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Determination of methylnaltrexone in clinical samples by solid-phase extraction and high-performance liquid chromatography for a pharmacokinetics study

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Abstract

A high-performance liquid chromatographic (HPLC) method with electrochemical detection and solid-phase extraction (SPE) using cartridges of weak cation-exchange capacity as the primary retention mechanism is described for the separation and determination of methylnaltrexone (MNTX) in small clinical samples of plasma or urine. The procedure was performed using a Phenomenex Prodigy ODS-2, 5 μm, 150×3.2 mm analytical column and 50 m*M* potassium acetate buffer, with 11% methanol as organic modifier at pH* 4.5 at a flow-rate of 0.5 ml/min. The detection potential was 700 mV. The six-point standard calibration curves were linear over three consecutive days in the range from 2 to 100 ng/ml. The average goodness of fit (*r*) was 0.9993. The lower limit of detection (LOD) and limit of quantification (LOQ) were found to be 2.0 and 5.0 ng/ml, respectively. At the LOQ, the coefficient of variation for the entire method was 8.0% and the accuracy was 10.0% ($n=10$). Recovery of the drug from plasma was in the region of 94%. The method was applied to a pharmacokinetics study of methylnaltrexone after subcutaneous administration and in numerous assays of analytes in blood plasma and urine. The pharmacokinetics parameters for a single dose of 0.1 or 0.3 mg/kg in plasma were $C_{max}=110$ (±55) and 287 (±101) ng/ml and $t_{max}=16.7$ (±10.8) and 20.0 (±9.5) min, respectively. The method is simple, yet sensitive for the detection and determination of methylnaltrexone in biological samples at the level of the physiological response.
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Keywords: Electrochemical detection; Methylnaltrexone; Naltrexone

1. Introduction

Opioid compounds are widely used as clinical analgesics. Unfortunately, their administration is often accompanied by side-effects, such as constipation, urinary retention, pruritus, nausea, and vomiting. These side-effects are often severe enough to limit the use of opiates even when medically indicated [1–3]. In the last two decades, significant progress has been achieved in understanding the mechanism of action of opioids [4]. These advances, however, have only yielded a few new approaches to the management of the side-effects of opioids.

Methylnaltrexone (MNTX), or *N*-methylnaltrex-

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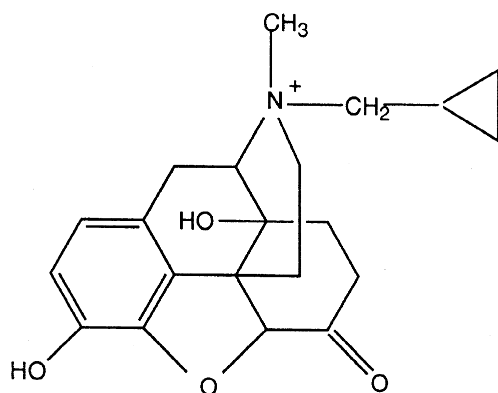

Fig. 1. Structure of methylnaltrexone.

one bromide, or naltrexone methobromide (Fig. 1) is a unique opioid antagonist [5]. It is a quaternary derivative of the pure opioid antagonist naltrexone. As tertiary compounds, opioid antagonists, such as naloxone, naltrexone and nalmefene, are fairly lipid soluble and readily cross the blood–brain barrier. Quaternary antagonists, on the other hand, having the general structure of a cationic detergent, are compounds of greater polarity and lower lipid solubility. Therefore, methylnaltrexone does not cross the human blood–brain barrier [5,6]. These properties provide methylnaltrexone with the potential to block the undesired side-effects of opioid pain medications predominantly mediated by receptors located peripherally (e.g., in the gastrointestinal tract) while sparing opioid effects mediated at receptors in the central nervous system, most importantly analgesia [7,8].

Kim et al. [9] analyzed methylnaltrexone in rat brain regions and serum by HPLC with coulometric electrochemical detection. The method involved CN solid-phase extraction and separation on an Ultrasphere ODS HPLC column. However, the work lacked the sensitivity required to study the effects of MNTX at a lower level of physiological response. An improvement of an order of magnitude or more was needed for plasma samples. There was another limitation of the method, i.e. it did not include a procedure for extraction of the analyte from the urine matrix. Urine is an important route of elimination of the drug from the organism and this deficiency of the method had to be overcome. Foss et al. [10] determined levels of methylnaltrexone in plasma and urine by HPLC using a modified version of the method described by Kim et al. [9]. The extraction process was completed on a phenyl solid-phase extraction column using the internal standard quantification technique. The lower limit of sensitivity was up to 100 ng/ml. This modification, which comprised both plasma and urine matrices, was far too insufficient in terms of sensitivity. An improvement of two orders of magnitude was necessary.

When studying the in vivo behavior of methylnaltrexone, very often at the lower level of physiological response, we had to improve the sensitivity of the analysis by refining the method of extraction, increasing the accuracy, lowering the variability of the assay and addressing environmental issues (reduction of generated organic wastes). We have employed this method in our animal and human studies [11–21].

In this paper, we present a method for the separation and determination of methylnaltrexone by applying a solid-phase extraction technique with a primary weak cation-exchange retention mechanism. The chromatography was performed on an ODS-2 reversed-phase column with thermal peak shape correction. The mobile phase is simple, easy to prepare and recyclable, thus helping to save chemicals and reduce waste. With the high-quality components of the mobile phase, the well-adjusted potentials of the electrodes and continuous electrocleansing of the mobile phase, the detector produced low currents, thus improving peak detection and quantification. Quantification was performed using an internal standard technique with naltrexone as standard. This simple, yet sensitive and reliable, method was applied to the analysis of MNTX in diverse biological matrices, specifically blood plasma and urine.

2. Experimental

2.1. Instrumentation

The Shimadzu Liquid Chromatography System (Shimadzu, Kyoto, Japan) was comprised of a LC-10AD pump, a SIL-10A automatic sample injector with the sample cooler set to 4 °C, a CTO-10A column oven and a SCL-10A VP system controller. The Shimadzu Class-VP Chromatography Laboratory Automated Software System, Version 5.03, with a Micron Electronics (Nampa, ID, USA) Micron Client

Pro computer was employed for system operation, data collection and reprocessing. Chromatography was performed on a Phenomenex Prodigy ODS-2, 5 μm, 150×3.2 mm analytical column protected by a Phenomenex ODS-2, ODS-2, 4×3 mm, guard column (Phenomenex, Torrance, CA, USA). We used a Model 5100A coulochem detector with a Model 5020 guard cell and a Model 5011 high-sensitivity analytical cell, containing a large surface area coulometric electrode and a high-efficiency amperometric electrode made from porous graphite [22]. All electrochemical equipment was from ESA (Chelmsford, MA, USA). For solid-phase extraction, we used a vacuum manifold from Burdick and Jackson (Muskegon, MI, USA). For pipetting, we used electronic motorized microliter pipettes from Rainin Instruments (Wolburn, MA, USA).

2.2. Reagents and procedures

Drug purity grade crystalline methylnaltrexone bromide was obtained from Mallinckrodt (St. Louis, MO, USA). Naltrexone hydrochloride, trifluoroacetic acid, acetic acid, and potassium hydroxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Potassium phosphate (MicroSelect) was obtained from Fluka (Ronkonkoma, NY, USA). Methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). SPE cartridges BondElut CBA were purchased from Varian (Harbor City, CA, USA). Propanol was procured from EM Science (Gibbstown, NJ, USA). Water was purified and deionized in house using a PURELAB Plus UV/UF system from US Filter (USA). Gasses (helium, nitrogen) were requisitioned from local sources. Syringe filters, Millex-LCR, were purchased from Millipore (Bedford, MA, USA) and membrane filters, Nylaflo, were purchased from Pall Gelman Sciences (Ann Arbor, MI, USA). Other chemicals were of reagent grade purity.

The mobile phase was prepared from the highest purity reagents available in order to obtain low currents. Purified, deionized and UV-treated water was filtered through a 0.22 μm nylon membrane filter, which reduced the iron concentration by removing hydrolyzed, non-ionic iron [22]. The potassium acetate concentrate solution was filtered separately through a nylon membrane. The solution of buffer, water and methanol (PTFE filtered) was mixed in a volumetric flask and filled to the line with water. The potassium acetate concentrate solution was 500 m*M* and the pH was adjusted to 4.1. The final composition of the mobile phase was 50 m*M* potassium acetate, 11% methanol and the hydrogen ion concentration was adjusted after addition of organic modifier to pH* 4.5. Prior to use, the mobile phase was degassed by helium sparging for 5 min and used for about 1 week with continuous recycling and electrochemical cleansing.

Stock solutions of methylnaltrexone bromide and naltrexone hydrochloride were prepared by accurately weighing and dissolving the compounds in deionized water to obtain a concentration of 1 mg/ml. The solution was stored in Eppendorf vials at −80 °C until needed. Appropriate concentrations of calibrators containing internal standard were prepared by diluting the stock solutions with mobile phase. All calibrators were filtered through 0.45 μm hydrophilic LCR (PTFE) syringe filters (Millex LCR) before injection.

2.3. Collection and preparation of clinical samples

With approval from the Institutional Review Board at the University of Chicago, eight males and four non-pregnant female volunteers were enrolled in this clinical trial. Subjects were screened for drug abuse disorders or medical contraindications that would keep them from participating in the study. In addition, they underwent a physical examination, received a resting electrocardiogram and laboratory blood and urine tests. Each participant completed a health history questionnaire.

In each session, an intravenous catheter was placed for administration of morphine and for drawing blood. At the onset of the session, subjects received a subcutaneous injection of methylnaltrexone or placebo (saline) into the inner thigh. Methylnaltrexone was administered at doses of 0.1 or 0.3 mg/kg. After 15 min, intravenous morphine or placebo (saline) was administered over 1 min. Vital signs were monitored after injections. The sessions lasted approximately 7 h.

Venous blood samples were drawn for plasma drug level determination at 0, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240 and 360 min. The samples were centrifuged at 1430 *g* and plasma was transferred to polypropylene tubes. Urine samples were

collected at time 0, and at intervals of 0–3 and 3–6 h. All samples were immediately chilled on ice and frozen (−20 °C). For prolonged storage, samples were kept at −80 °C.

2.4. Solid-phase extraction

Solid-phase extraction was performed using columns with a primary weak cation-exchange mechanism [23]. The calibration standard mixtures contained 100 μl plasma or urine obtained at time 0, MNTX standard solution at volumes to obtain a series of concentrations, 50 μl of internal standard (100 μl in the case of urine) and water up to 1 ml. The sample extraction mixtures consisted of 100 μl plasma or urine, internal standard and water up to 1 ml. The blanks were mixtures of water and an appropriate matrix. Extraction solutions were prepared in glass tubes.

Solid-phase extraction was performed using a 24-port extraction manifold. The SPE columns (Varian, CBA columns, 100 mg) were conditioned with 1 ml methanol and 1 ml 10 m*M* potassium phosphate buffer at pH 6.8. The extraction mixtures were drawn through the columns slowly. The columns were dried for 1 min and washed using 2 ml aliquots of 10 m*M* potassium phosphate buffer at pH 6.8 or D.I. water, respectively, for plasma and urine. The columns were dried under full vacuum for 2 min. The analytes were eluted with 2 ml of a freshly prepared eluting mixture of *n*-propanol–25 m*M* trifluoroacetic acid (2:1). The eluants were collected into new glass tubes, and evaporated under a gentle stream of nitrogen at about 55 °C. Dry residues were reconstituted in 1 ml of mobile phase by vortexing for 2×15 s at setting 8 under vacuum. Samples were filtered using Millipore Millex-LCR syringe filters and kept in the automatic sample injector at 4 °C until injected. Aliquots of 50 μl were injected into the HPLC column.

3. Results and discussion

3.1. Peak distortion

During method development and early use, severe peak fronting of methylnaltrexone, as well as naltrexone, was present. This peak distortion presented a major obstacle for a proper evaluation of the chromatograms, for example it influenced the selectivity, accuracy and precision of the assay. Examination of published chromatograms of MNTX and methylnaloxone [9] revealed a similar phenomenon, although not so acute. We hypothesized that an anti-Langmuir adsorption isotherm governs the chromatographic process or a chemical reaction is taking place on the column [24–26]. Elevation of the column temperature to 50 °C produced symmetrical peaks for both analytes. The peak shape change as a function of temperature is presented in Fig. 2. The C_{18} ODS-2 column (Phenomenex, Prodigy) was placed in the column oven and heated. The mobile phase consisted of 11% methanol in an aqueous solution of 50 m*M* potassium acetate buffer at pH* 4.5. The clearly visible fronting subsided at 40 °C. At 50 °C, fronting was not observed. The retention time changed. There are additional peaks observed to the left of MNTX present in all chromatograms, which are artifacts.

Recently, Brogle et al. [27] presented an explanation for this phenomenon in the case of oxycodone hydrochloride. It was established that water and methanol present in the mobile phase interact with the ketone group at C-6, resulting in the formation of a gem-diol and a hemiketal. These substances tend to exist only in aqueous solution in equilibrium with oxycodone and are not degradation products. Elevating the temperature to 60 °C eliminated the fronting, lead to a good peak shape, and produced two additional adduct bands. Methylnaltrexone, naltrexone and methylnaloxone are similar to oxycodone and contain a C-6 ketone group. We hypothesize that, in a mobile phase of water and methanol, similar species are formed. Comparing the peaks for methylnaltrexone and methylnaloxone published by Kim et al. [9] and our data in Fig. 2 indicates that the peaks of Kim et al. are less skewed than ours. This difference may be attributed to the partial use of ACN (10%) as an organic modifier. Methanol was used only at 5% concentration. In contrast to Brogle et al. [27], no evidence of adduct formation was observed in our experiments. This again might be explained by differences in the injected mass. We injected 5 and 2.5 ng of MNTX and NTX, respectively, compared with 4 μg of oxycodone (our calculation, based on Brogle's experimental con-

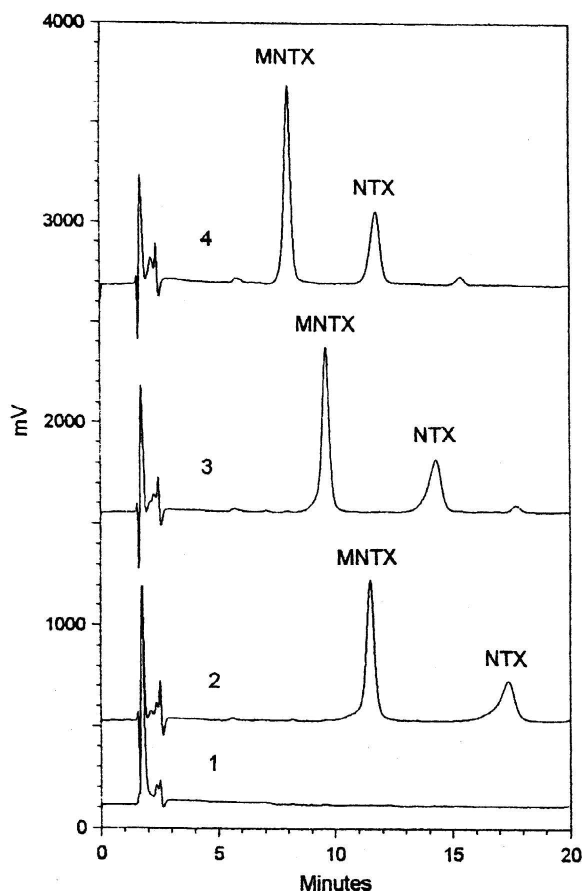

Fig. 2. Thermal peak shape (fronting) correction of methylnaltrexone and naltrexone at concentrations of 100 and 50 ng/ml, respectively. Experimental conditions: column, Phenomenex Prodigy, ODS-2, 5 μm, 150×3.2 mm; guard column, Phenomenex Prodigy, ODS-2, 5 μm, 4×3 mm; buffer, 50 m*M* potassium acetate, 11% methanol, pH 4.5*; flow-rate, 0.5 ml/min; concentration of MNTX and NTX, 100 and 50 ng/ml, respectively; potentials, electrode 1 350 mV, electrode 2 700 mV, guard cell 750 mV; gain, 1500. The chromatograms were run at temperatures of (1,2) 30, (3) 40, and (4) 50 °C.

ditions). Thus, the concentration of adducts may be below our detection limit. This phenomenon of adduct formation at the ketone group might be of much broader significance in HPLC.

3.2. Hydrodynamic voltammograms

Electrochemical detection, one of the most sensitive in separation science, needs careful selection of the working potentials. The hydrodynamic voltammograms of methylnaltrexone and naltrexone are presented in Fig. 3. It can be seen that the electrochemical behaviors of the analyte and internal standard are similar, thus fulfilling the requirement of proper selection of the internal standard. Both substances are detected by an oxidation reaction occurring at the phenol group. There is no observable detection response at potentials lower than 350 mV. The detected peak currents increase sharply when the potential reaches 400 mV. At potentials above 600 mV, the peaks tend to plateau. The graph presents another characteristic of the electrochemical detector, namely the current of the working electrode, which is the oxidation current of the mobile phase, in our case an aqueous solution of 50 m*M* potassium acetate, 11% methanol at pH 4.5*. Here, the readings are comparatively stable until the potential reaches 750 mV. Both the detector response and the current were taken into account in the process of selecting the working conditions. Potentials of 350, 700 and 750 mV were chosen, respectively, as the optimum for the screening, working and guard electrodes. Under these conditions, the detector maintained

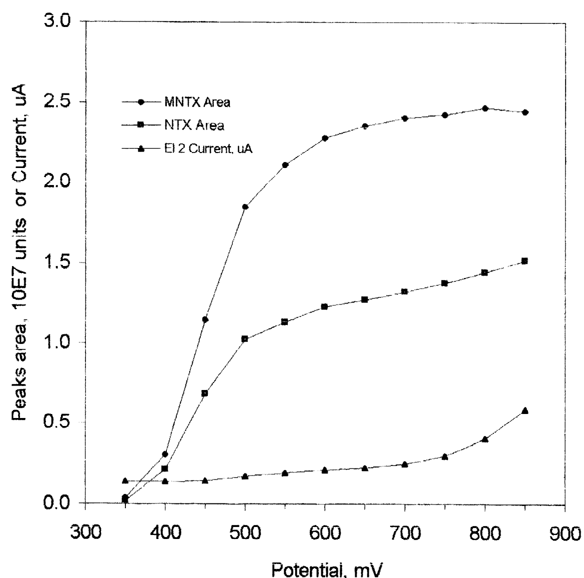

Fig. 3. Hydrodynamic voltammograms and electrode 2 current. Experimental conditions as in Fig. 2; temperature, 50 °C; optimized potentials, electrode 1 350 mV, electrode 2 700 mV, guard cell 750 mV; gain, 1500.

stability and reproducibility for a long period of time.

3.3. Solid-phase extraction, chromatography and method validation

Our expectations of analyzing MNTX in plasma and urine at the sensitivity level of the physiological response were met using this method. The combined selectivity of the weak ion-exchange extraction mechanism and electrochemical detection produced clean and reproducible chromatograms. Extraction recovery was in the region of 95%. The applied weak ion-exchange extraction mechanism demonstrated much merit in comparison with C_{18} and the strong cation-exchange process. C_{18}, being more inclusive, produced chromatograms with more endogenous peaks, while the latter lacked in extraction efficiency.

Chromatograms of a typical extract of a standard mixture of MNTX and NTX from a plasma matrix are presented in Fig. 4. The analytes are at the level of 100 and 50 ng/ml, respectively. There are no endogenous substances interfering with the peaks. They are well shaped and separated. Fig. 5 shows chromatograms of a set of plasma samples after subcutaneous injection. The traces represent time points of 0, 2, 15, and 90 min. The chromatograms are clean and contain, in addition to the analytes, one

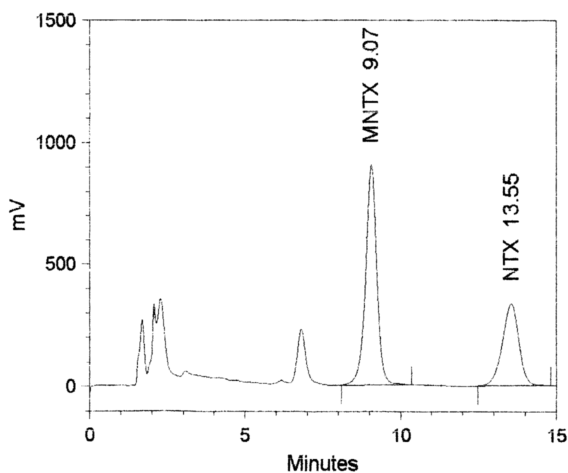

Fig. 4. Chromatograms of a standard plasma extract of methylnaltrexone and naltrexone. Experimental conditions as in Figs. 2 and 3.

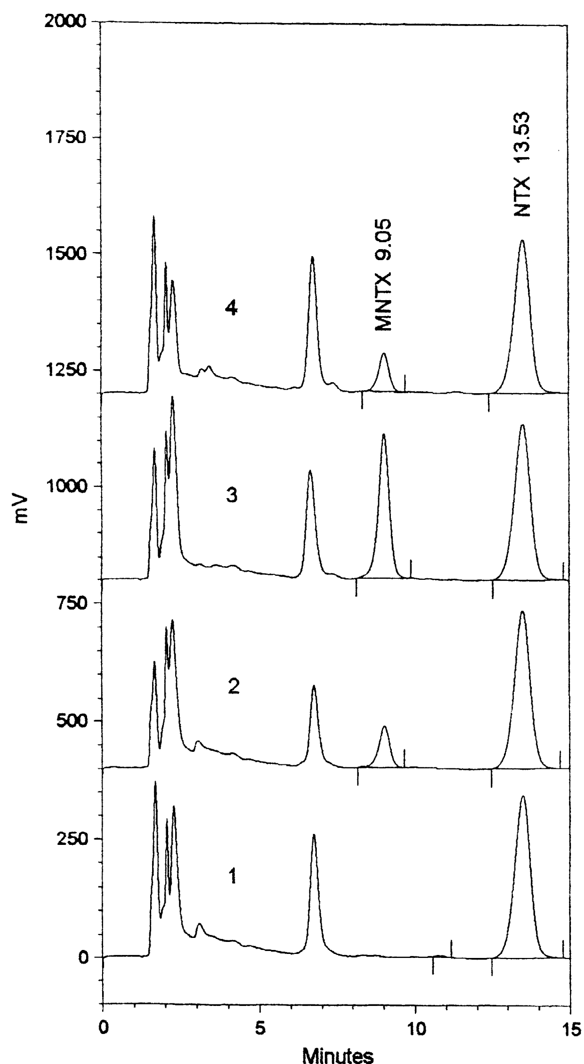

Fig. 5. Chromatograms of methylnaltrexone and naltrexone in plasma. Experimental conditions as in Figs. 2 and 3. The chromatograms from bottom to top are representative of samples at time (1) 0, (2) 2, (3) 15, and (4) 90 min.

unknown substance. The MNTX peak at 90 min is equivalent to 8.6 ng/ml and is still readily observed. Fig. 6 depicts the chromatograms of a set of urine samples. They are more complex, although the selectivity of the method helped to eliminate most undesirable admixtures. The time 0 sample contains the undiluted urine matrix, while the remaining samples were diluted five times to obtain readings within the calibration range.

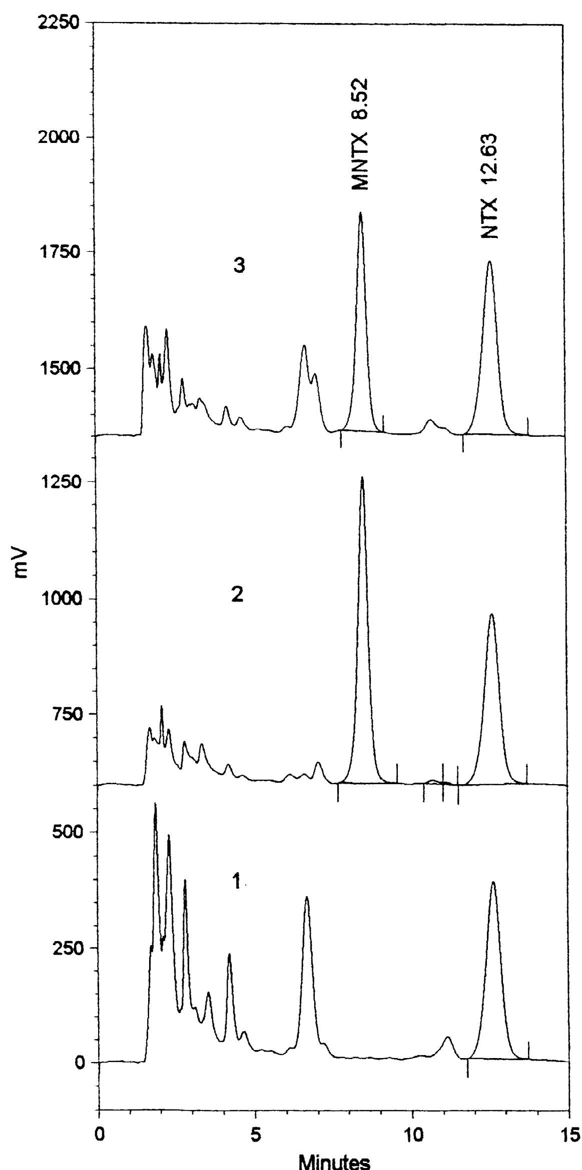

Fig. 6. Chromatograms of methylnaltrexone and naltrexone in urine. Buffer, 50 m*M* potassium acetate, 11.5% methanol, pH 4.5*; gain, 800. Other experimental conditions as in Fig. 3. The chromatograms from bottom to top are representative of samples at time (1) 0 and (2) intervals 0–3, and (3) 3–6 h. The last two samples were diluted five times to obtain readings within the calibration range.

Intra-assay statistics at the lower limit of detection (LOD), the lower limit of quantification (LOQ) and at 100 ng/ml were obtained according to the guidelines presented by Bressolle et al. [28]. The evaluation was performed for the instrumental part of the assay, as well as for the whole method, including extraction. The performance of the method was checked each day by measuring the concentration of the pool sample.

Intra-assay statistics for the instrumental part of the assay for five runs at the LOD and LOQ yielded an accuracy better than 4% and a coefficient of variation better than 2.5%. The plasma intra- and inter-day validation data for the whole assay were obtained for five parallel samples independent of standards at levels of 2 and 5 ng/ml (assumed to be LOD and LOQ) and 100 ng/ml for two consecutive days. The extraction efficiency data at a level of 100 ng/ml are also presented. The extraction efficiency data were obtained by a comparison of the peak areas of MNTX and NTX from five parallel extracted plasma calibration standards at the 100 ng/ml level with the MNTX/NTX peak areas of standards prepared in mobile phase at the same level. The intra-assay data for the complete method were an accuracy better than 15, 10 and 2.9%, and a coefficient of variation of 15.1, 8 and 4.3%, respectively, for LOD, LOQ and 100 ng/ml. The extraction efficiency was in the region of 94% for MNTX and 92–94% for NTX. The pool sample coefficient of variation for 12 working days was 2.2%. A six-point calibration was run for three consecutive days. The individual six-point calibration in the range 2–100 ng/ml yielded a linear response with a goodness of fit of $r=0.9993$. The extraction efficiency for both analytes from the urine matrix was in the region of 95%.

3.4. Application in pharmacokinetics study

The analytical method was applied successfully to a pharmacokinetics study of methylnaltrexone after subcutaneous administration of a single dose of 0.1 or 0.3 mg/kg in 12 clinical subjects. The free, unchanged drug concentration was measured in plasma and urine. The concentration versus time profile in plasma is presented in Fig. 7. All data points are an average of six values obtained from six subjects.

The presented data indicate fast absorption of the drug when injected subcutaneously. The maximum

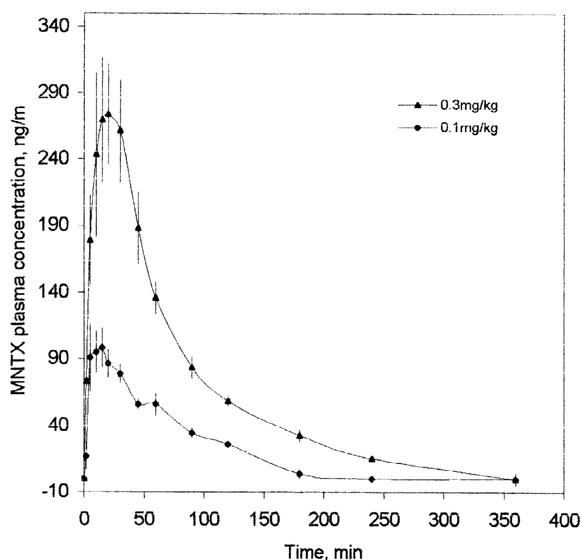

Fig. 7. MNTX plasma concentration versus time profile after subcutaneous administration of two single doses of 0.1 and 0.3 mg/kg. All data represent the average value for six patients (±SE).

plasma concentration (C_{max}) was reached in 16.7 (±10.8) and 20.0 (±9.5) min, respectively, for doses of 0.1 and 0.3 mg/kg. The C_{max} was 110 (±55) and 287 (±101) ng/ml and the range oscillated between 64 and 465 ng/ml. After reaching the maximum value, the drug plasma concentration decreased steadily. The concentration of the drug after 6 h was often still in the range of the limit of detection.

The measurement of MNTX in urine was conducted in the time brackets of 0–3 and 3–6 h. The bulk of the drug was eliminated in 3 h. The total amount of unchanged compound excreted in urine in 0–6 h was 51.7% (±8.8) for the 0.1 mg/kg dose group and 47.3% (±6.6) for the 0.3 mg/kg dose group. The obtained results correspond to our previous data [12].

4. Conclusion

The presented method for the determination of methylnaltrexone in plasma and urine is a major improvement over the published procedures in terms of sensitivity and variability. All the methods discussed here use electrochemical detection as the detection method [9,10]. The method of Kim et al. [9] for serum (plasma) was calibrated in the range 5–40 ng of mass injected, thus assuming 5 ng as the lower limit of quantification. At this level, the within-assay coefficient of variation was 4.6%. The accuracy of the method was not reported. The extraction efficiency using CN solid-phase extraction was 89%. The urine matrix was not investigated. The method of Foss et al. [10] was applied to plasma and urine. It was calibrated in the range 25–250 ng/ml. The limit of sensitivity was 100 ng/ml, or 2 ng mass injected. Data for the extraction efficiency, accuracy and variability of the method were not reported.

The present method was calibrated for plasma and urine in the range 2–100 and 2–200 ng/ml, respectively. This translates to 0.1–5 ng for plasma and 0.1–10 ng for urine (50 μl injection). The analyte was extracted from a 100 μl plasma or urine sample. The lower limit of quantification (LOQ) was 5 ng/ml or 250 pg. At this level, the accuracy was better than 10.0% and the coefficient of variation 8.0% for 10 assays from a single pool. The application of a more selective solid-phase extraction mechanism (weak ion exchange) proved to be beneficial in terms of the purity of the injected preparations. The extraction efficiency was in the region of 95%. The overall improvement in terms of sensitivity in comparison with the method of Kim et al. [9] is 10-fold and for Foss et al. [10] 80-fold. This method for the determination of MNTX was used successfully in the current study and numerous assays of the analyte at the level of the physiological response.

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