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Simultaneous determination of nifedipine and dehydronifedipine in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

Quantitative analysis of therapeutic compounds and their metabolites in biological matrix (such as plasma, serum or urine) nowadays requires sensitive and selective methods to allow the determination of concentrations in the ng/ml range. A new on-line LC–MS–MS method using atmospheric pressure chemical ionisation (APCI) as interface for the simultaneous determination of nifedipine (NIF) and its metabolite in human plasma, dehydronifedipine (DNIF) has been developed. The compounds were extracted from plasma using solid-phase extraction (SPE) on disposable extraction cartridges (DECs). The SPE operations were performed automatically by means of a sample processor equipped with a robotic arm (ASPEC system). The DEC filled with phenyl modified silica was first conditioned with methanol and water. The washing step was performed with water. Finally, the analytes were successively eluted with methanol and water. The liquid chromatographic (LC) separation of NIF and DNIF was achieved on a RP-18 stationary phase (4 μm). The mobile phase consisted of methanol–50 m*M* ammonium acetate solution (50:50, v/v). The LC was then coupled to tandem mass spectrometry with an APCI interface in the positive ion mode.

The method developed was validated. The absolute recoveries evaluated over the whole concentration range were 95±2% and 95±4% for NIF and DNIF, respectively. The method was found to be linear in the 0.5–100 ng/ml concentration range for the two analytes (r^2=0.999 for both NIF and DNIF). The mean R.S.D. values for repeatability and intermediate precision were 2.9 and 3.0% for NIF and 2.2–4.7% for the metabolite.The method developed was successfully used to investigate the plasma concentration of NIF and DNIF in the pharmacokinetic studies.

Keywords: Nifedipine; Dehydronifedipine

1. Introduction

Nifedipine (NIF), 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine-dicarboxylic acid dimethyl ester (Fig. 1) belongs to the dihydropyridine class of calcium entry antagonist which selectively dilates peripheral arteries [1]. It is frequently used in the treatment of hypertension and angina pectoris [2–6]. NIF is predominantly metabolized by oxidative mechanism to dehydronifedipine (DNIF) (Fig. 2).

The study of pharmacokinetics parameters in human beings in various physiological and

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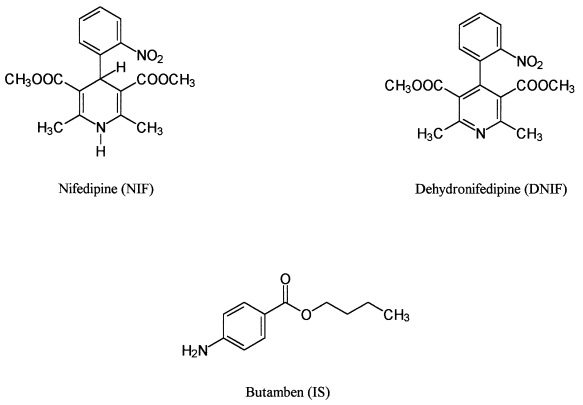

Fig. 1. Chemical structures of nifedipine, its metabolite dehydronifedipine and butamben (internal standard).

pathological situations requires a sensitive analytical method. The typical plasmatic concentration range of NIF and DNIF after an oral dose of 30 mg (control released formulation) ranges from 1 to 100 ng/ml [7]. Numerous methods for the determination of NIF in biological samples have been reported [8]. Most of them involved gas chromatography (GC) and liquid chromatography (LC). The GC methods reported involved electron-capture detection (ECD) [8–12], flame ionization detection (FID) [13], nitrogen–phosphorus detection (NPD) [14] and mass spetrometric (MS) detection [15,16]. Among the LC procedures, most of them are based on the combination of LC separation and UV detection [7,8,17–22] and electrochemical detection [4,8,23–26].

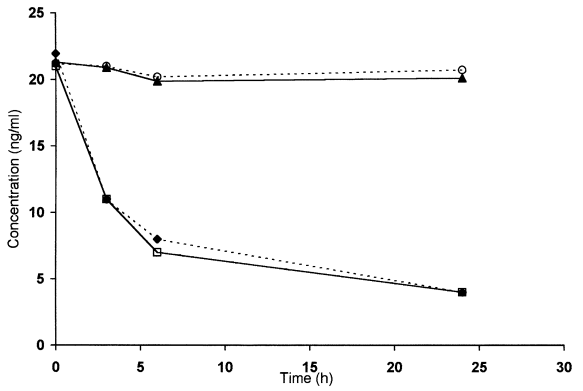

Fig. 2. Stability of plasma solutions of NIF and DNIF (20 ng/ml) after day light and non-actinic light exposure. ♦, Eluate exposed to daylight; ▲, eluate exposed to non-actinic light; □, plasma solution exposed to daylight; ○ plasma solution exposed to non-actinic light.

In the last few years, LC coupled to MS detection has been widely used in biomedical fields for both identification and quantitation of drugs and metabolites in biological fluids at very low concentrations [27–37]. The use of atmospheric pressure interfaces (API) based on electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) allowed the coupling of LC to MS. While the mobile phase flow-rates recommended with ESI interfaces are lower than 200 μl/min, the APCI can manage LC flow-rates in the range of 1 ml/min without splitting the effluent [38]. In the APCI source, the liquid is sprayed into a heated quartz tube causing the finely dispersed sample drops to vaporize. The vaporized sample molecules are carried through an ion molecule reaction region by means of a flow of heated gas. The primary ionization, initialized by a corona discharge, creates reagent ion from the solvent vapor. The reagent ions consist of protonated solvent molecules in the positive mode and solvated oxygen ions in the negative mode. For instance, in reversed-phase LC systems, mobile phases containing modifiers such as ammonium acetate can lead to protonated ammonia or acetate ion as primary reagents in the positive and negative mode, respectively. Sample molecules are therefore ionized by proton or electron transfer and attracted inside the mass spectrometer. APCI is particularly suited to the analysis of low or moderately polar compounds introduced at typical flow-rates ranging from 0.2 to 1.0 ml/min [39–41].

The preparation of plasma samples containing NIF generally consists of a liquid–liquid extraction (LLE) of the analyte using organic solvents [7,8,13,16,17,21,22,25]. In some case, sample clean-up is performed using off-line [18] or on-line [24] solid-phase extraction (SPE). The method reported in the present paper involves off-line sample handling by SPE using disposable extraction cartridges (DECs), liquid chromatography and subsequent MS detection using APCI as the interface. The SPE procedure was performed automatically by means of an ASPEC system. The selection of the type of SPE sorbent has been optimized with respect to analyte recovery. The influence of daylight on stability of NIF and DNIF solutions was also investigated.

The LC–APCI-MS–MS method was developed and validated in order to study the pharmacokinetics of an oral formulation of NIF. The method reported was successfully used to perform the determination of NIF and DNIF in real human plasma samples and was found to be applicable for the quantification of NIF and DNIF in pharmacokinetics studies which required high sensitivity and selectivity.

2. Experimental

2.1. Chemical and reagents

NIF and DNIF were obtained from the European Pharmacopoeia (Strasbourg, France). Butyl-4-aminobenzoate (Butamben), used as the internal standard (I.S.), was supplied by Aldrich (Gillingham, Dorset, UK). Isobutylamine (99%) was obtained from Janssen (Geel, Belgium). Ammonium acetate, urea and glacial acid acetic were all of analytical grade from Merck (Darmstadt, Germany). Methanol and water were of HPLC grade from Merck. Nitrogen (99.999%) was purchased from Air liquide (Air Liquide, Voisins-Le-Bretonneux, France).

Isolute DECs (1 ml capacity) filled with 50 mg phenyl silica (Ph) were obtained from IST (International Sorbent Technology, Mid-Glamorgan, UK). Other Isolute DECs filled with 50 mg of end-capped octadecyl (C_{18}^{EC}), end-capped octyl (C_8^{EC}), phenyl (Ph^{EC}), cyanopropyl (CN) or end-capped ethyl (C_2) silica were also tested.

The LiChroCart analytical and guard columns were respectively prepacked with Superspher 100 RP-18 (particle size: 4 μm) and LiChrospher 100 RP-18 (particle size: 5 μm) from Merck.

2.2. Instrumentation

The ASPEC system (automated sample preparation with extraction cartridges) from Gilson (Villiers-le-Bel, France) consisted of an automatic sampling injector module, a model 401 dilutor–pipettor and a set of racks and accessories for handling DECs, plasma samples and solvents.

The LC system consisted of a Model 1100 series liquid chromatograph equipped with a binary pump, a vacuum degasser, a thermostatted column compartment and an autosampler, all from Hewlett-Packard (Palo Alto, CA, USA). A Manu-Cart system which consisted of a LiChroCart analytical column (125×4 mm I.D.) and a short LiChroCart guard column (4×4 mm I.D.) from Merck was thermostated at 35°C. The mobile phase consisted of methanol–50 m*M* ammonium acetate (50:50, v/v). Before use, the mobile phase was degassed for 15 min in an ultrasonic bath. The flow-rate was 1.0 ml/min.

Mass spectrometric detection was carried out using a Perkin Elmer Sciex API 300 triple quadrupole instrument (Thornhill, Toronto, Canada) equipped with an APCI interface.

The heated nebuliser temperature was 400°C and the auxiliary gas (N_2) flow-rate was 2 l/min. The curtain gas flow-rate was 1.2 l/min at 60 p.s.i. (1 p.s.i.= 6894.76 Pa). The mass spectrometer was set to generate and to select the protonated pseudomolecular ion ($[M+H]^+$) (corona discharge 3.2 μA—orifice voltage 3 eV for NIF and 5 eV for DNIF and the I.S.) at m/z 347 for NIF, m/z 344.8 for DNIF and m/z 194 for the I.S. via the first quadrupole mass filter (Q_1). The MS–MS fragmentation was achieved by introducing the pseudomolecular ions into the collision cell (Q_2) with a collision energy of 13 eV (collision gas: N_2) for NIF and I.S. and 30 eV for DNIF. Signals for product ions at m/z 315 for NIF, 284 for DNIF and 137.5 for I.S. were monitored via the third quadrupole mass filter (Q_3).

A Power Mac 8600 (200 MHz) computer from Apple Macintosh (Austin, TX, USA) equipped with a SAMPLE CONTROL version 1.3 and a MAC QUAN version 1.3 software from P.E.Sciex was used to control the LC–MS–MS system and to collect and treat the data.

2.3. Standard stock solutions

The stock standard solutions of NIF, DNIF and the I.S. were prepared by dissolving appropriate amounts of the compounds in methanol to give final concentrations of 10 μg/ml for each compound. The NIF and DNIF solutions were then successively diluted with water to achieve concentrations of 2 μg/ml, 200 ng/ml and 20 ng/ml. The stock solution of the I.S. was diluted with water to reach a final concentration of 150 ng/ml.

The aqueous solutions were used to spike plasma

samples (2.0 ml) either for calibration curves (from 0.50 to 100 ng/ml) for both compounds of interest (NIF and DNIF) or for quality control during the pharmacokinetic study (0.5 to 100 ng/ml).

2.4. Sample preparation

Blood samples were collected in tubes containing heparin. After centrifugation at 3000 *g* for 10 min at 4°C, the separated plasma was collected and stored at −80°C. All manipulations of plasma and stock solutions of NIF and DNIF were performed under non-actinic light. Before analysis, the plasma samples were thawed at 18°C.

A 1.20-ml volume of sample was transferred manually into a vial on the appropriate rack of the ASPEC system and 600 μl of the I.S. solution (150 ng/ml) was added. The DEC sorbent was first treated with 1.50 ml of methanol and then with 1.50 ml of water. A 1.50-ml volume of plasma sample was aspirated by the autosampler needle from the corresponding vial and applied onto the DEC. The washing step was then performed by dispensing 1.5 ml volume of water twice. A 0.5-ml volume of methanol was then dispensed on the DEC and the eluate was collected in the tube positioned under the DEC. A 0.5-ml volume of a 0.05 *M* ammonium acetate solution containing 0.2% of isobutylamine was passed through the DEC and the resulting eluate was successively aspirated and dispensed twice in the collection tube. Finally, the final extract was manually transferred to an autosampler vial for analysis and a 100-μl volume was injected into the chromatographic system. The sample extraction procedure was performed automatically by the ASPEC system in the batch mode.

2.5. Pharmacokinetics study

The LC–MS–MS procedure developed was used to investigate the plasma profile of NIF and its metabolite DNIF after multiple oral dose of a controlled release formulation of NIF (Procardia®, 30 mg). A clinical study on ten healthy male volunteers was conducted. The subjects received successively for 5 days one NIF dosage form at 7.00 a.m. One blood sample was obtained on day four at 7.00 a.m. ($t-24$ h) and fourteen blood samples were obtained on day five at different times until 36 h after the last administration of the medication.

3. Results and discussion

3.1. Selection of SPE sorbent

Six different kinds of DECs containing bonded silicas with various polarities were tested. Spiked plasma solutions were used as samples and the corresponding recoveries of NIF and DNIF were determined (Table 1).

The recoveries were calculated by comparing peak areas obtained from freshly prepared samples extracts with those found by direct injection of aqueous solutions at the same concentration into the LC–MS–MS system, using the same autosampler. As can be seen in Table 1, very low recoveries for NIF and DNIF were observed with the CN phase. This can be explained by analytes losses during the loading and the washing steps. Except for the C_2 phase, the recoveries obtained for the other phases tested (C_8^{EC}, C_{18}^{EC}, Ph^{EC}, Ph) were around 90–95%. Taking into account the slightly higher recovery observed with

Table 1
Types of sorbents used in the disposable extraction cartridges (DECs)[a]

Sorbent	Recovery of NIF (mean±S.D., %; $n=3$)	Recovery of DNIF (mean±S.D., %; $n=3$)
CN	10±1	9±1
C_2	66±2	80±5
C_8^{EC}	91±1	89±2
Ph	95±2	95±2
Ph^{EC}	92±1	92±1
C_{18}^{EC}	93±1	93±3

[a] DECs: Isolute (50 mg); Conditioning: methanol–water (1.5 ml of each); Washing: 1.5 ml water; Elution: 0.5 ml of methanol; Buffer addition: 0.5 ml of ammonium acetate solution containing isobutylamine; Sample: spiked plasma solution of NIF and DNIF (20 ng/ml).

DECs filled with phenyl silica (95%), the latter phase was finally selected.

3.2. Analyte stability

NIF and DNIF are known to be sensitive to light in the solid state and in aqueous solution [42,43]. The stability of plasma and aqueous solutions treated by the SPE procedure was therefore investigated in order to be sure that this step could not influence the assay. This study was carried out by comparing the concentrations of the same solutions maintained for 0, 3, 6 and 24 h at a daylight exposure and under non-actinic light. The concentrations of plasma solutions and eluate used in this study were 20 ng/ml of NIF and DNIF. Fig. 2 shows clearly the rapid instability of the two compounds when they are exposed to light. On the contrary, no significant decrease in the NIF and DNIF concentration was observed when the solutions were stored under non-actinic light for 24 h. Results obtained from these experiments demonstrate that the analysis of NIF and DNIF must be performed under non-actinic light.

3.3. Optimization of MS conditions

The LC–MS–MS method for the detection of NIF and DNIF in human plasma was investigated. Each compound was first directly introduced in mass spectrometry using APCI ionisation and parameters such as corona discharge, orifice voltage, ring voltage, flow of nebulizer and auxilary gas (N_2) and temperature of auxilary gas (N_2), were optimized in order to obtain the protonated pseudomolecular ions of NIF and DNIF. Fig. 3 shows the full-scan Q1 mass spectra of the two compounds of interest where the pseudomolecular ion [MH^+] was m/z 347 for NIF and m/z 344.8 for DNIF. The most suitable collision energy was determined by observing the response obtained for the fragment ion peak m/z. The product ion mass spectra presented in Fig. 4 illustrate a predominant fragment ion peak m/z 315 for NIF and 284 for DNIF, observed when the

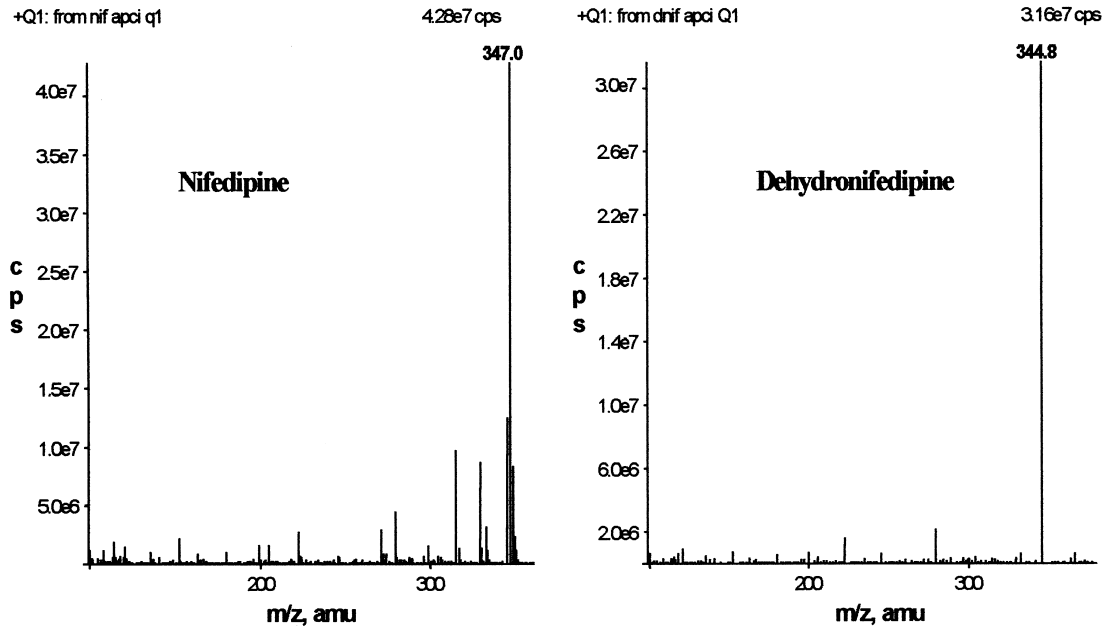

Fig. 3. Mass spectra of NIF and DNIF illustrating the base peak ions m/z 347 and m/z 344.8 as the protonated molecular ions of nifedipine and dehydronifedipine, respectively, (cps) counts per second.

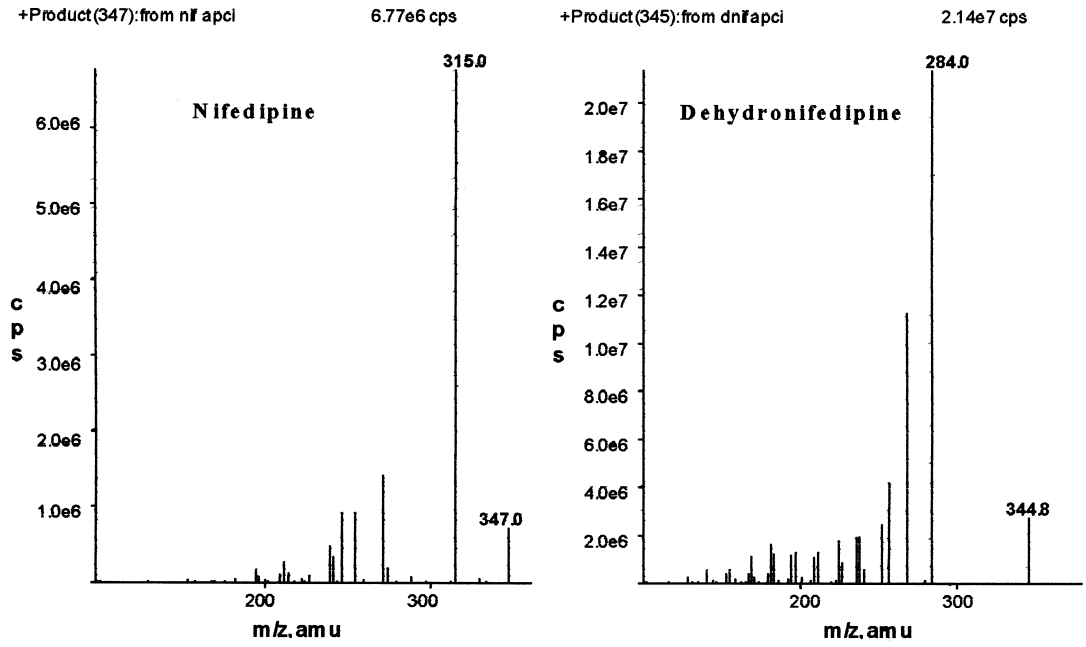

Fig. 4. Production mass spectra of protonated molecular ions of NIF and DNIF, (cps) counts per second.

collision energy was 13 eV for NIF and I.S. and 30 eV for DNIF.

Selective reaction monitoring mode (SRM) ion chromatograms were used to determine NIF and its metabolite in plasma. SRM spectra selectively filter out ions not related to the target compounds and a very clean ion chromatogram can thus be obtained due to the great selectivity and sensitivity of this operational mode.

3.4. Validation

3.4.1. Stability

Table 2 shows the stability of the sample processing, chromatography and storage of processed spiked samples. No significant degradation of both compounds of interest was observed after stability tests.

3.4.2. Selectivity

The coupling of LC with MS–MS detection in the selective reaction monitoring mode (SRM) provides a highly selective method for the determination of drugs in biological samples. Typical chromatograms obtained with a blank plasma and a plasma containing 0.5 and 10 ng/ml of NIF and DNIF are presented in Fig. 5. No endogenous sources of interferences were observed at the retention times of the analyte.

3.4.3. Absolute recovery

The absolute recovery of NIF and DNIF over the whole concentration range was determined by comparing peak areas obtained from plasma sample and those found by direct injection of an aqueous standard solution at the same concentration, using the same autosampler equipped with the same loop [44]. The mean recoveries of NIF and DNIF were 95±2% and 95±4%, respectively (Table 3).

3.4.4. Linearity

The linear regression analysis of NIF and DNIF was made by plotting the peak area ratio (y) versus analyte concentration (x) in ng/ml. The following equations were obtained (concentration range: 0.5–100 ng/ml):

Nifedipine:	$y=0.0298x+0.0092$	$r^2=0.9999$
Dehydronifedipine:	$y=0.0414x+0.0097$	$r^2=0.9998$

The linearity of the relationship between peak area

Table 2
Stability of nifedipine and dehydronifedipine in plasma control samples

	NIF (ng/ml)			DNIF (ng/ml)		
	1.0	20	100	1.0	20	100
Stock solution (n=6)						
24 h, 18±2°C						
ng/ml	–	18.9	–	–	19.2	–
% of initial	–	97.6	–	–	100.3	–
Plasma sample (n=3)						
24 h, 18±2°C						
ng/ml	1.10	20.7	99.7	–	20.5	100.5
% of initial	103.2	103.5	99.7	–	103.2	100.5
Freeze–thaw (n=3)						
2ce cycle (% of initial)	–	105.5	–	–	102.3	–
3rd cycle (% of initial)	–	102.7	–	–	109.4	–
Plasma sample storage (n=3)						
20 ng/ml, 42 days, −80±10°C						
ng/ml	1.0	20.5	101.9	1.2	20.2	100.7
% of initial	92.2	105.6	101.9	101.2	104.8	100.6

ratios and concentration is demonstrated by the determination coefficients (r^2) obtained for the regression lines in the case of both analytes.

3.4.5. Detectability

The limits of detection (LOD), and quantification (LOQ), for the two analytes were calculated from regression lines [45]. The LODs and LOQs were found to be 0.08 and 0.24 ng/ml for NIF and 0.09 and 0.26 ng/ml for DNIF, respectively (Table 3).

3.4.6. Precision

The precision of the bioanalytical method was determined by measuring repeatability and intermediate precision for both compounds at three different concentrations levels ranging from 1.0 to 100 ng/ml. The mean values for repeatability and intermediate precision were 2.9 and 3.0 % for NIF and 2.2 and 4.7 % for DNIF, respectively (Table 3).

3.4.7. Accuracy

The overall accuracy of the procedure was assessed by calculating the ratio between the analyte amount found versus the amount spiked in the plasma, ranging from 1.0 to 100 ng/ml. The accuracy, defined as mean%±interval of confidence ($p>$ 0.05), shows that the LC–MS–MS procedure developed for the determination of NIF and DNIF can be considered as accurate and linear within the concentration range investigated (Table 3).

3.5. Pharmacokinetics

The LC–MS–MS procedure developed was used to investigate the plasma profile of NIF and its metabolite DNIF after multiple oral dose of a controlled release formulation of NIF (Procardia®, 30 mg). Plots of the plasma NIF and DNIF levels (ng/ml) versus post-dose sampling time (h) are presented in Fig. 6. Pharmacokinetic parameters ($AUC_{0\rightarrow24}$, $AUC_{0\rightarrow36}$, C_{max}) calculated from these data are presented in Table 4.

4. Conclusions

A sensitive, accurate and precise procedure based on LC–MS–MS has been developed for the simultaneous determination of NIF and its metabolite DNIF in human plasma with lower quantifiable limits of 0.2 ng/ml. The method was validated to meet the requirements of the pharmacokinetic investigation of these two compounds. The procedure developed was successfully applied to the determination of NIF and DNIF plasma levels for investigating a pharmacokinetic study.

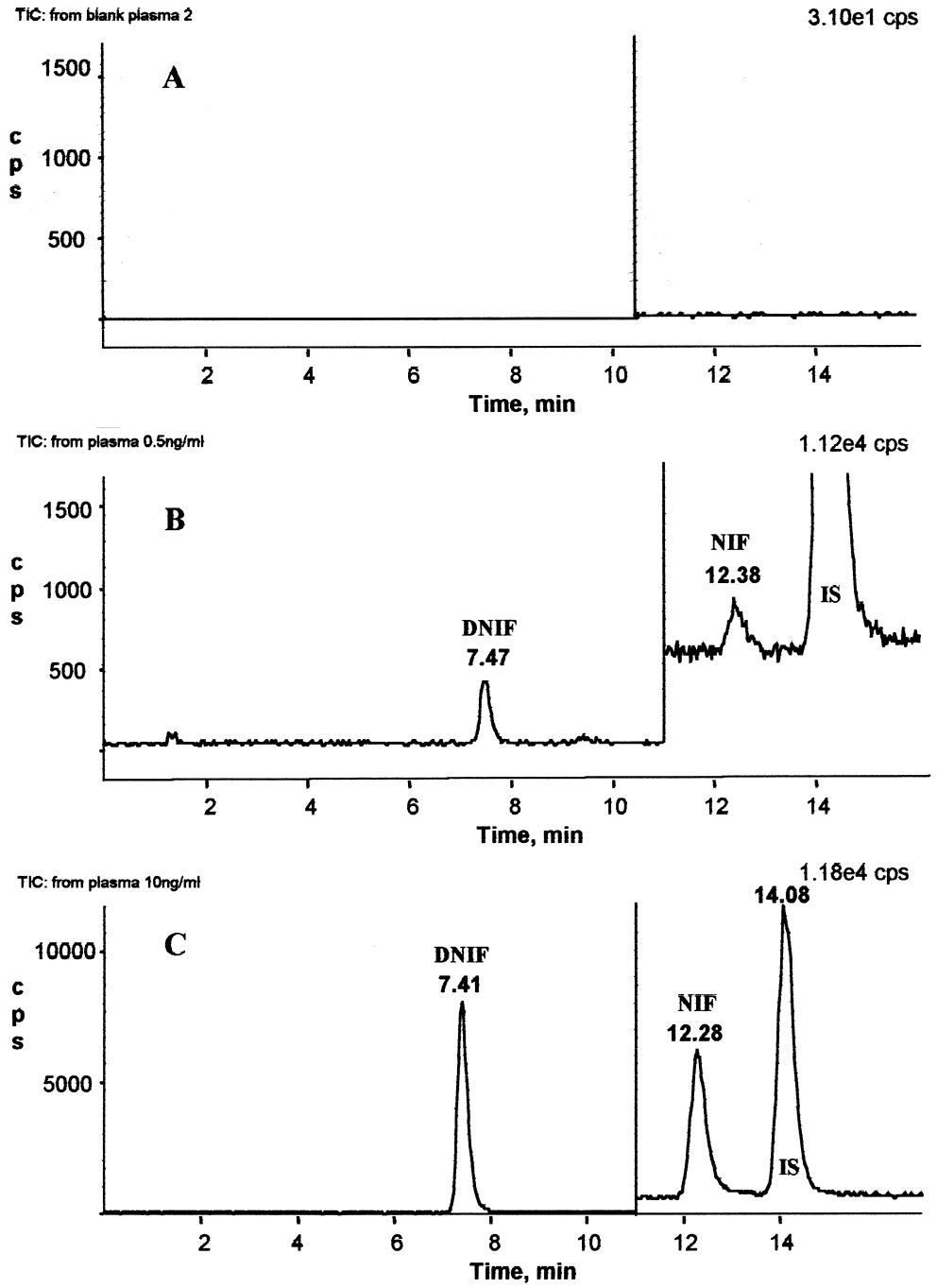

Fig. 5. SRM ion chromatograms of blank plasma (A) and spiked plasma samples with 0.5 (B) and 10.0 ng/ml (C) of NIF and DNIF, (cps) counts per second.

Table 3
Validation of the LC–MS–MS method for the determination of NIF and DNIF

Validation criterion		NIF	DNIF
Absolute recovery	(mean±S.D., n=7)	95±2 %	95±4 %
Linearity (n=7, k=1)	0.5–100 ng/ml	y=0.0298x+0.0092 r^2=0.9999	y=0.0414x+0.0097 r^2=0.9998
LOD		0.08 ng/ml	0.09 ng/ml
LOQ		0.24 ng/ml	0.26 ng/ml
Repeatability			
	(n=6)		
	1.0 ng/ml	5.5	2.4
	20 ng/ml	2.5	3.4
	100 ng/ml	0.7	0.7
	Mean	2.9	2.2
Intermediate precision			
	(n=6; 3 days)		
	1.0 ng/ml	5.6	9.4
	20 ng/ml	2.7	3.8
	100 ng/ml	0.9	0.8
	Mean	3.0	4.7
Accuracy (recovery±IC, %; n=6)			
	1.0 ng/ml	95.7±5.8	101.6±2.3
	20 ng/ml	99.7±0.8	99.8±1.8
	100 ng/ml	100.0±0.7	99.9±0.7

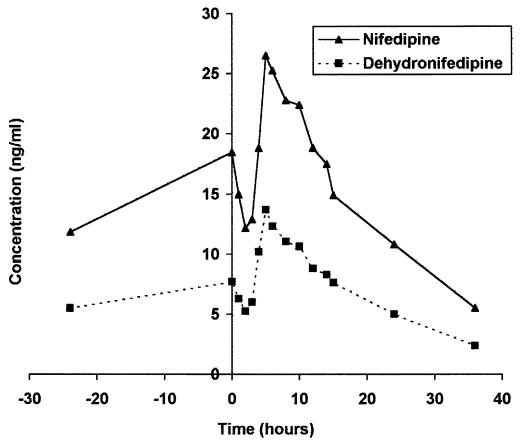

Fig. 6. Plasma concentration time profile of NIF and DNIF following a multiple oral dose of 30 mg nifedipine (Procardia®).

Table 4
Pharmacokinetic parameters

Parameters	NIF	DNIF
C_{max} (ng/ml)	30.14	14.59
$AUC_{0\rightarrow24}$ (ng/ml/h)	409.04	197.14
$AUC_{0\rightarrow36}$ (ng/ml/h)	506.79	241.27

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