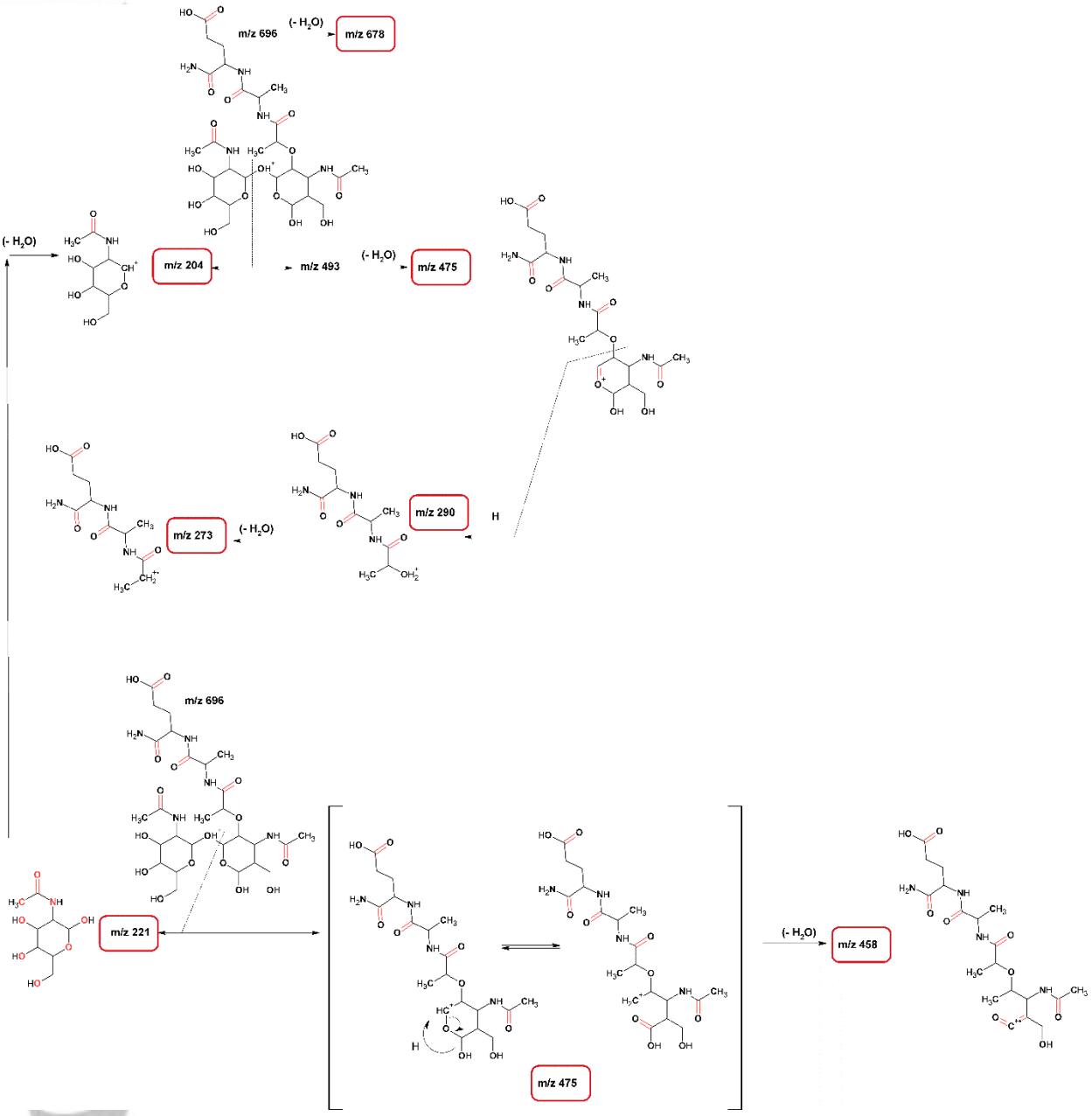


Figure 3. *GMDP fragmentation patterns.*

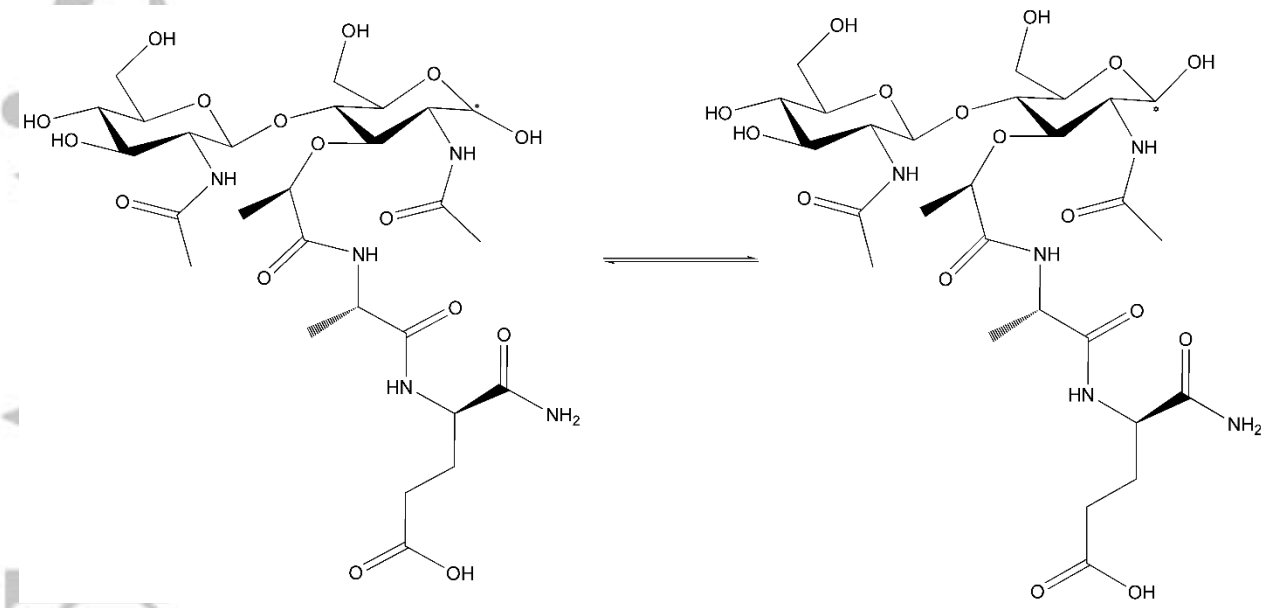


Figure 4. *Dynamic equilibrium of α- and β- GMDP anomers in solution.*

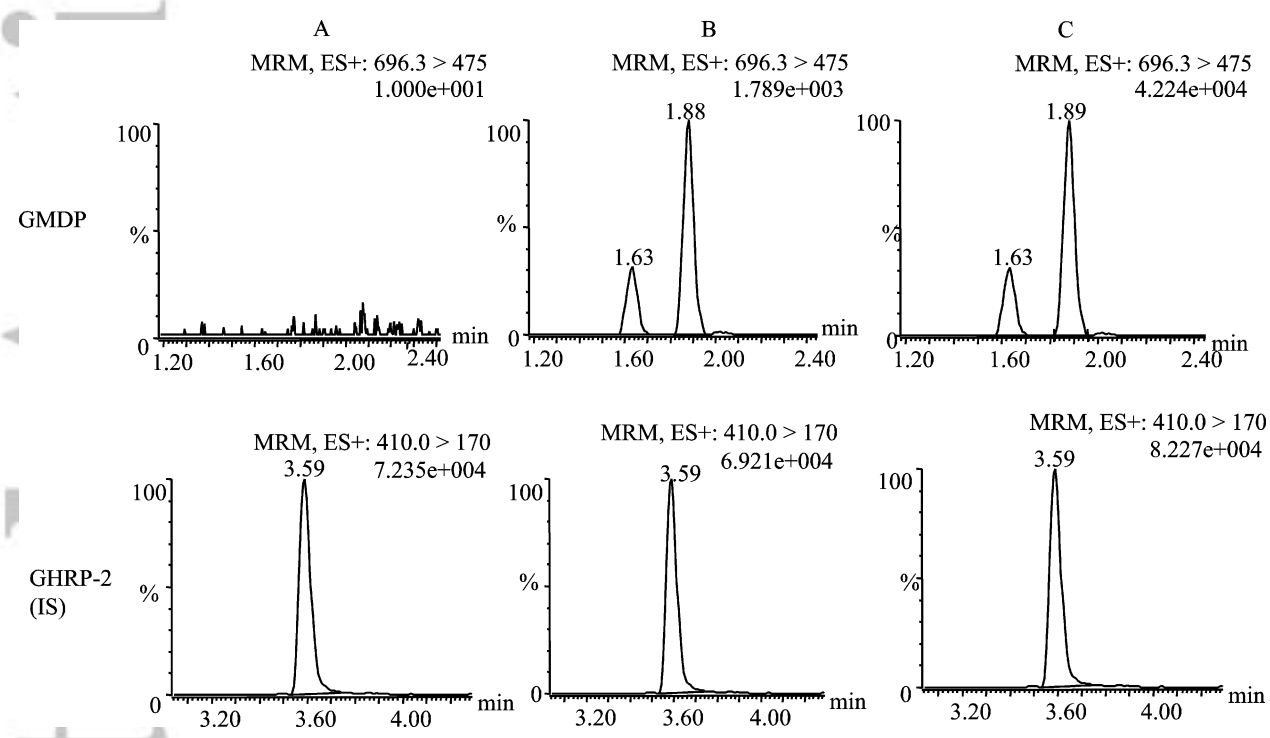


Figure 5. *Typical MRM chromatograms of GMDP and IS (a) blank plasma with IS, (b) the lowest calibration sample (LOQ), and (c) a plasma sample obtained from a patient after 2mg of Licopid®.*

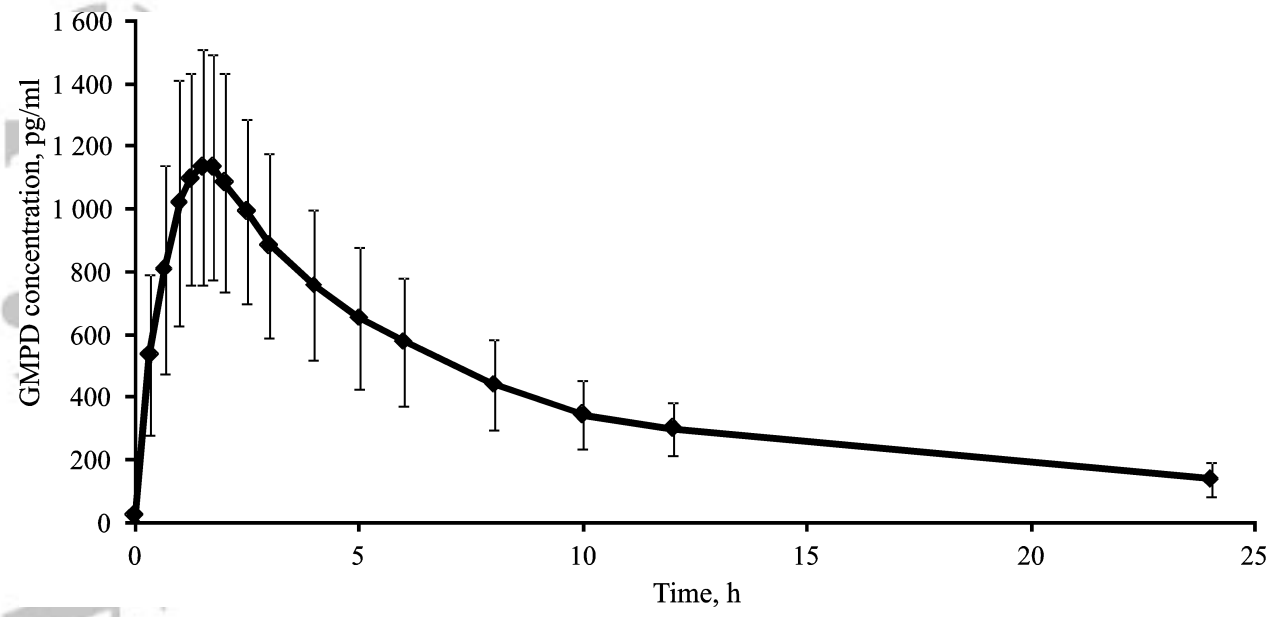


Figure 6. *Mean plasma concentration–time profiles of GMPD following oral administration of Licopid® (2 mg) to healthy volunteers (data are means ± SD, n=40).*