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Quantitative determination of methylnaltrexone in human serum using liquid chromatography–tandem mass spectrometry

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ABSTRACT

Methylnaltrexone (MNTX) is a novel peripherally acting μ-opioid antagonist that prevents peripheral side effects of opioid drugs such as constipation without affecting the analgesia. We developed a selective and sensitive assay to measure MTNX concentrations in human serum.

The drug was measured after protein precipitation with perchloric acid using naltrexone as internal standard and liquid chromatography–tandem mass spectrometry (LC–MS/MS) for detection. The chromatography was performed isocratically on a RP18 column using 25 mM ammonium acetate buffer (pH 4)/acetonitrile (90%/10%; flow rate 200 μl/min) as mobile phase. The MS/MS analysis was performed in positive ionization mode monitoring the m/z transitions 356.4/284.2 for MNTX and 342.4/324.2 for naltrexone.

The method was validated according to selectivity, linearity, accuracy, precision, recovery, matrix effects and stability. The validation range for MNTX in serum was 0.5–250 ng/ml. The developed LC–MS/MS was shown to be valid and successfully applied to measure serum-concentration–time curves of MNTX in a pilot study in healthy volunteers.

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1. Introduction

Opioid drugs are widely used in clinical practice as moderate to strong analgesics. Unfortunately, chronic treatment with opioid analgetics is frequently associated with several side effects including nausea, vomiting, constipation and urinary retention [1,2]. Methylnaltrexone (Fig. 1, MNTX) is a pure antagonist of μ-opioid receptors and approved for the prevention of opioid-induced constipation [3]. Due to its quaternary structure, MNTX is not capable to penetrate across the blood–brain-barrier. Thus, the drug exclusively antagonizes the unwanted gastrointestinal opioid effects without affecting the central analgetic effects [4–6]. In order to quantify serum concentrations of MNTX within a clinical study in healthy volunteers, a sensitive and specific bioanalytical method was needed. So far only a few methods have been published on this topic.

A method by Kim et al. quantified MNTX in rat serum and brain tissue after sample preparation using solid-phase extraction (SPE) and HPLC with coulometric electrochemical detection (LLOQ: 25 ng/ml) [7]. A modified version of the previously mentioned method to quantify MNTX in urine and plasma was published by Foss et al. (LLOQ: 100 ng/ml) [8]. However, both assays lack sufficient method validation data (e.g. accuracy, precision, stability and recovery).

Another quantification method was reported by Osinski et al. to quantify the drug in urine and plasma with a substantially lower LLOQ of 5 ng/ml [9]. In this study, sample preparation was also done by SPE using a primary weak cation-exchange retention and electrochemical detection. Very recently, two papers from the Pfizer laboratories described the use of liquid chromatography–mass spectrometry (LC–MS) and LC–MS/MS for the identification and quantification of MNTX and its major metabolites in biological samples from in vitro and in vivo studies [10,11]. However, these methods were not useful for our approach as they predominately focused on the identification of metabolic pathways and did not provide sufficient analytical details for a quantitative analysis of MNTX. Finally, Yu et al. published the first detailed LC–MS/MS method for the analysis of plasma samples [12]. Here, the biological samples were worked up by protein precipitation with acetonitrile and analyzed via MRM-based mass spectrometric detection with a LLOQ of 1 ng/ml. However, although it was stated by the authors that this assay was validated and found to be specific, accurate and precise, the applied validation program was somewhat spartan and does not fulfilled the current requirements of a bioanalytical method validation. In detail, no data on recovery, stability and matrix effects were provided. Especially the latter aspect is of main importance when using electrospray ionization based

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower limit of quantification; MNTX, methylnaltrexone; MRM, multiple reaction monitoring.

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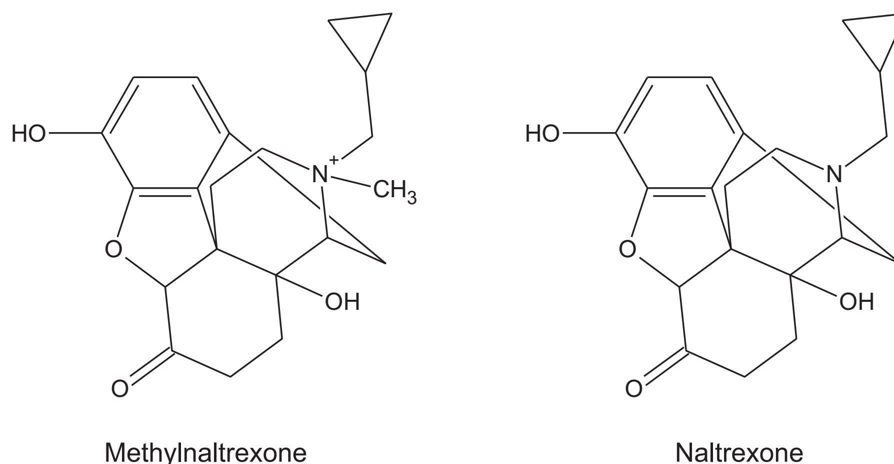

Fig. 1. Structural formulas of methylnaltrexone (left) and the internal standard naltrexone (right).

LC–MS/MS. Because no chromatographic retention was realized by this method, one has to assume considerable ion suppression by the complex biological matrix.

Under consideration of the mentioned methods we decided to develop a new and easy applicable LC–MS/MS method due to the following reasons: (1) electrochemical detection is not available in our and many other laboratories, (2) nowadays analysis by tandem-mass spectrometry is much more common and even more specific than coulometry, (3) sample preparation by SPE represents a time consuming and expensive technique, (4) given the published pharmacokinetic data of MNTX, the so far described LLOQ of 1–5 ng/ml were expected to be not sufficient to monitor the serum concentration–time profiles over 24 h after subcutaneous and oral single-dose administration of 12 mg methylnaltrexone, which was the aim of our current clinical study [4,9,12,13], and (5) the so far published LC–MS/MS method was expected to be not useful due to substantial deficits in method validation, lower sensitivity and no sufficient chromatographic retention of the analyte to prevent significant matrix effects. This paper describes the development and validation of this method according to the FDA guideline bioanalytical method validation [14] and its successful application in a clinical trial in humans.

2. Materials and methods

2.1. Reagents

Acetonitrile was purchased in LC–MS quality (Chromasolv®, Sigma–Aldrich, Taufkirchen, Germany). Deionized water (conductance: ≤0.055 μS/cm, pH 5.0–6.0) was generated with the system SG 2800 (S.G. Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel, Germany). MNTX was a kind gift from Develco Pharma GmbH (Schopfheim, Germany) and the internal standard naltrexone was purchased from U.S. Pharmacopeia (Basel, Switzerland). Ammonium acetate, ammonium hydroxide and perchloric acid were from Merck (Darmstadt, Germany). Stock solutions of MNTX and naltrexone were prepared in ammonium acetate buffer (25 mM, pH 4)/acetonitrile (1 + 1, v/v) and stored at −20 °C. Working solutions for both compounds were made weekly from stock solutions by adequate dilution and stored at 4 °C.

2.2. Sample preparation

Serum samples arrived at the laboratory deeply frozen at −20 °C. All sample preparations were carried out in one step and at room temperature. After thawing and shaking, 200 μl serum samples were mixed with 50 μl of the internal standard solution (naltrexone, 100 ng/ml). For protein precipitation 100 μl perchloric acid (10%, v/v) were added and the samples were mixed for 30 s and subsequently centrifuged for 5 min at 11,000 rpm. The supernatant was separated and mixed with 20 μl ammonia solution (30%, v/v) for neutralization. 100 μl of the resulting solution was transferred into sample vials of which 10 μl were injected into the chromatographic system.

2.3. LC–MS/MS analysis

The LC–MS/MS system consisted of the Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with the API4000 mass spectrometer equipped with the Analyst 1.4 software (AB Sciex, Darmstadt, Germany).

Chromatography was performed isocratically using 25 mM ammonium acetate buffer (pH 4.0) and acetonitrile (90%/10%) as mobile phase at a flow rate of 200 μl/min and the column XTerra® MS (C_{18}, 2.1 mm × 100 mm, Waters, Milford, USA). To avoid contamination by particles, the chromatographic flow was filtered through a 0.5 μm filter device (PEEK, Supelco, Bellefonte, USA). The mass spectrometer was equipped with the TurboIon® interface and operated in the positive ion mode monitoring the *m*/*z* transition 356.4–284.2 for MNTX and 342.4–324.2 for naltrexone. The optimized gas and MS parameters are given in Table 1. All chromatograms were evaluated with the validated Analyst 1.4 software using the internal standard method and peak-area ratios for calculation (linear regression, 1/*x* weighting).

2.4. Validation

The developed method was validated according to the current FDA guideline for bioanalytical method validation [14].

Selectivity of the LC–MS/MS method was confirmed by measuring and comparing in each case six blank human serum samples from different donors before and after the addition of MNTX and naltrexone, respectively.

Linearity of the method was studied by adding increasing amounts of MNTX to drug-free serum. Each calibration curve ($N = 6$) consisted of a double blank matrix sample without analyte and without internal standard, a blank matrix sample spiked with internal standard only and nine calibration values (0.5, 1, 2.5, 5, 10, 25, 50, 100 and 250 ng/ml). The validation range was 0.5–250 ng/ml.

For the evaluation of accuracy and precision, in each case six quality control (QC) sample sets consisting of blank serum samples spiked with 1.5, 50 and 250 ng/ml MNTX were prepared

Table 1
Gas- and mass spectrometry parameters for the determination of methylnaltrexone and naltrexone using the AB Sciex API4000 mass spectrometer. Nitrogen was used as nebulizer, curtain and collision gas (1 psi = 6894.8 Pa). CAD, collision-activated dissociation.

Gas parameters	MS/MS parameters	Methylnaltrexone	Naltrexone
Curtain gas: 20 psi	Q1/Q3 mass	356.4/284.2	342.4/324.2
CAD gas: 35 psi	Declustering potential (V)	80	70
Nebulizer gas: 60 psi	Entrance potential (V)	12	14
Heater gas: 70 psi	Collision energy (V)	35	30
Temperature: 450 °C	Collision cell exit potential (V)	20	25
Needle voltage: 5000 V	Dwell time (ms)	250	250

and analyzed. Between-day accuracy and precision was assessed by comparing the measured concentrations in QC samples (six separately prepared sets measured on different days) with the respective nominal concentrations, expressed as relative error (accuracy) and the respective coefficients of variation of the mean values (precision). Within-day accuracy and precision was determined by six-fold measuring of the respective QC samples on one day.

To investigate the loss of analyte during sample preparation (recovery), MNTX concentrations obtained from serum samples (each $N = 6$) after protein precipitation were compared with MNTX concentrations obtained from samples without preparation (diluted stock solution, 100% values).

To assess significant matrix effects in terms of ion suppression or enhancement in the ionization source, concentrations of MNTX measured in matrix-free samples (diluted stock solution) were compared with drug concentrations obtained from serum samples which were spiked with respective amounts of the analyte only after protein precipitation. Determination of recovery and matrix effects was performed on the low, middle and high validation ranges using the spiking concentrations of QC samples.

Stability of MNTX in serum samples was determined with respect to short-term, post-preparative, freeze–thaw, and long-term stability by using in each case six QC sample sets. Short-term (bench-top) stability was tested after storing the samples at room temperature for 4 h prior sample preparation and measurement. Post-preparative (rack) stability was assessed by storing the prepared sample extracts in the cooled autosampler (5 °C) for 24 h. To assess freeze-and-thaw stability, the samples were thawed and frozen up to three times prior analysis. For long-term stability check, serum samples spiked with low, middle and high concentrations of MNTX were measured before and after storage at −20 °C for 4 weeks. In each case, stability was assumed if the drug content after the given storage condition was within the acceptable range of accuracy, i.e. ±15%.

2.5. Measurement of biological samples

On each day of analysis, calibration curves were freshly prepared using blank human serum samples as mentioned above. QC samples represented at least 10% of all analytical samples and were measured during the entire analytical run. The criterion of acceptance for an analytical run was if at least 4 of 6 of all QC samples were within an accuracy range of ±15% of the nominal values as suggested by the respective FDA guideline.

2.6. Clinical study

The pharmacokinetic pilot study was performed according to current international and national regulations in three male healthy subjects (age 23–26 years; body mass index 20.3–24.9 kg/m^2) who gave informed written consent. The study was approved by the local ethical committee and the German Federal Institute for Drugs and Medical Devices (BfArM). On the pharmacokinetic study day, a single dose of 12 mg MNTX bromide (Relistor, Pfizer Pharma, Berlin, Germany) was administered subcutaneously. Venous blood (5 ml) was sampled from a forearm vein before and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 12, 16, and 24 h after drug administration. Serum was stored at −20 °C until quantitative analysis. For pharmacokinetic evaluation, maximum serum concentrations (C_{max}) were taken from the concentration–time curves and the area under the concentration–time curve (AUC) was calculated by the trapezoidal rule.

3. Results and discussion

3.1. LC–MS/MS analysis

Because stable-isotope labeled MNTX was commercially not available, naltrexone was used as the internal standard because of its close structural similarity and a distinct mass difference of 15 Da. In the positive ionization mode, both compounds generated higher signals for the protonated molecule peaks ($[M+H]^+$) than for the respective molecular ion produced by hydride abstraction ($[M-H]^-$) in the negative mode. Beyond the major peak from the protonated species of MNTX, a weak mass peak occurred at m/z 374.4 representing the ammonium adduct ($[M+NH_4]^+$) (data not shown). MNTX was substantially fragmented to the following product ions 302.1, 227.3, 338.2 and 284.2, the latter of which generated the highest signal intensity. The finally used mass to charge transitions, i.e. 356.4/284.2 for MNTX and 342.4/324.2 for naltrexone, were optimized in order to obtain maximum mass peak intensities (Table 1). The observed values are in good agreement with the literature [10,15]. Isocratic elution with the reversed phase column resulted in retention times of 3.9 min for MNTX and 4.5 min for the internal standard (Fig. 2). The substantial retention of the quaternary compound MNTX was surprising and obviously caused by the formation of a neutrally charged ion pair with acetate ions from the mobile phase which enabled interaction with the C18 material of the analytical column. This mechanism was also reported by Nunez et al. for the determination of quaternary biocides [16]. Because one analytical run could be finished within 6 min, our method enables a sample throughput of at least 200 samples per day.

3.2. Validation

The analytical method was shown to be selective for MNTX as concluded from the absence of analytical signals in different blank serum samples and no interferences between the analyte and the internal standard (Fig. 2).

There was a linear correlation between MNTX concentrations and the analytical signal for the entire validation range (0.5–250 ng/ml) observed. Mean correlation coefficient (r) of all calibration curves ($N = 6$) was 0.9985 ± 0.0014. The LLOQ of our analytical method was 0.5 ng/ml. Here, the analytical signal was at least >5 times above the signal of blank serum samples as requested by current bioanalytical guidelines.

Within-day as well as between-day accuracy was 97.5–108.4% of the nominal concentrations, whereas within-day and

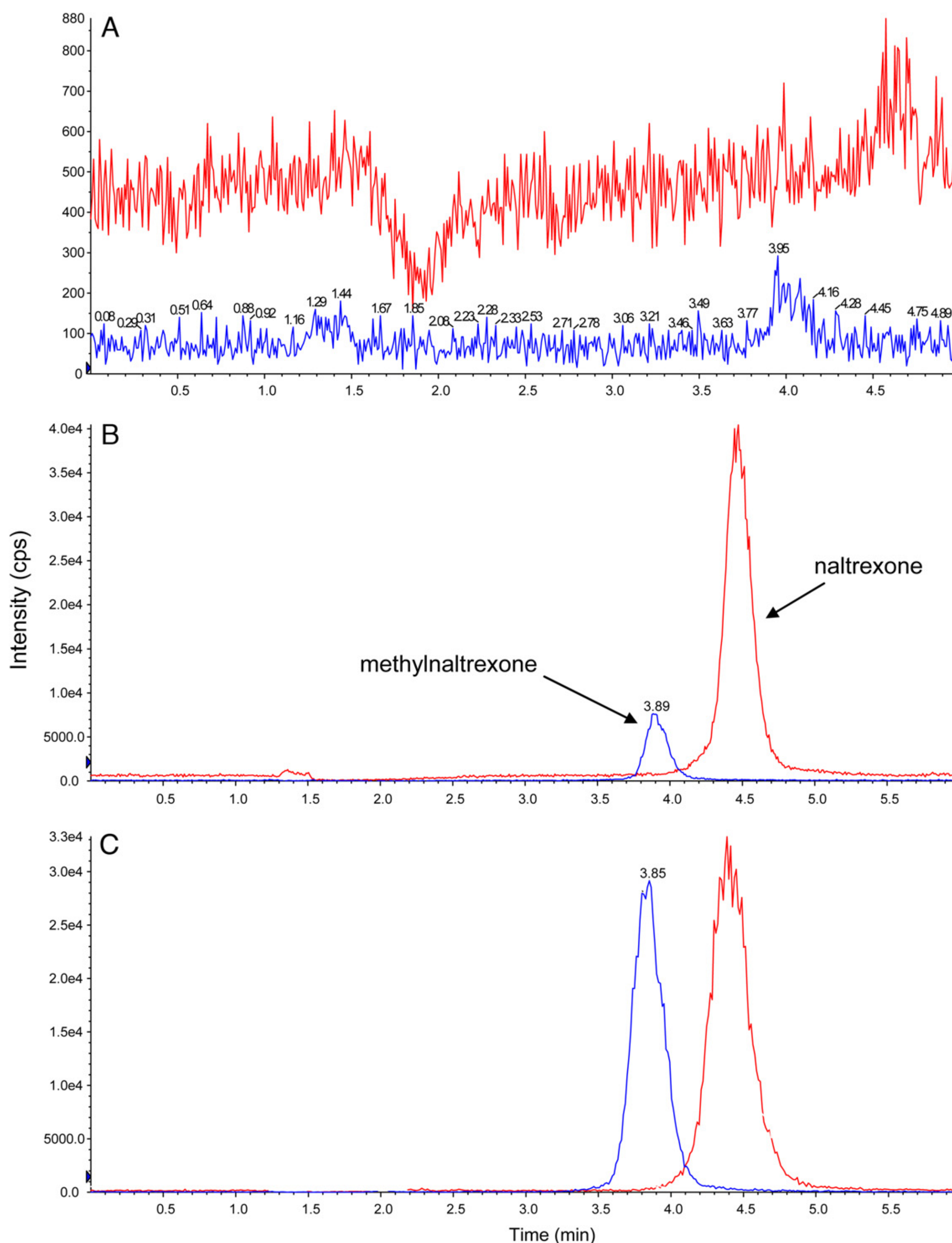

Fig. 2. Total ion chromatograms of a blank serum sample (A), of a serum calibrator spiked with 5 ng/ml methylnaltrexone and the internal standard naltrexone (B), and a serum sample taken 1 h after subcutaneous administration of 12 mg methylnaltrexone from of a healthy volunteer (C). All chromatograms were obtained by monitoring the m/z transitions 356.4/284.2 for methylnaltrexone and 342.4/324.2 for naltrexone.

Table 2
Within- and between-day accuracy and precision for the determination of methylnaltrexone in human serum. Data are given as relative error (accuracy) or coefficients of variation (precision) of nominal and respective mean concentrations.

Concentration	n	Within-day data			between-day data		
		Mean ± SD	Precision (%)	Accuracy (%)	Mean ± SD	Precision (%)	Accuracy (%)
Q1 (1.5 ng/ml)	6	1.63 ± 0.09	5.4	8.4	1.49 ± 0.06	4.1	−0.4
Q2 (50 ng/ml)	6	50.6 ± 1.23	2.4	1.2	46.6 ± 6.12	7.4	−2.5
Q3 (250 ng/ml)	6	239 ± 5.87	2.5	4.4	259 ± 15.8	6.1	3.4

Table 3
Validation data for the determination of methylnaltrexone in human serum. Mean values for matrix effects, recovery, short-term stability (4 h, room temperature), rack stability (24 h, 5 °C), freeze–thaw stability (−20 °C) and long-term stability (4 weeks, −20 °C) are given in percent compared to initial (stability) or matrix-free samples (matrix effects, recovery).

Validation parameter	*n*	Q1 (1.5 ng/ml)	Q2 (50 ng/ml)	Q3 (250 ng/ml)
Matrix effect (%)	6	102.9 ± 3.2	95.1 ± 3.3	90.6 ± 4.5
Recovery (%)	6	99.3 ± 3.6	94.6 ± 7.2	92.8 ± 5.6
Short term stability (%)	6	105.2 ± 5.8	101.0 ± 2.9	99.7 ± 3.9
Rack stability (%)	6	100.9 ± 8.3	106.5 ± 3.2	105.0 ± 3.58
Freeze–thaw stability (%)				
1st cycle	6	96.9 ± 8.6	106.7 ± 2.8	108.0 ± 6.6
2nd cycle	6	96.1 ± 10.1	111.1 ± 5.4	108.4 ± 3.8
3rd cycle	6	95.5 ± 6.0	99.8 ± 4.5	98.0 ± 3.1
Long term stability (%)	6	89.5 ± 6.4	105.7 ± 2.2	111.9 ± 4.5

Table 4
Comparison of so far available methods for the quantification of methylnaltrexone in human plasma (Foss et al., Osinski et al., Yu et al.) and serum (Kim et al., Oswald et al.). HPLC, high performance liquid chromatography; LC–MS/MS, HPLC coupled with tandem-mass spectrometry; LLOQ, lower limit of quantification; PFP, pentafluorophenyl, SPE, solid phase extraction.

	Kim et al. [7]	Foss et al. [8]	Osinski et al. [9]	Yu et al. [10]	This study
Sample preparation	SPE	SPE	SPE	Protein precipitation	Protein precipitation
Analytical principle	HPLC with electro-chemical detection	HPLC with electro-chemical detection	HPLC with electro-chemical detection	LC–MS/MS	LC–MS/MS
Chromatography	Isocratic, C_{18} column	Isocratic, C_8 column	Isocratic, C_{18} column	Isocratic, PFP propyl column	Isocratic, C_{18} column
Internal standard	Methylnaloxone	Methylnaloxone	Naltrexone	Ketamine	Naltrexone
Run time	15 min	–	15 min	2 min	6.0 min
Elution time	9.4 min	–	9 min	1 min	3.9 min
LLOQ	25 ng/ml	100 ng/ml	5 ng/ml	1 ng/ml	0.5 ng/ml
Validation data	Linearity, precision, recovery	None	Linearity, accuracy, precision, recovery	Linearity, accuracy, precision	Linearity, accuracy, precision, recovery, stability, matrix effects

between-day precision ranged from 2.4 to 7.4% for the respective coefficients of variation of the observed mean values (Table 2).

Although the used protein precipitation with perchloric acid represents no classical sample extraction as liquid–liquid or solid-phase extraction, loss of analyte may have occurred by protein binding. Thus, the recovery of the analyte was studied. MNTX containing serum samples can be reliably processed with protein precipitation as the recovery ranged from 92.3 to 99.3% among the entire validation range (Table 3). This is in line with the low binding of MNTX to human plasma proteins (<10–15%) [13].

Matrix effects were found to be negligible in our assay because the observed concentrations of MNTX in serum samples differed only by 2.9–9.4% compared to matrix-free samples of the same concentrations (Table 3). The considerable retention of the analyte and its internal standard most likely contributed to this desirable lack of ion suppression or enhancement.

The comprehensive analysis of MNTX stability was necessary because this information is totally missing from the so far published analytical methods (Table 3). Our experiments clearly indicated that MNTX is stable in serum at room temperature for at least 4 h (99.7–105.2%). The prepared samples were also shown to be stable for at least 24 h when stored at 5 °C in the autosampler (100.9–106.5%). The results for freeze–thaw stability are given in Table 3 and indicate that MNTX may undergo up to three freeze–thaw cycles without any significant influence on its stability. Serum samples can be stored at −20 °C for at least four weeks because the analyte was shown to be stable over this time.

3.3. Application of the method

The validated analytical assay enabled the quantitative determination of MNTX in serum samples from a pilot study in three healthy volunteers. The mean serum concentration–time profile for all volunteers after subcutaneous administration of 12 mg MNTX is shown in Fig. 3. A single dose of MNTX caused a fast appearance of the drug in the systemic circulation with mean serum peak concentrations of 111 ± 9 ng/ml reached after 20 ± 8.6 min and which generated an average AUC of 166 ± 19.6 ng h/ml. The increased sensitivity of our method enabled accurate quantitative determination of MNTX over 24 h. With the previously published methods (LLOQ: 1–5 ng/ml) only up to 4–12 h could have been monitored. Thus, a substantial faction of the serum AUC (~10%) would have been missed after subcutaneous administration. After oral administration of MNTX, the serum concentrations were shown to be considerably lower [5,17]. Consequently, the higher sensitivity appears to be an essential prerequisite to characterize the serum concentrations of MNTX after oral absorption, which was also the aim of our current pharmacokinetic study. In our pilot study,

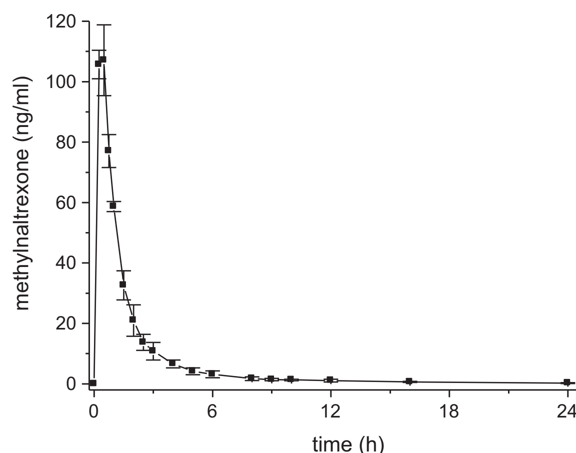

Fig. 3. Mean serum concentration–time profile of three healthy volunteer after subcutaneous administration of 12 mg methylnaltrexone (mean ± SD are given).

the measured mean serum concentrations over 24 h after oral administration of 12 mg MNTX as immediate and extended release dosage form ranged between 1.66–29.0 ng/ml and 0.41–6.32 ng/ml, respectively.

Although unchanged MNTX was shown to be the major drug-related compound in human plasma, one has to consider that MNTX was also shown to undergo a considerable metabolism in humans. In this context, sulfation to M2, and reduction to M4 and M5 was reported accounting for a drug exposure in plasma for up to 25%, 12% and 7%, respectively, 4 h postdose in healthy volunteers [10,11].

4. Conclusions

The developed method was shown to be specific, sensitive, precise and accurate for the quantification of MNTX in human serum. The method validation indicated stability of the analyte and good linearity over a wide concentration range. Sample preparation using protein precipitation with perchloric acid did not affect recovery of the analyte and caused no detectable matrix effects. The developed LC–MS/MS method was successfully applied to monitor serum-concentration–time profiles of MNTX over 24 h after oral and subcutaneous administration of a low dose of 12 mg in a pilot study in healthy volunteers. The investigated 12 mg (∼0.15 mg/kg) dose administered as subcutaneous injection represents the labeled standard dose to treat an adult (62–114 kg). There are so far no published pharmacokinetic data referring this dose. However, the study by Osinski et al. investigated the 0.1 mg/kg and 0.3 mg/kg dose after subcutaneous administration in 12 healthy volunteers. The C_{max} in this study was 110 ± 55 ng/ml reached after 17 min for the 0.1 mg/kg dose and 287 ± 101 ng/ml reached after 20 min for the 0.3 mg/kg dose, which fits quite well to our data.

The developed method represents an improvement compared to the so far published procedures due to the following reasons (Table 4): (1) sample preparation was done by fast and cheap protein precipitation instead of SPE, (2) mass spectrometric detection increased the selectivity compared to electrochemical detection, (3) the method features the so far highest sensitivity with a LLOQ of 0.5 ng/ml, (4) the run time of 6 min enables a high sample throughput but prevents significant matrix effects because of a sufficient chromatographic retention (elution time ∼4 min), and (5) in contrast to all other published methods, our method was comprehensively validated according to the current FDA guideline for bioanalytical method validation.

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