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Enantiospecific determination of nimodipine in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A direct enantiospecific HPLC assay using tandem mass spectrometric (MS–MS) detection via pneumatically-assisted electrospray has been developed for the determination of the calcium antagonist nimodipine in human plasma. By the addition of ammonium acetate (2 m*M*) to the purely organic eluent ethanol–*n*-heptane (20:80, v/v) charged species ($M+NH_4^+$) were producible by electrospray ionization at sufficient sensitivity. Routine determination of nimodipine enantiomers in human plasma in the working range of 0.5–75 μg/l plasma for each isomer with an accuracy $\leq \pm 10\%$ and a precision of about 10% (20% close to the limit of quantification) was possible. This was comparable to the available LC–GC–MS assay, however, the time required for routine analysis of ca. 150 unknowns could be reduced from 4 weeks to 1 week.

1. Introduction

The calcium antagonist nimodipine (Nimotop®) is known for its preferential action on cerebral blood vessels and its suggested cytoprotective effects by reducing calcium influx into nerve cells [1,2]. It is approved for the prevention or treatment of delayed ischaemic dysfunction following subarachnoid haemorrhage, and is currently widely studied in impaired brain function in old age and in senile dementia.

With its asymmetrical substitution at the dihydropyridine ring nimodipine is a racemate (Fig. 1). A stereospecific assay to describe the pharmacokinetic behaviour of its enantiomers has recently been reported [3,4]. The liquid chromatography–gas chromatography–mass spectrometry (LC–GC–MS) assay is based on liquid–liquid extraction with a 1:1 mixture of diethyl ether–*n*-heptane, separation of racemic nimodipine into its enantiomers via chiral stationary phase HPLC, and collection of the two isomer fractions followed by off-line GC–MS for quantification.

To increase the sample throughput, especially by simplifying this tedious procedure and reducing manual operations, a direct HPLC assay using tandem mass spectrometry (MS–MS) detection in the single reaction monitoring (SRM) mode via pneumatically-assisted electrospray (IonSpray®) has been developed. Details of the method and performance data in routine use are presented.

2. Experimental

2.1. Reference compounds and chemicals

Nimodipine, isopropyl-2-methoxyethyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-

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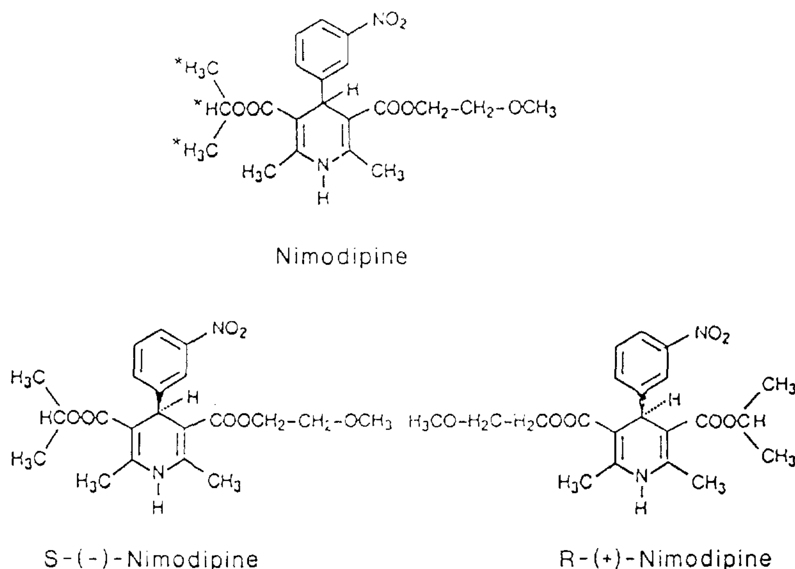

Fig. 1. Structure and absolute configuration of Nimodipine and its enantiomers (positions of deuterium-labelling for the ISTD are marked with an asterisk).

pyridine dicarboxylate (BAY e 9736, Nimotop), its *R*-(+)- and *S*-(−)-enantiomer (nominal molecular mass M_r 418) and racemic [$^2H_7$]nimodipine (deuterium-labelling located within the isopropyl side chain; used as internal standard (ISTD); M_r 425) were used as certified reference compounds (Bayer AG, Wuppertal, Germany; Fig. 1). The grade of impurity by not deuterated nimodipine has been found to be 0.2–0.3%. The optical purity of the enantiomers was determined by chiral HPLC to be better than 99%.

Stock solutions of nimodipine and the ISTD and their dilutions at appropriate concentrations were prepared in acetonitrile. HPLC-grade acetonitrile and *n*-heptane were obtained from Riedel-De-Haen (Seelze, Germany) and Merck (Darmstadt, Germany), respectively. All other solvents and chemicals were of analytical-grade purity and purchased from Merck. Nitrogen (99.999% purity) was used as the curtain gas for the API source and as nebulizer gas for the IonSpray interface, and argon (99.999% purity) was used as the collision-induced dissociation (CID) gas (all delivered from Messer-Grießheim, Krefeld, Germany). Fresh heparinized pool plasma for calibration and quality control (QC) samples was obtained from in-house volunteers.

As nimodipine is sensitive to light-induced degradation, sample preparation and instrumental analyses were carried out under yellow light (light source L 40W/62, Osram, Berlin, Germany).

2.2. Preparation of calibration and QC samples

To 1.0 ml blank plasma, 25 μl of the solution containing the reference substance at defined concentrations were added. The following concentration levels were used for the calibration curve: 0.25, 0.5, 1, 2.5, 5, 10, 20, 37.5, 50 and 75 μg/l of each enantiomer. These calibration samples were prepared freshly for each run.

Before starting the analysis QC sample pools at defined concentration levels were prepared independently by spiking 20 ml blank plasma with appropriate working solutions of the reference compounds. The concentration levels used were: 5, 50 and 100 μg/l racemic nimodipine, 5.4, 18 and 60 μg/l *S*-nimodipine and 5.4, 18 and 60 μg/l *R*-nimodipine. The pools were divided into aliquots of 1.0 ml and stored deep-frozen until analysis. QC samples were used to de-

termine inter-assay accuracy and precision of the assay for each enantiomer and as a tool for quality assurance during routine analysis of the unknowns.

2.3. Sample preparation

A 1.0-ml aliquot of plasma from the unknowns (or a 1.0-ml calibration sample spiked as described above, or a 1.0-ml QC sample), 30 μl (24 ng) of ISTD solution, 0.3 ml of 1 *M* sodium hydroxide and 5 ml of diethyl ether–*n*-heptane (1:1, v/v) were thoroughly mixed for 5 min at 400 rpm on a mini-shaker. The samples were centrifuged for 5 min at ca. 3000 *g*, 4°C. The upper organic layer was transferred to another glass tube and was evaporated in a metal heating block at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 200 μl of mobile phase and transferred to the autosampler vials for HPLC analysis.

2.4. Instrumentation and operating conditions

A modular instrument HP 1050 (Hewlett-Packard, Waldbronn, Germany) was used in conjunction with an API III$^+$ tandem mass spectrometer (Sciex, Toronto, Canada). The injection volume was 50 μl. Nimodipine enantiomers were separated with a Chira OJ MOD column (250 × 2 mm I.D., 8 μm) with a guard column (10 × 2 mm I.D.), supplied by Grom (Herrenberg, Germany). The column was operated at 35°C. The mobile phase consisted of ethanol–*n*-heptane (20:80, v/v) including 2 m*M* ammonium acetate, with a constant flow-rate of 300 μl/min. A split was applied to reduce the flow-rate into the IonSpray interface of the MS system to approximately 50 μl/min.

The API III$^+$ triple quadrupole mass spectrometer was operated with a standard atmospheric pressure ionization (API) source and the pneumatically-assisted electrospray (IonSpray) interface. The potential hazard by using flammable eluents and the occurrence of discharge at the tip of the sprayer has to be carefully addressed by creating an oxygen-free atmosphere within the API source (filling with nitrogen), and applying moderate voltage to the IonSpray interface to avoid discharge phenomena.

For optimizing the MS conditions a constant infusion of 1–2 mg/ml nimodipine dissolved in ethanol–*n*-heptane containing potassium acetate (Fig. 2A), sodium acetate (Fig. 2B), formic acid (Fig. 2C) and ammonium acetate (Fig. 3A) was used, delivered by a Harvard syringe pump (Harvard Apparatus, SouthNatick MA, USA) at a flow-rate of 10 μl/min. For the MS spectra the scan range was 200–500 amu with a step-size of 1 amu and a dwell time of 4 ms each; the result is the average of 15 scans. For the MS–MS spectra the scan range was 50–450 amu with a step-size of 1 amu and a dwell time of 4 ms each; the result is the average of 10 scans.

Both mass analyzers were always operated under unit mass resolution conditions (10% valley definition). The high vacuum in the analyzer region was $3 \cdot 10^{-5}$ Torr under MS–MS conditions. Collision-induced dissociation in the second quadrupole was obtained at $3.1 \cdot 10^{14}$ atoms/cm^2 argon gas thickness and a collision energy of 33 eV. The IonSpray interface was floated at +4.2 kV. Single reaction monitoring (SRM) was based on the observation of the parent ions $(M + NH_4)^+$ *m/z* 436 for nimodipine and *m/z* 443 for the ISTD and the common fragment *m/z* 301 (Fig. 3). The dwell time for each selected ion pair was 200 ms.

2.5. Assay calibration, quality control and validation

Each set of samples analyzed included a set of calibration samples in duplicate, a set of QC samples, analyzed in triplicate during routine analysis, and unknowns, all processed identically during sample pretreatment and LC–MS–MS analysis. Each set of samples included also a blank plasma sample to check for interferences.

The samples were processed as described and peak height ratios of analyte–internal standard were calculated. Calibration curves were constructed by weighted least squares linear regression analysis; as weights the reciprocals of the squared response (i.e., peak height ratio) were used.

Before starting the analysis of unknowns two validation runs with replicates of QC samples ($n = 5$) were performed. Overall assay performance was assessed by calculating inter-assay accuracy and precision of all QC samples analyzed ($n = 16$) for each concentration level.

3. Results and discussion

3.1. Optimization of MS conditions

Starting with the existing LC–GC–MS procedure, there were three options for a direct HPLC–MS assay:

(i) Changing the separation system completely to attain reversed-phase conditions commonly applied to electrospray ionization, e.g., a stationary-phase based on α_1-acid glycoprotein [5] combined with an appropriate buffer–organic modifier system

(ii) Keeping the successful separation, but changing the effluent before entrance into the API source by post-column addition–sheath flow of water–buffer–organic modifier amenable to electrospray ionization

(iii) Adapting the eluent to directly allow electrospray operation without sacrificing the enantiomeric separation.

Although the use of 100% organic mobile phases is not very common in LC–MS with electrospray ionization [6], the latter strategy was selected. While no signal could be acquired with pure ethanol–*n*-heptane (20:80, v/v), charged species of the type $(M + X)^+$, (where X = H, when formic acid was added, or Na, K, NH_4, when the respective acetate salts of these cations were added into the ethanol before mixing) were obtained. Fig. 2 and 3A show typical electrospray mass spectra of nimodipine under these conditions.

For the $(M + H)^+$ spectrum with formic acid the sensitivity was dependent on the amount of formic acid (0.1% vs. 1%); beyond 1%, however, no further increase was achieved. The $(M + Na)^+$ adduct ion was always present in the formic acid experiment, possibly due to the purity of the reagent used. The ratio of signal intensity of the ions at *m/z* 419 and *m/z* 441 could be controlled by the applied orifice voltage. The mass spectra of the ammonium acetate medium showed both the $(M + NH_4)^+$ adduct and the protonated ion $(M + H)^+$ depending on the orifice voltage (fragmentation energy) chosen. Fig. 3A shows the mass spectrum under conditions optimized for highest sensitivity (orifice voltage: 33 V).

Though all modifiers were able to produce charged nimodipine species in ethanol–*n*-heptane and showed satisfactory and comparable sensitivity for the $(M + X)^+$ ion, no adequate fragmentation could be obtained in the MS–MS experiment (collision-induced dissociation with argon) for the alkali adducts. Only the parent ion itself and the alkali ion at *m/z* 23 or *m/z* 39, respectively, could be detected, even at increased collision energy. In contrast, the CID spectrum of the $(M + NH_4)^+$ parent ion showed fragmentation with a dominant daughter ion at *m/z* 301 (or at *m/z* 343, depending on the applied collision energy). A detailed interpretation of the CID spectrum was not undertaken, but both fragments at *m/z* 343 and *m/z* 301 show loss of the labelled isopropyl side chain (Fig. 1). Fig. 3B shows the nimodipine MS–MS spectrum under final conditions (collision energy: 33 eV).

Based on these optimized MS and MS–MS conditions for $(M + NH_4)^+$, single reactions of both *m/z* 436 and *m/z* 443 (ISTD) to *m/z* 301 were selected to monitor the enantiomeric HPLC separation.

3.2. Chromatographic performance

Various aspects of the chromatographic performance have already been discussed together with the LC–GC–MS assay [3,4] and will not be repeated herein. All four considered modifiers, i.e., formic acid, sodium, potassium and ammonium acetate, were tested and did not change the separation, at least not in the concentrations mentioned. An example of the separation in the direct LC–MS–MS assay is given in Fig. 4, showing chromatograms of duplicate injections at various concentrations. The slightly reduced

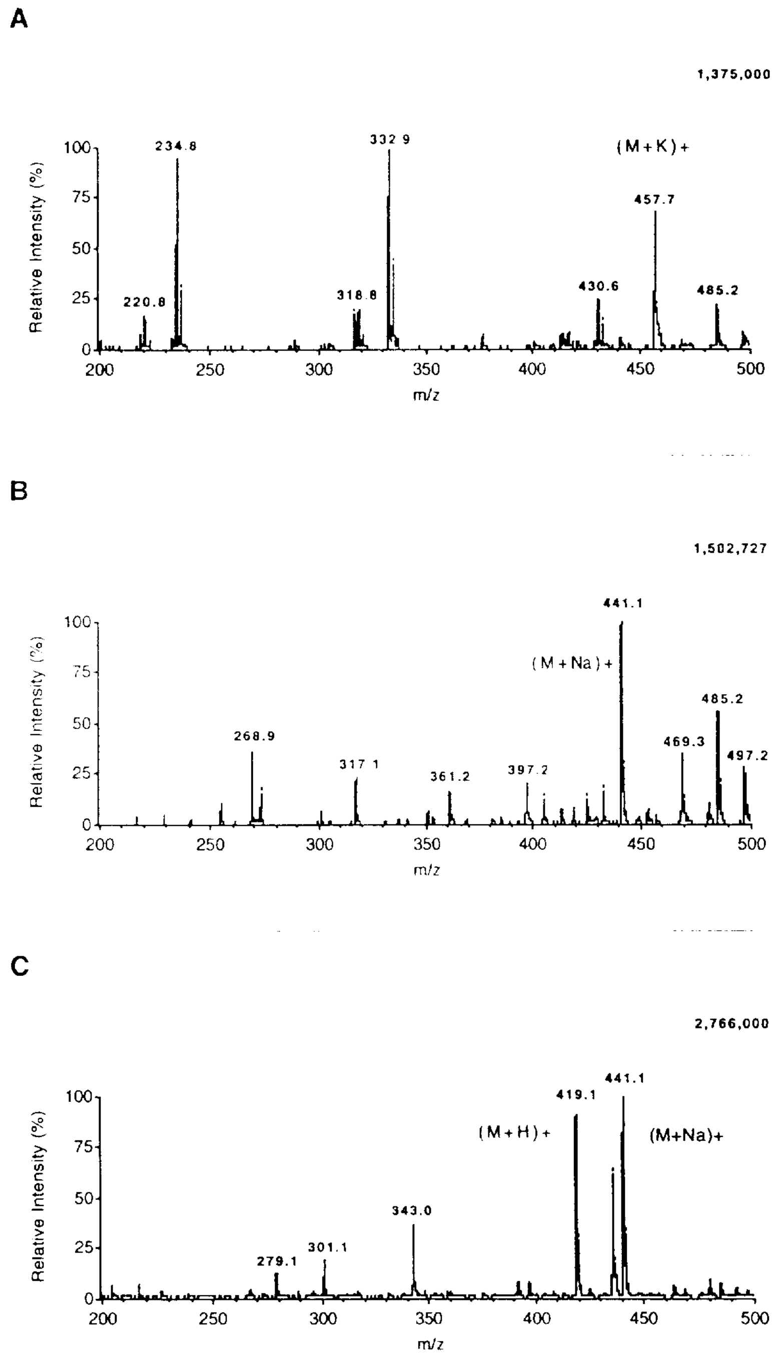

Fig. 2. Mass spectra of nimodipine (2 mg/l constant infusion) dissolved in ethanol–n-heptane (20:80, v/v) including 2 mM of (A) potassium acetate (B) sodium acetate or (C) 1% formic acid

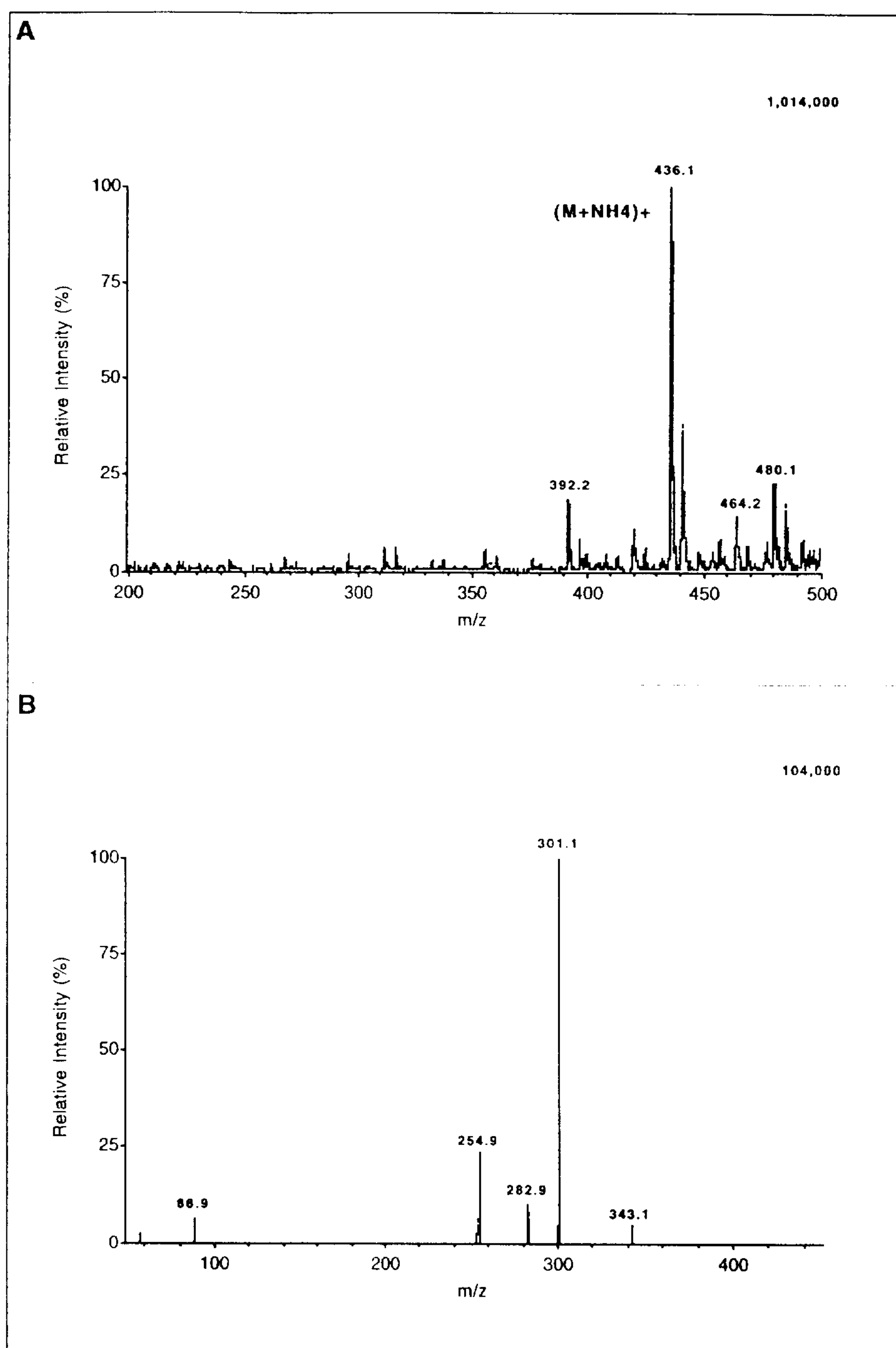

Fig. 3. Mass spectrum (A) and CID-spectrum (B) of nimodipine (1 mg/l constant infusion [dissolved in ethanol–*n*-heptane (20:80, v/v)] including 2 m*M* ammonium acetate).

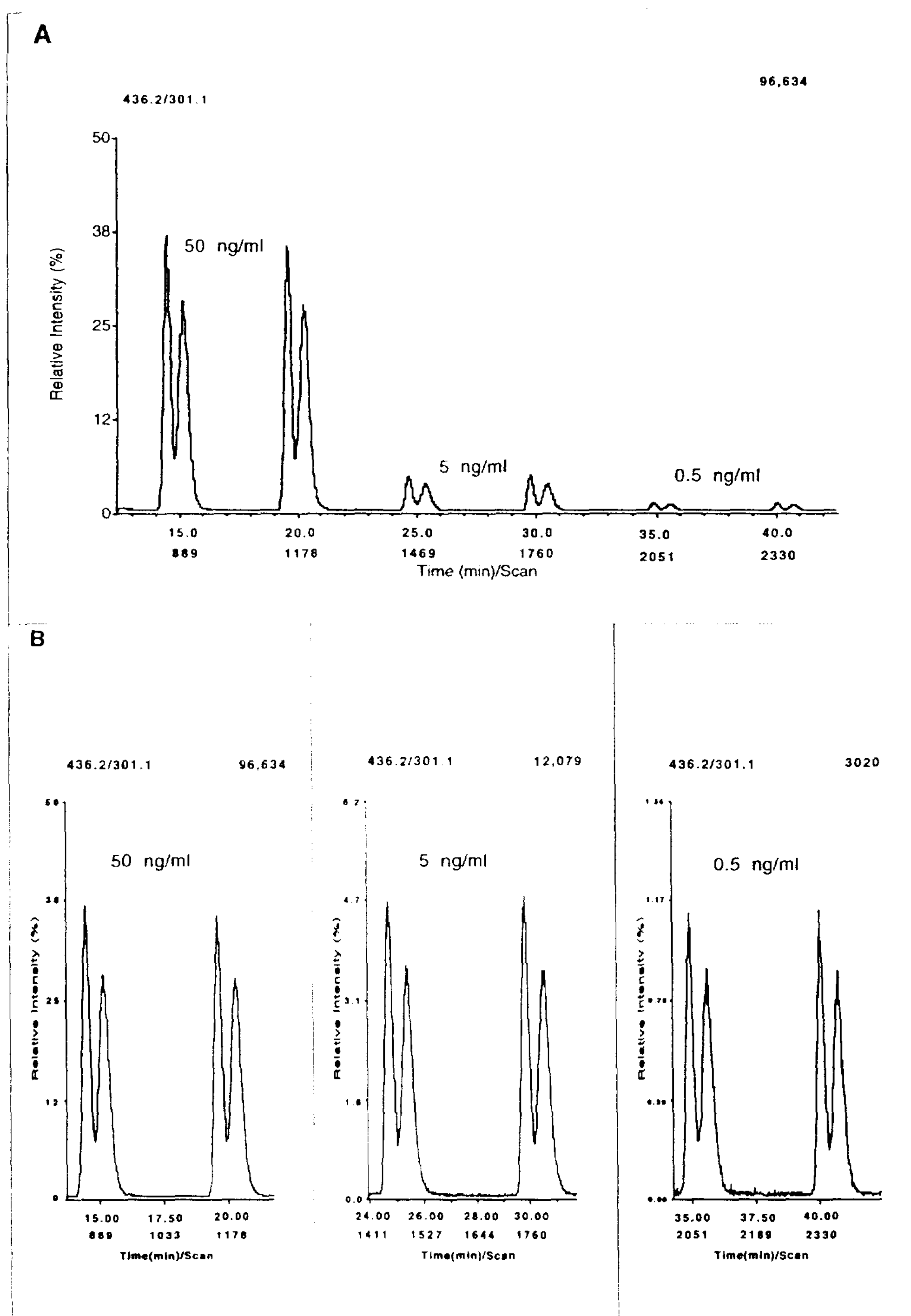

Fig. 4. (A) LC–MS–MS separation of nimodipine enantiomers (50, 5, and 0.5 μg/l of each enantiomer, duplicate injection). (B) Normalized ion chromatograms of each concentration level.

separation efficiency compared with chromatograms from the LC–GC–MS assay, may be due to the different column size (4.6 vs. 2 mm I.D.) or due to the different supplier used.

The effluent split of approx. 1:10 used before entering the atmospheric pressure ionization chamber resulted in a flow of ca. 50 μl/min. This flow-rate was used to keep the amount of non-aqueous solvent entering the API source as low as possible and was not due to sensitivity requirements. A separate experiment investigating the dependence of the SRM signal on the flow-rate exhibited no decrease in sensitivity at increasing flow-rates up to 250 μl/min (highest flow tested). On the contrary, a decrease in signal could be monitored at lower flow-rates than 30 μl/min, e.g., 10 μl/min. This needs further investigation, but an explanation may be found in the rapid desolvation of the ions in the non-aqueous medium.

3.3. Method specifications

The validity of the sample pretreatment with an extraction efficiency of about 90% has been previously presented [3].

For both enantiomers, linear calibration curves could be established in the range from 0.25–75 μg/l. Inter-assay precision and accuracy data were derived from all QC results (two validation runs and two series including unknowns, $n = 16$ each) at the defined six concentration levels. Accuracy, expressed as the deviation from the nominal value in percentage, was between −0.6% and −4.9% for *S*-nimodipine and between −0.3% and −7.0% for *R*-nimodipine. The precision (coefficient of variation CV) was between 8.2% and 13.7% for *S*-nimodipine and between 5.7% and 11.7% for *R*-nimodipine. Thus, over the whole working range an overall accuracy of ≤ ± 10% and a precision of ≤10% could be attained, comparable to the LC–GC–MS method [3]. No mutual interference of the enantiomers was observed. When comparing the sum of nimodipine enantiomers with the total (racemic) concentrations determined independently with the achiral GC assay with electron-capture detection [3], good agreement could be observed (see Fig. 5 as example): differences between both determinations were in general below 10%, only in a few cases higher up to 20%.

The limit of quantification (LOQ), i.e., the concentration level with an accuracy ≤15% and a precision ≤20%, was defined at 0.5 μg/l for both enantiomers in an additional experiment where six replicates of this concentration level showed an accuracy of −13.0% *S*-(−) and +16.8% *R*-(+), respectively, and a precision of

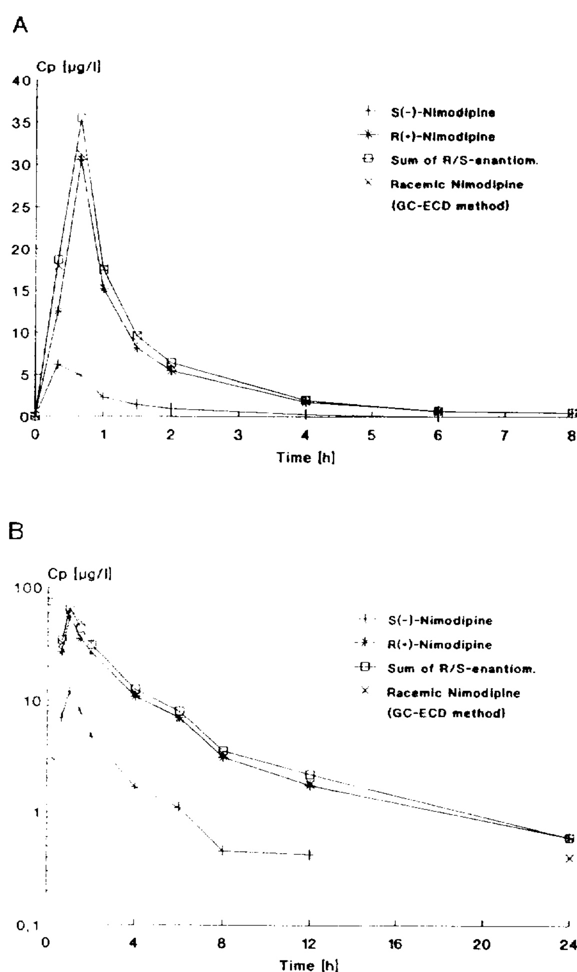

Fig. 5. Plasma concentration–time profiles of nimodipine enantiomers, their sum, and of racemic nimodipine (determined previously by an achiral GC–ECD assay). (A) after a single oral dose of 30 mg to a fasting healthy volunteer (linear scale); (B) after a single oral dose of 60 mg to a fasting healthy volunteer (semi-logarithmic scale).

14.9% and 20%, respectively. The limit of detection (LOD) was estimated at 0.25 μg/l for each enantiomer (an even lower LOD was restricted by the $^{2}H_{0}$ impurity from the ISTD which was observed in the blank samples in the order of ca. 0.1 μg/l).

3.4. Application

Fig. 5 shows representative plasma concentration–time data obtained from a healthy volunteer using this LC–MS–MS assay. Plasma concentrations were higher for *R*-(+)-nimodipine than for *S*-(−)-nimodipine, suggesting similar stereoselective disposition of nimodipine to that reported earlier [7].

The gain in time required for routine analysis of 150 study samples — one working week with the new LC–MS–MS assay — was considerable when compared to the four weeks needed with the established LC–GC–MS procedure. When comparing the flow charts of both assays, however, this gain in sample throughput is not unexpected since the tedious time-consuming steps of fractionation, collection of isomer fractions, and GC analysis could be omitted.

4. Conclusions

Based on the existing LC–GC–MS assay for determination of nimodipine enantiomers, the development of a direct enantiospecific HPLC assay with MS–MS detection via pneumatically-assisted electrospray (IonSpray) was straightforward. By the addition of suitable modifiers to the non-aqueous eluent, charged species could be produced by electrospray with sufficient sensitivity. This makes electrospray, in principal, compatible with 'normal-phase' separation conditions, provided that any potential hazard by using flammable eluents and the occurrence of discharge at the tip of the sprayer can be excluded.

The reported stereospecific LC–MS–MS assay is not only successful for the quantification of nimodipine in human plasma, but allows fast method development and highly specific and sensitive routine determination of enantiomers in biological matrix in general. A method transfer to other dihydropyridines where successful separations using the described chromatographic system have already been reported, e.g., nitrendipine, felodipine, nisoldipine [8–10], should be straightforward. In addition, similar chromatographic separation systems should also be amenable to MS detection via the outlined approach. Since the gain in sample throughput may be considerable, the efforts should be worth undertaking.

Acknowledgement

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