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Determination of deramciclane and *N*-desmethylderamciclane in human plasma by liquid chromatography–tandem mass spectrometry using off-line robotic sample pretreatment

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Abstract

A rapid and highly sensitive LC–MS–MS method using deuterium-labelled internal standards was developed and evaluated for the simultaneous determination of deramciclane and its pharmacologically active metabolite (*N*-desmethylderamciclane). The sample preparation based on liquid–liquid extraction was carried out with an off-line robotic system. Evaluation of this analytical method shows that samples can be assayed with acceptable accuracy and precision in the 0.1 to 50 ng/ml concentration range for both compounds. The method was applied for the quantitative determination of deramciclane and its metabolite in human plasma samples during a food interaction pharmacokinetic study.

Keywords: Sample handling; Validation; Deramciclane; Desmethylderamciclane

1. Introduction

Deramciclane {(1*R*,2*S*,4*R*)-(−)-*N*,*N*-dimethyl-2-((1,7,7-trimethyl-2-phenylbicyclo[2.2.1]hept-2-yl)-oxy) ethane amine-2-(*E*)-butenedioate, EGIS-3886, CAS 120444-78-8} is a newly developed, highly efficient anxiolytic drug, acting on the 5-HT2 receptors [1,2].

The absorption and pharmacokinetics of deramciclane has been studied in rat, dog, rabbit and man [3–8,15,16]. The in vivo biotransformation was also investigated in different species [9–12].

N-Desmethylderamciclane was found to be a pharmacologically active metabolite [7].

Different highly sensitive analytical methods have been used for the quantitation of deramciclane and the metabolite. The pharmacokinetics of deramciclane have been studied in many species using GC–NPD (gas chromatography with nitrogen–phosphorous detection) [4,5,8]. Other pharmacokinetic studies used GC with mass-selective detection to quantitate plasma concentrations [6].

In order to carry out pharmacokinetic studies on deramciclane and its active metabolite (*N*-desmethylderamciclane) in humans, we have developed a highly sensitive reversed-phase liquid chromatographic method based on a previously elaborated method [8,13], using a tandem mass spectrometer as

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detector. The novelty in our approach is using two deuterated internal standards (each is the corresponding deuterated version of the analyte to be quantitated) instead of one. This approach highly enhances the precision of the determination. We observed that preparing these analytes from plasma matrix in the ng/ml–sub-ng/ml range there is a substantial adsorbance of the compounds to the glass tubes, which reduces recovery. However deramciclane and *N*-desmethylderamciclane adsorb to different extents on glass surfaces, therefore their recoveries are different. When using only deuterated deramciclane as an internal standard for both compounds, the quantitation of *N*-desmethylderamciclane is highly irreproducible. This problem is overcome by using the corresponding deuterated compound in the quantitation of each analyte, since they adsorb similarly to their respective non-deuterated pair. In our work the liquid–liquid extraction of the plasma samples is automated using a laboratory robot, which considerably decreases the occurrence of human errors, therefore making the analysis more reliable. The elaborated method was validated and successfully applied to a human food interaction study.

2. Experimental

2.1. Chemicals and reagents

Deramciclane (M_r 301.5), *N*-desmethylderamciclane (M_r 287.4), [$^2H_6$]deramciclane (M_r 307.5) and [$^2H_5$]*N*-desmethylderamciclane (M_r 292.4) were synthesised at EGIS Pharmaceuticals (Budapest, Hungary) and their chemical structures are shown in Fig. 1. These compounds were provided as fumarate salts. The physico–chemical properties, identity and the purity of the test substances were checked by thin-layer chromatography (TLC), IR spectroscopy, melting point determination, high-performance liquid chromatography (HPLC) and titrimetry according to quality assurance regulations of EGIS Pharmaceuticals. In the case of the deuterated compounds the chemical purity was checked by HPLC and the isotope purity was determined by electron impact ionisation (EI) mass spectrometry (MS). Other reagents and solvents used in this study were of

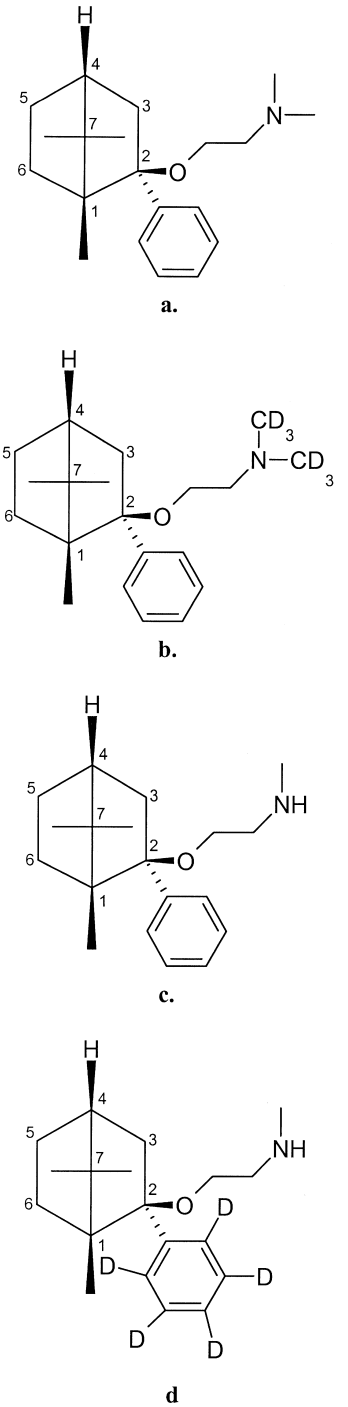

Fig. 1. Structures of the compounds measured in this assay. Deramciclane (a), [$^2H_6$]deramciclane (b), *N*-desmethylderamciclane (c) and [$^2H_5$]*N*-desmethylderamciclane (d).

analytical- or HPLC-grade and were obtained commercially.

2.2. Plasma samples

Blood samples of 7.5 ml volume were collected in 100 μl of 12% EDTA anticoagulant using heavy-wall centrifuge tubes with a calibration mark at 7.5 ml. The samples were centrifuged at 1500 *g* (3000 rpm) for 10 min at room temperature. The plasma was transferred to 10-ml Wassermann tubes and stored at −20°C until analysis. For method validation pooled plasma collected from 20 individuals was used, prepared in a similar way as described above.

2.3. Apparatus

The LC instrument consisted of two pumps and an autosampler Series 200 LC (Perkin-Elmer, Norwalk, CT, USA). In this system, an Asahipak ODP-50 (125×4 mm, 5 μm) poly(vinyl alcohol)-based column (Hewlett-Packard, Palo Alto, CA, USA) was used as the analytical column. To protect the analytical column during measurements a Purospher RP-18e pre-column (4×4 mm, 5 μm) (Merck, Darmstadt, Germany) was installed. The elution was performed using 25% 2 m*M* ammonium acetate, 70% methanol, 5% acetonitrile with 0.1% acetic acid content at 1 ml/min flow-rate. The organic and aqueous components were mixed on-line. The eluent was split 1:1 before entering the mass spectrometer. The injected sample volume was 50 μl.

Electrospray ionisation (ESI) MS–MS was carried out on an API 365 tandem mass spectrometer (Perkin-Elmer Sciex Instruments, Foster City, CA, USA). The TurboIon Spray™ source was operated at 4.5 kV, the nebulizer N_2 gas flow was 12 units, the curtain gas flow-rate was fixed at 12 units and the heater gas, set at 350°C had a flow-rate of 7 l/min.

The spectrometer was operated in the multiple reaction monitoring (MRM) mode and was set to admit the protonated molecules $[M+H]^+$ at *m/z* 302.2 (deramciclane), 288.2 (*N*-desmethylderamciclane), 308.2 ($[^2H_6]$deramciclane) and 293.0 ($[^2H_5]$*N*-desmethylderamciclane) via the first quadrupole (Q1). After collision-induced fragmentation of these ions in quadrupole Q2 (N_2 gas flow of 2 units) the *m/z* 213.0 (deramciclane, *N*-desmethylderamciclane, $[^2H_6]$deramciclane) and the *m/z* 218.2 ($[^2H_5]$*N*-desmethylderamciclane) product ions were monitored. Since three compounds out of four had the same product ion mass there was a chance of ''cross talk'' during MRM experiments, i.e., the Q2 quadrupole where fragmentation takes place could still contain some products of a previous parent ion when the other is being fragmented and detected. This type of cross talk could be avoided by using the ''Q2 purge'' option in the controlling software (MassChrom 1.1 software, Perkin-Elmer Sciex Instruments).

For sample preparation a Movemaster RV-M1 laboratory robot (Mitsubishi Electric Europe, Ratingen, Germany) was used. The environment of the robot consisted of two automatic burettes, Radelkis OP-930 (Budapest, Hungary) and Metrohm 665-Dosimat (Herisau, Switzerland) and a Reax2000 laboratory vortex mixer (Heidolph, Kelheim, Germany). A Sigma 204 (Sigma, Osterode am Harz, Germany) laboratory centrifuge was used to separate the two phases after liquid–liquid extraction. The organic solvent was evaporated in a P207 type test tube thermostat (MTA Kutesz, Budapest, Hungary).

2.4. Preparation of deramciclane and N-desmethylderamciclane standard solutions

A 25.0-mg amount of deramciclane and 25.0 mg *N*-desmethylderamciclane were dissolved in 25 ml of methanol. The standard solutions (2,10, 50, 100, 200, 500 and 1000 ng/ml) used for spiking calibration samples were prepared from this 1 mg/ml stock solution by dilution with methanol. During the calibration 50 μl of the standard solutions was added to 950 μl plasma.

A similarly prepared separate stock solution of the same concentration was used for the preparation of quality control samples after dilution to 1 μg/ml concentration with methanol. Quality control (QC) samples were prepared in the following way. The highest concentration quality control sample (25 ng/ml) was prepared by a 40-fold dilution of the 1 μg/ml standard solution with blank pooled plasma. QC samples of 5 and 0.5 ng/ml were prepared by consecutive dilution with blank plasma starting from the 25 ng/ml QC.

2.5. Preparation of [$^2H_6$]deramciclane and [$^2H_5$]N-desmethylderamciclane standard solutions

A 1 mg/ml stock solution of the two internal standards was prepared in the same way as the deramciclane and *N*-desmethylderamciclane stock solution. By further dilution with methanol a 100 ng/ml standard solution was obtained, which was used for spiking the calibration, quality control and human samples during the pharmacokinetic study.

2.6. Sample preparation

The sample preparation was carried out with an earlier developed automated, robot-based set-up [14]. The sample preparation involved the following steps:

(i) A 200-μl volume of 0.25 *M* ammonium hydroxide was added to 1 ml sample to adjust the pH to 9.6.

(ii) A 50-μl volume of internal standard solution (100 ng/ml) was pipetted into the sample.

(iii) The mixture was homogenised in a vortex mixer for 10 s.

(iv) A 3-ml volume of extraction solvent mixture (*n*-hexane–ethyl acetate, 95:5, v/v) was added and the sample was vortex-mixed for efficient extraction for 5 min.

(v) Samples were centrifuged at 1400 *g* for 15 min at room temperature.

(vi) A 2-ml volume of the organic phase was transferred to a silylated test tube and evaporated under an N_2 atmosphere at 50°C.

The robot carried out all the above steps except for centrifugation. The final dried sample was dissolved manually in 200 μl of the chromatographic eluent and transferred to the autosampler.

3. Method of validation

3.1. Linearity of calibration curve

Linearity was studied by analysing spiked plasma samples at seven (0.1, 0.5, 2.5, 5, 10, 25, 50 ng/ml) concentrations on 6 different days. The ratio of the peak areas of the analytes relative to their respective internal standard was plotted against the plasma concentration ratios. A straight line was fitted to the calibration points using the least-square method with $1/y^2$ weighting. For integration of peak areas and regression analysis of the calibration curves MacQuan 1.6 software (Perkin-Elmer Sciex Instruments) was used.

3.2. Intra-day variability

Intra-day repeatability and accuracy were determined analysing five replicate plasma standards at three concentration levels (0.5, 5, 25 ng/ml). The average, the relative standard deviation (RSD) and accuracy were calculated at each concentration.

3.3. Inter-day variation

Inter-day precision data were calculated from the results of the calibration standards measured on 6 different days.

3.4. Extraction recovery

Percentage recovery of the liquid–liquid extraction was determined by comparing the detector response of an extracted plasma sample with that of a directly injected aqueous standard. Recoveries were determined at the 0.5, 5, 25 ng/ml concentration levels.

3.5. System suitability test

This test was performed by making five replicate injections into LC–MS–MS from extracted plasma samples at three concentration levels (0.5, 5, 25 ng/ml).

3.6. Stability of deramciclane and its metabolite in extracted samples and after multiple freezing and thawing cycles

The concentration of deramciclane and its metabolite was determined in extracted samples at three concentration levels (0.5, 5, 25 ng/ml) with three replicates, immediately following their preparation and after storage at room temperature (20°C) for 12 h.

The stability of the compounds was determined also after three and five freeze and thaw cycles at three concentration levels (0.5, 5, 25 ng/ml) with three replicates. The stability of deramciclane and its metabolite was calculated by comparing the con-

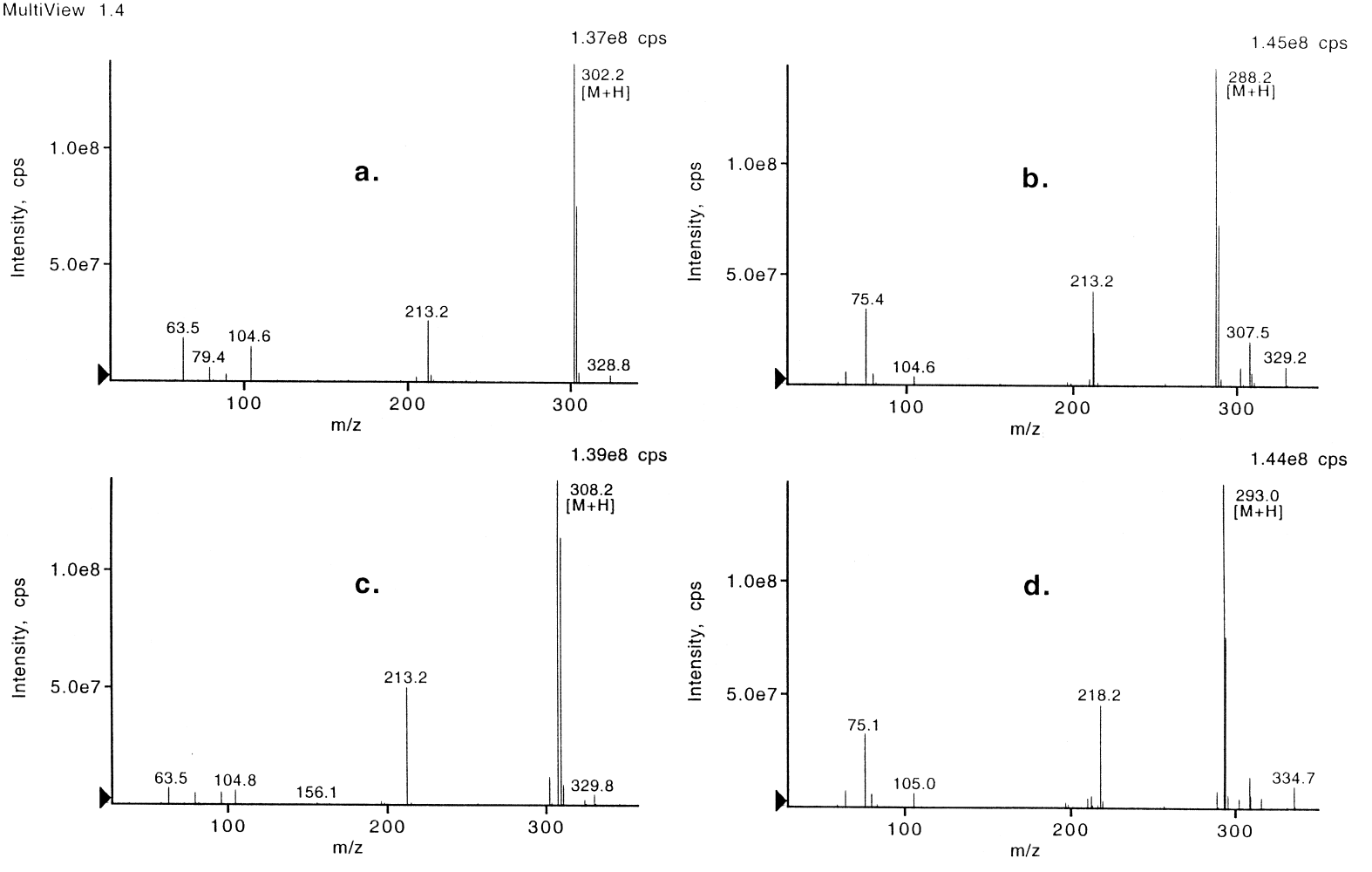

Fig. 2. The ESI-MS spectra of deramciclane (a), *N*-desmethylderamciclane (b), [$^2H_6$]deramciclane (c) and [$^2H_5$]*N*-desmethylderamciclane (d).

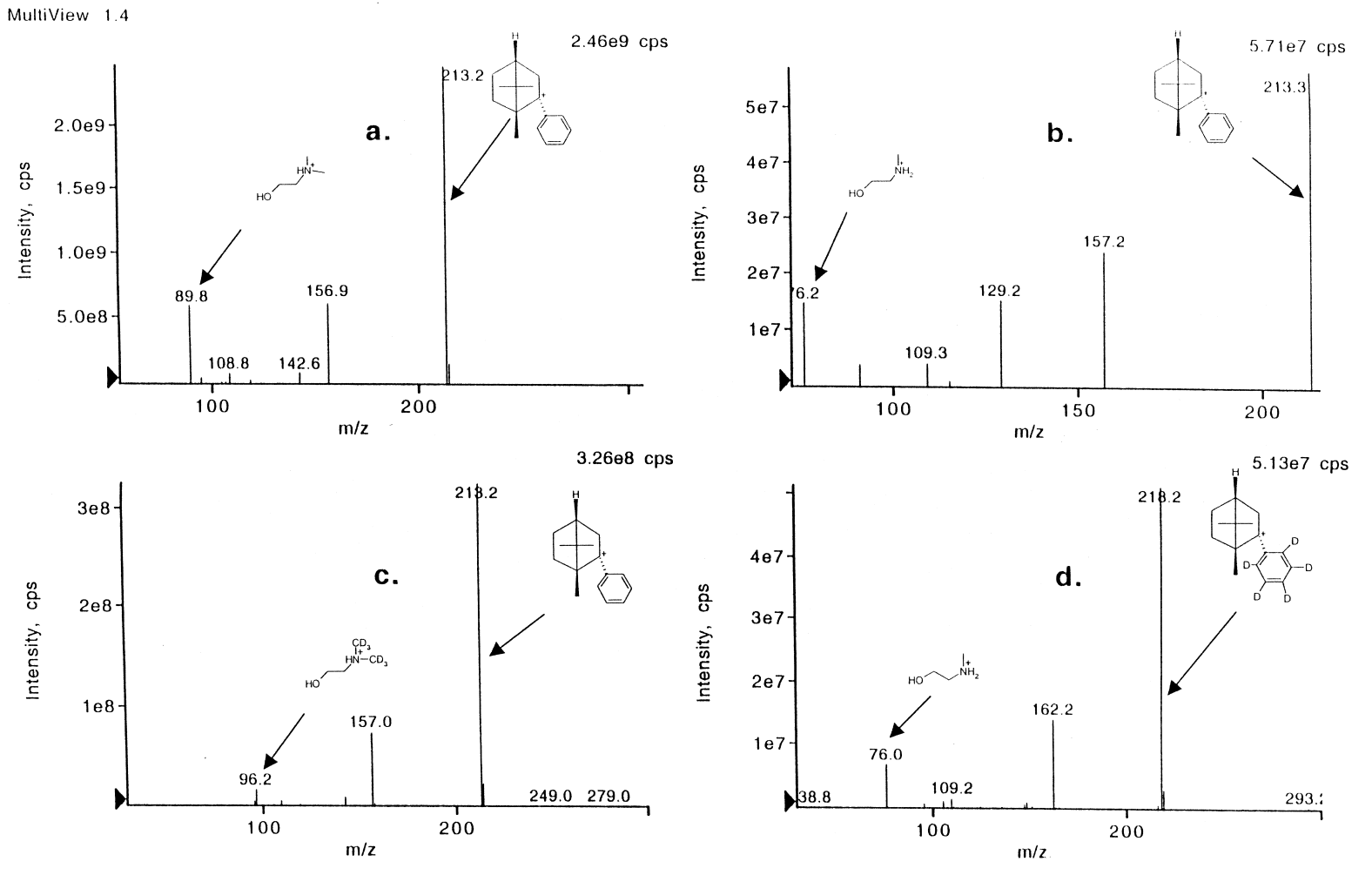

Fig. 3. The product ion spectra of deramciclane (a), *N*-desmethylderamciclane (b), [$^2H_6$]deramciclane (c) and [$^2H_5$]*N*-desmethylderamciclane (d).

Table 1
Linearity data ($n=6$) for deramciclane and *N*-desmethylderamciclane

	Slope		Intercept		Correlation coefficient	
	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
Deramciclane	1.03	4.10	0.02	46.00	1.000	0.05
N-Desmethylderamciclane	0.77	10.40	0.01	29.60	0.999	0.04

centrations of the freshly prepared samples and the samples involved in the stability study.

3.7. Extension of the calibration by diluting with plasma

In order to test the possibility of measuring samples that exceed 50 ng/ml, the following experiments were carried out. Three replicates were spiked to 75 ng/ml and 100 ng/ml concentrations and these samples were diluted with equal volume of blank plasma. Each sample was analysed and its concentration was determined using the daily calibration.

4. Results and discussion

The ESI-MS spectra of deramciclane, *N*-desmethylderamciclane and the deuterated internal standards

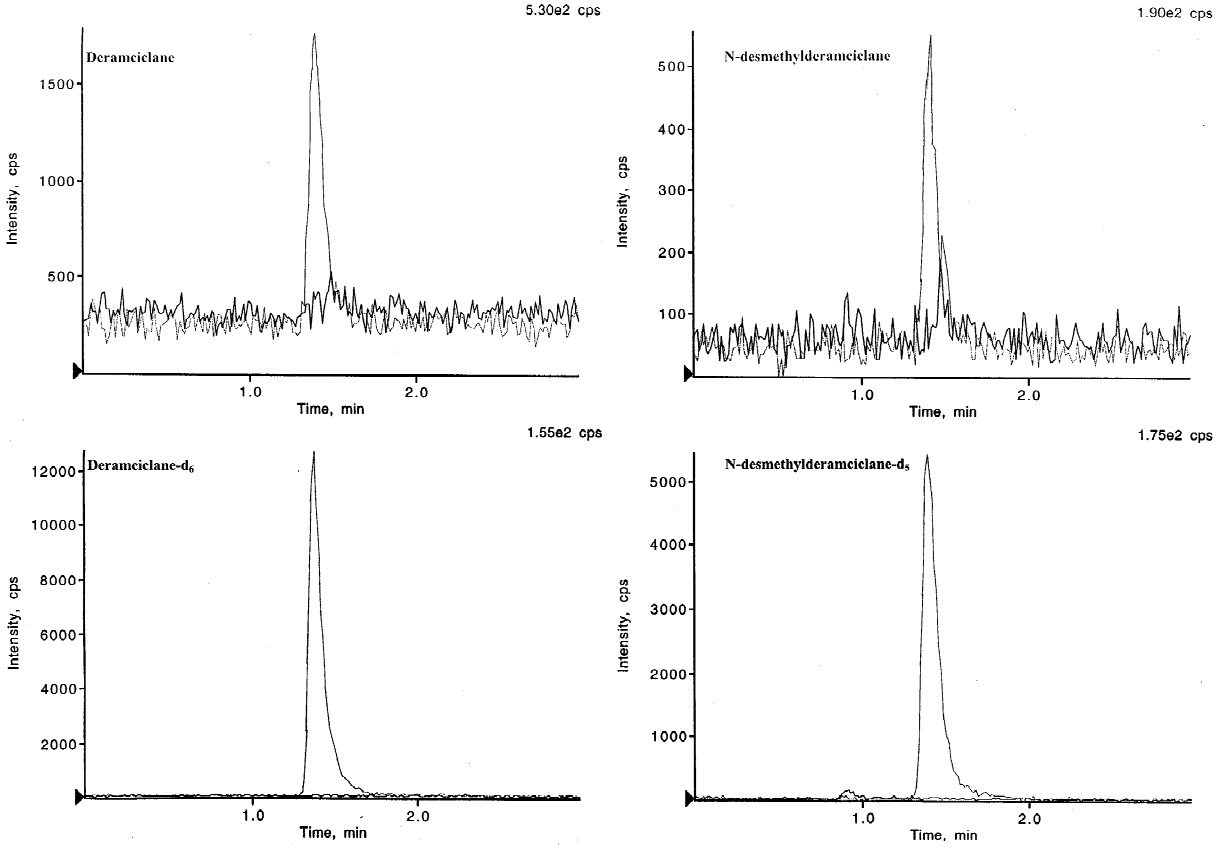

Fig. 4. Ion chromatograms (XIC) of blank plasma (solid line) and a plasma spiked to 0.1 ng/ml with deramciclane and *N*-desmethylderamciclane, and 5 ng/ml internal standards (dashed line).

are shown in Fig. 2. The fragment ions, which were generated by collision-induced fragmentation of the molecular ions are shown in Fig. 3. The mass spectra were obtained by infusing 1 μg/ml standard solutions into the MS–MS system. The identified fragments of the studied compounds are shown in Fig. 3.

The response of both deramciclane and *N*-desmethylderamciclane were linear with respect to their concentrations in plasma within the range of 0.1 ng/ml to 50 ng/ml.

The statistics of the calibration data for the two compounds are shown in Table 1.

In Fig. 4 the extracted ion chromatogram (XIC) of blank plasma and a plasma sample spiked to 0.1 ng/ml deramciclane and *N*-desmethylderamciclane, and 5 ng/ml of each internal standard are presented. It can be seen that the chromatogram of the blank plasma does not contain any measurable interfering peaks at the retention times of the four compounds. This proves that the method is appropriately selective. Fig. 5 presents the ion chromatograms of a blank plasma sample spiked only with the internal standards (5 ng/ml each) and Fig. 6 shows the ion chromatograms of a high concentration (50 ng/ml) plasma standard without the addition of internal standards. These chromatograms prove that neither the analytes contain any measurable internal standard (or other compounds interfering with the measurement of the internal standards), nor the internal standards contain any measurable quantities of the analytes.

Intra-day precision and accuracy data for the two compounds are listed in Table 2. Good precision was obtained for both compounds (<5%) in three different concentrations and the intra-day accuracy was smaller than the level of acceptance (15%). Intra-day

Table 2
Intra-assay validation data (n=5)

	Nominal concentration (ng/ml)	Average calculated concentration (ng/ml)	Precision (%)	Accuracy (%)
Deramciclane	0.5	0.569	2.07	113.76
	5	5.154	1.85	103.08
	25	24.530	2.57	98.12
N-Desmethylderamciclane	0.5	0.535	2.24	107.05
	5	5.053	2.04	101.06
	25	24.694	2.59	98.78

Table 3
Inter-assay validation data (n=6)

	Nominal concentration (ng/ml)	Average calculated concentration (ng/ml)	Precision (%)	Accuracy (%)
Deramciclane	0.1	0.098	1.53	101.67
	0.5	0.519	3.84	96.13
	2.5	2.527	2.78	98.93
	5	7.858	1.75	102.84
	10	9.888	3.25	101.12
	25	25.470	1.03	98.12
	50	49.802	2.04	100.40
N-Desmethylderamciclane	0.1	0.099	0.83	101.33
	0.5	0.526	3.81	94.80
	2.5	2.515	4.51	99.42
	5	4.837	3.01	103.26
	10	9.758	1.80	102.42
	25	25.578	2.15	97.69
	50	50.211	1.43	99.58

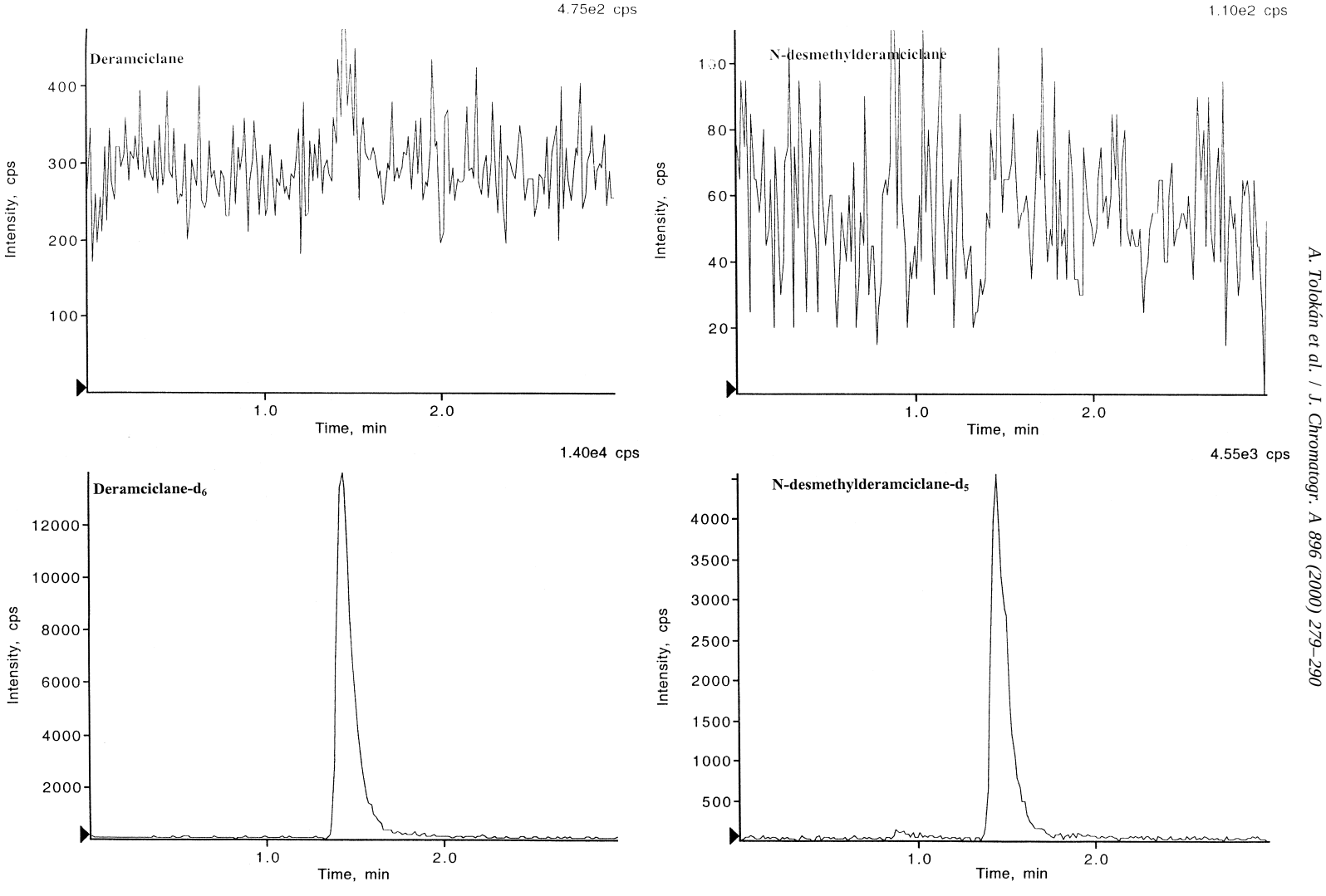

Fig. 5. Ion chromatograms (XIC) of a blank plasma spiked with 5 ng/ml internal standards.

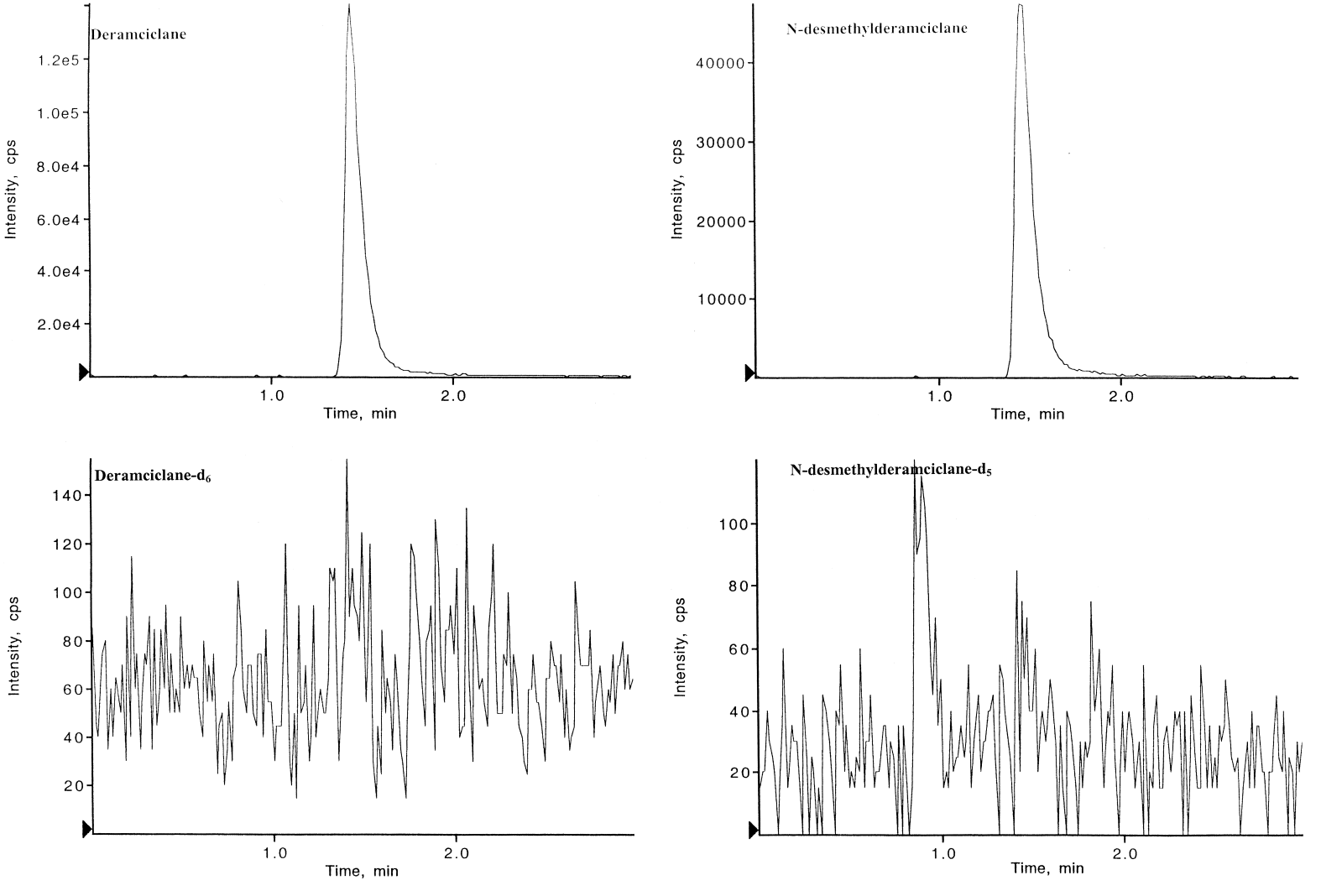

Fig. 6. Ion chromatograms (XIC) of a blank plasma spiked with 50 ng/ml deramciclane and *N*-desmethylderamciclane.

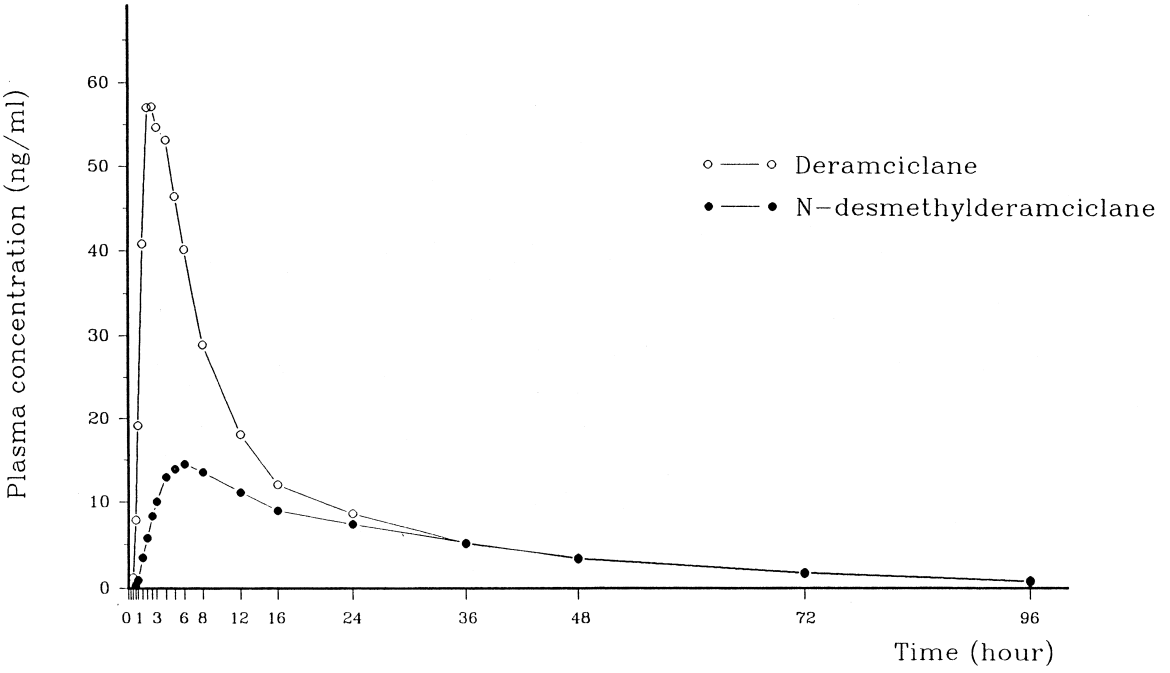

Fig. 7. Pharmacokinetic curves of deramciclane and *N*-desmethylderamciclane in a healthy volunteer after oral administration of a 30 mg deramciclane tablet.

precision was between 1.85 and 2.57% for deramciclane and between 2.04 and 2.59% for the metabolite, while the accuracy values fell between 98.12 and 113.76% and between 98.78 and 107.05%, respectively.

Using the data generated in the linearity study, the inter-day precision and accuracy of the method were also calculated. The inter-day precision of the method for both compounds is smaller than the level of acceptance (15%). Inter-day precision values were between 1.03 and 3.84% for the deramciclane and between 0.83 and 4.52% for the metabolite. Inter-day accuracy values were in the range of 96.13–102.84% for deramciclane and 94.80–103.26% for the metabolite. In Table 3 the inter-day validation data are summarised for the two compounds.

Based on the results of the assay validation, the lower limit of quantitation (LLOQ) for both deramciclane and *N*-desmethylderamciclane was 0.1 ng/ml. At this concentration level the precision and accuracy values are lower than the limit of acceptance (20%).

The recovery values for deramciclane were between 61.6 and 88.5% and 49.6 to 64.0% for the metabolite.

The system suitability test performed at three concentration levels gave good precision values for both compounds, between 1.20 and 3.87%.

The stability tests showed that both compounds are stable in extracted samples at room temperature for 12 h. The difference between the concentration of freshly prepared samples and stored samples was smaller than 3% for both compounds, which is far below the limit of acceptance (±10%). The results of the freeze–thaw stability test show that both compounds were stable even after five freeze–thaw cycles. The concentration relative to freshly prepared samples was in the range of −7.48 to 7.97% for deramciclane and between −6.16 and 7.61% for the metabolite.

The extension of calibration can be achieved by dilution with blank plasma; the accuracy values of the 75 ng/ml (−6.64 and −10.28%) and 100 ng/ml (−5.22 and −9.02%) were lower than the limit of acceptance (±15%).

A food interaction study of deramciclane has been carried out on 18 healthy male, non-smoking volunteers. The variation of the individual plasma levels of deramciclane and *N*-desmethylderamciclane with time is shown in Fig. 7, in the case of one volunteer.

5. Conclusion

The LC–ESI-MS–MS method described in this paper is suitable for the determination of low levels of deramciclane and *N*-desmethylderamciclane in human plasma. The sample preparation was successfully automated using a laboratory robot. The method was validated and is sufficiently sensitive and specific for the quantitation of plasma deramciclane and *N*-desmethylderamciclane levels. The elaborated method was successfully used in a human food interaction study and in a human and mouse pharmacokinetic study.

Acknowledgements

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