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First enantioseparation and stereoselective metabolism of carisoprodol in rat liver microsomes

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ABSTRACT

Carisoprodol, a widely prescribed muscle relaxant, is primarily metabolized into its main active metabolites, meprobamate and hydroxy carisoprodol. Despite its frequent use, the pharmacokinetics of carisoprodol have not been extensively studied, with the majority of research focusing on meprobamate. Moreover, even though carisoprodol has a stereogenic center, no chiral separation of carisoprodol has ever been reported in the literature. The present study describes the first enantioseparation of carisoprodol by a heart-cut bidimensional achiral-chiral chromatographic method in reversed-phase mode with a high-resolution mass spectrometry detector. The method was applied to the calculation of the depletion kinetics of the two enantiomers after incubation in rat liver microsomes. The results revealed that hepatic microsomes metabolize one enantiomer at a significantly faster rate than the other. The work provides valuable insights into the underexplored metabolism of carisoprodol, laying the ground for future pharmacological studies on the single enantiomers.

1. Introduction

Carisoprodol is a muscle relaxant developed in 1959 by Dr. Frank M. Berger as a safer, less addictive alternative to meprobamate, its active metabolite with sedative properties known for its abuse potential [1]. Chemically designated as (2*RS*)-2-[(carbamoyloxy)methyl]-2-methylpentyl(1-methylethyl)carbamate, carisoprodol's safety profile has raised concerns. Indeed, it has been classified as a Schedule IV controlled substance since 2012 in the United States. Moreover, several European countries—beginning with Sweden in 2007—have withdrawn it from the market due to its involvement in impaired driving and overdose cases, prompting the European Medicines Agency (EMA) to recommend its suspension [2–4]. More recently, the WHO's 47th ECDD proposed adding carisoprodol to Schedule IV of the 1971 Convention on Psychotropic Substances [3].

Carisoprodol is administered orally, with typical adult doses of 250–350 mg taken up to four times daily. It has a rapid onset of action (30–60 min), reaches peak plasma levels within 1.5–1.7 h, and has high oral bioavailability (~92 %) [5–7]. Its primary route of metabolism is via the hepatic enzyme CYP2C19, which transforms it into meprobamate—a pharmacologically active metabolite with a much longer half-life (~10 h) [8]. Interindividual variability in CYP2C19 activity significantly affects both carisoprodol exposure and meprobamate formation [9–11], and additional factors such as drug interactions (e.g., with oral contraceptives or enzyme inducers/inhibitors) can further alter its pharmacokinetics [12].

Although meprobamate is thought to mediate much of carisoprodol's effect through GABA-A receptor modulation, carisoprodol itself also exerts direct CNS activity independent of its metabolite [13,14]. Another metabolite, hydroxy carisoprodol, has been detected in both plasma and urine, but little is known about its pharmacological role or structure [3,15–17]. Analytical challenges, such as low UV absorbance and thermal instability, have made detection of carisoprodol and its metabolites difficult. Modern techniques like LC-MS/MS and HRMS are

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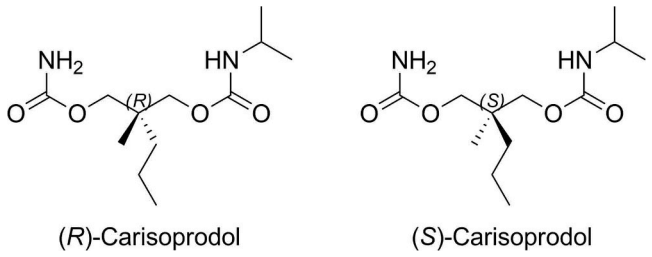

Fig. 1. Structure of carisoprodol enantiomers. The absolute stereochemistry is indicated on the stereogenic centre.

now preferred for analyzing these compounds in biological matrices [3, 18].

Importantly, carisoprodol contains a chiral center, existing as a racemic mixture of two enantiomers, (*R*)-carisoprodol and (*S*)-carisoprodol (Fig. 1) [3]. Despite the well-known influence of chirality on drug efficacy and safety, no previous studies have reported the enantioseparation of carisoprodol or investigated the individual behavior of its enantiomers. Given the potential for stereoselective metabolism by CYP2C19, such studies are critically needed.

In a previous work (data submitted), the metabolic profile of carisoprodol has been elucidated by means of LC-HRMS/MS and software-assisted metabolite identification following incubation in rat liver microsomes. In this context, the present work aimed to develop a bidimensional achiral-chiral LC-HRMS method, which enabled for the first time the enantioseparation of carisoprodol. Moreover, a microsomal incubation assay allowed to investigate the stereoselective metabolism of this drug by monitoring the depletion of the individual enantiomers over the time.

2. Materials and methods

2.1. Chemicals and materials

LC-MS grade acetonitrile (ACN) and formic acid (FA) were bought from Honeywell (Charlotte, NC, USA). A water purification system (Direct-Q 3UV, Merck Millipore, Milan, Italy) was employed to obtain ultrapure water for LC-HRMS analyses. Analytical standard of racemic carisoprodol, racemic carisoprodol-d_7 (internal standard), and testosterone (positive control) were purchased from Sigma Aldrich Merck (Darmstadt, Germany).

Stock solutions of KH_2PO_4 and K_2HPO_4 (0.2 M) (Sigma Aldrich Merck) were used to prepare a 0.1 M potassium phosphate buffer by adding an appropriate amount of Milli-Q water. The cofactor nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from BLD Pharmatech GmbH (Reinbek, Germany).

Rat (Sprague Dawley) liver microsomes (0.5 mL) pooled from twenty-five donors (protein concentration 20 mg/mL), was purchased from the Gibco product line of Thermo Fisher Scientific (Carlsbad, CA, USA) and stored at −70°C.

2.2. Analytical conditions

2.2.1. Achiral LC-HRMS analysis

LC-HRMS analyses were performed on a Thermo Fisher Scientific (Waltham, MA, USA) Ultimate 3000 ultra-high performance liquid chromatograph (UHPLC) interfaced to a heated electrospray ionization (HESI) source of a Q-Exactive quadrupole-Orbitrap high-resolution mass

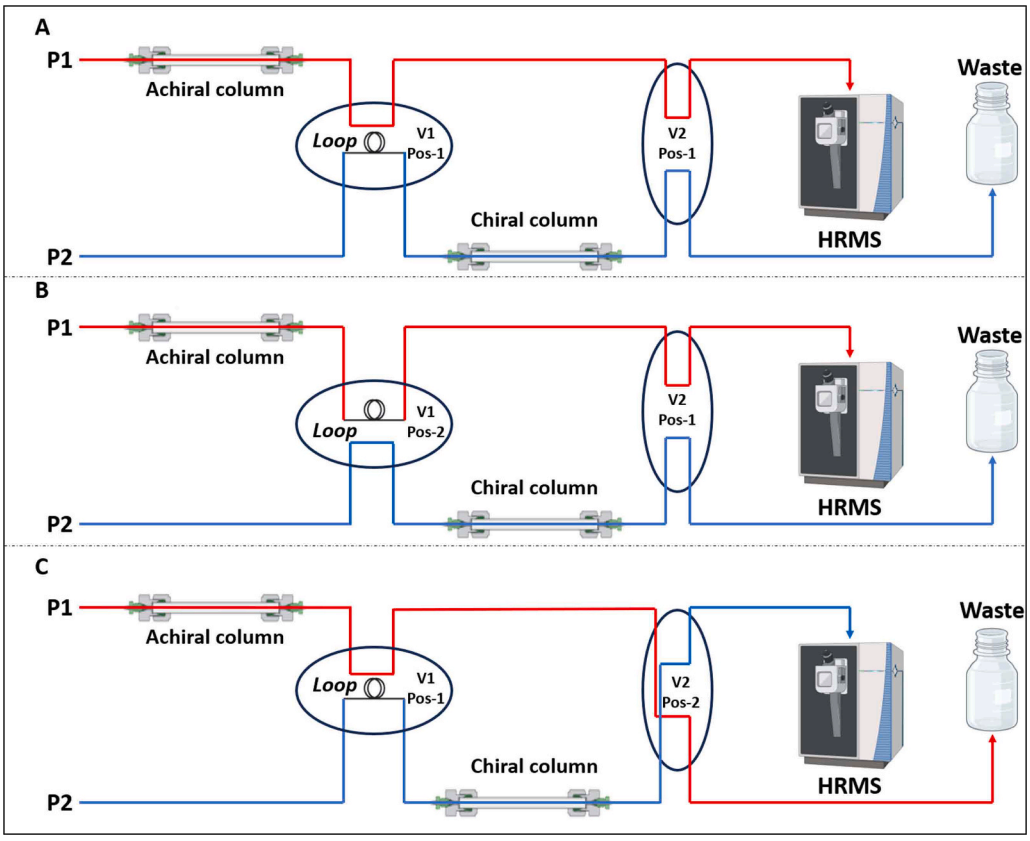

Fig. 2. Three-step configuration of the divert valves of the bidimensional heart-cut achiral-chiral LC-HRMS method. A) with V1 and V2 in Pos-1 the eluent from P1 is directed to the HRMS detector and that from P2 to the waste; B) At 12.2 min V1 changes to Pos-2 to trap the peak into the 100-µL loop, while V2 remains unchanged; C) at 12.4 min V1 is switched back to Pos-1 to connect the loop to P2 and simultaneously V2 is switched to Pos-2 to carry the eluent from the chiral column to the HRMS detector and the one from the C_{18} column to the waste.

A

Run	ACN (%)	T (°C)	Resolution	ACG Target	IT Target (ms)	R_T (min) t_1	R_T (min) t_2	k_1	k_2	α	R_S
1	50	25	70000	3E+06	250	5,28	5,86	0,53	0,70	1,32	1,57
2	50	25	140000	3E+06	500	5,30	5,87	0,54	0,71	1,31	1,72
3	50	37	140000	3E+06	500	5,18	5,63	0,51	0,64	1,26	1,52
4	50	37	140000	1E+06	500	5,00	5,35	0.45	0.56	1,22	1,42
5	50	8	140000	1E+06	500	5,67	6,61	0,65	0,92	1,42	1,19
6	40	37	140000	1E+06	500	6,74	7,48	0,96	1,17	1,22	1,85
7	**40**	**37**	**140000**	**3E+06**	**500**	**6,63**	**7,33**	**0,93**	**1,13**	**1,22**	**1,98**
8	40	45	140000	3E+06	500	6,28	6,79	0,83	0,97	1,18	1,61

B

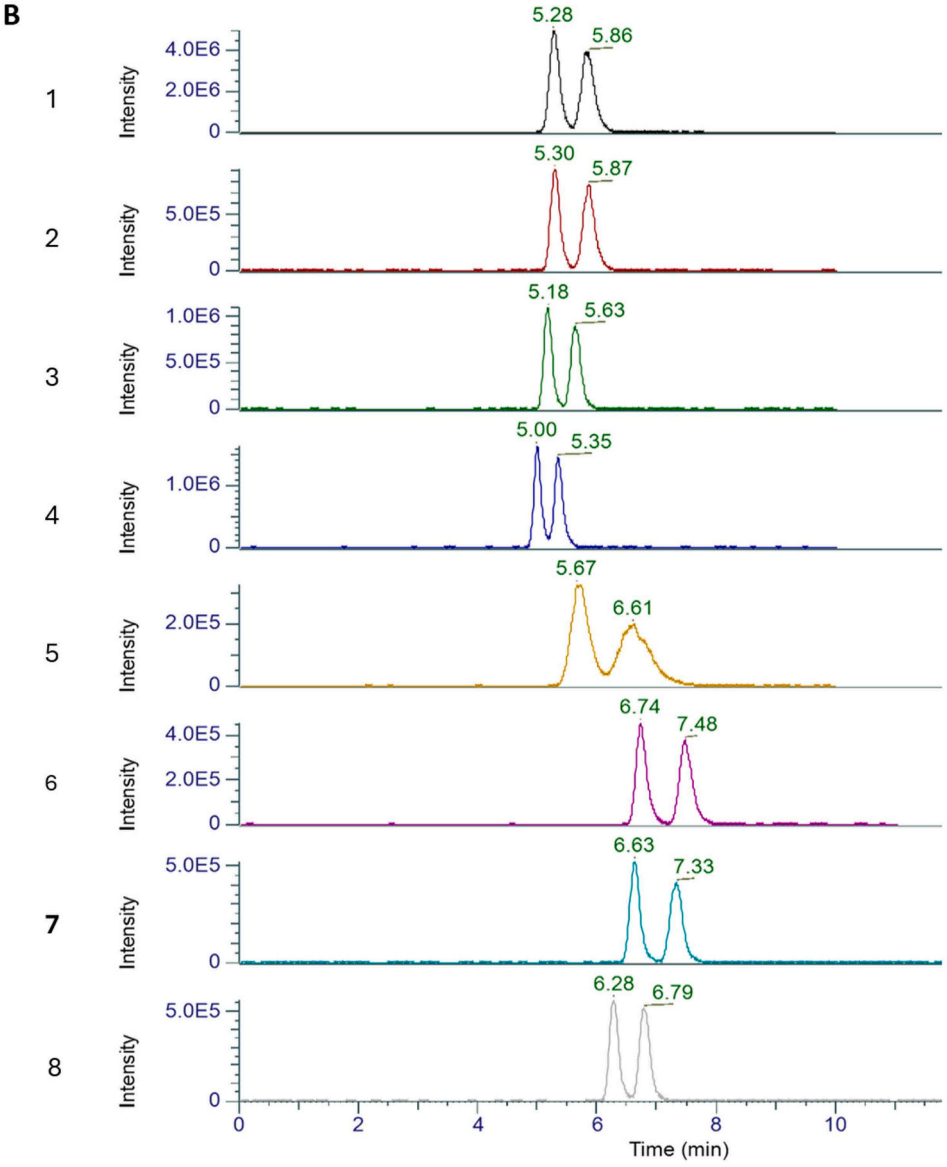

Fig. 3. Optimization of the chromatographic conditions for the offline chiral separation of carisoprodol. A) Parameters evaluated during the optimization step. B) LC-HRMS traces ($[M+H]^+$ at m/z 261.1804) for each optimization run.

spectrometer. The UHPLC system included a binary pump, a vacuum degasser, a thermostated autosampler set at 7 °C, and a thermostated column compartment set at 25 °C.

The achiral chromatographic separations were achieved on a Poroshell 120 EC-C18 (100 × 3.0 mm I.D., 2.7 μm) with a guard (5 × 3 mm I.D., 2.7 μm) (both from Agilent Technologies, (Santa Clara, CA, USA) using a flow rate of 0.5 mL/min. A gradient elution of water and ACN (both with 0.1 % formic acid) was employed for the chromatographic separations according to the following program: linear increase from 5 % to 95 % ACN (2.0–20.0 min), 95 % ACN isocratic elution

(20.1–25.0 min), and re-equilibration at 5 % ACN (25.1–30.0 min).

The HESI source parameters were set as follows: capillary temperature, 320 °C; vaporizer temperature, 300 °C; voltage, 3.8 kV; sheath gas, 55 relative intensity of the ions; auxiliary gas, 30 au; S lens RF level, 55 au. Analyses were acquired using Xcalibur 3.0 software (Thermo Fisher Scientific) in full-scan (FS) mode over an *m/z* range of 66.7–750, while QC samples were analysed in data-dependent acquisition (DDA) to generate fragmentation spectra. The automatic gain control (AGC) target in FS mode was set to 3×10^6, with a resolution of 140,000 at full width at half maximum (FWHM) at 15 s. The maximum injection time (IT) was 500 ms and the isolation window was set at *m/z* 0.7. In DDA mode the AGC target was set to 5×10^5, with a minimum threshold of 1×10^3, a resolution of 17,500, a maximum IT of 100 ms, and an isolation window of *m/z* 0.4. The normalized collision energy (NCE) was set to 20 AU, with a loop count of 2 and a dynamic exclusion duration of 5.0 s. The raw data was processed using FreeStyle 1.8 (Thermo Fisher Scientific).

2.2.2. Enantioseparation of carisoprodol

The optimization of the chromatographic conditions for the enantioseparation of carisoprodol was carried out offline on the same LC-HRMS system described above by screening various chiral stationary phases (CSPs) including CHIRALCEL OD-R [cellulose tris (3,5-dimethylphenylcarbamate)] (250 × 4.6 mm I.D., 10 μm), CHIRALCEL OB-H [cellulose tribenzoate] (250 × 4.6 mm I.D., 5 μm), CHIRALCEL OJ-H [cellulose tris (4-methylbenzoate)] (250 × 4.6 mm I.D., 5 μm), CHIRALPAK AD [amylose tris (3,5-dimethylphenylcarbamate)] (250 × 4.6 mm I.D., 10 μm), and CHIRALPAK AD-RH [amylose tris (3,5-dimethylphenylcarbamate)] (150 × 4.6 mm I.D., 5 μm) (all from Daicel Chiral Technologies Europe S.A.S, Illkirch, France). The pure analytical standard at 10 μg/mL in microsomal matrix was used for the optimization step and the enantiomers were eventually separated with 40 % ACN with 0.1 % FA at a flow rate of 0.5 mL/min on the Chiralpak AD-RH at 37 °C.

2.2.3. Bidimensional heart-cut achiral-chiral LC-HRMS analysis

An online heart-cut bidimensional LC-HRMS/MS method was developed for the evaluation of the stereoselective metabolism of carisoprodol.

The microsomal sample was injected under the conditions previously described (Section 2.2.1) into the C_{18} achiral column. With both valve 1 (V1) and valve 2 (V2) in position 1 (Pos-1) the eluent from pump 1 (P1) was directed to the HRMS detector and that from pump 2 (P2) to the waste as shown in Fig. 2A. At the retention time (R_T) of carisoprodol the position of V1 was changed to position 2 (Pos-2) to trap the peak into the 100-μL loop, while the position of V2 remained unchanged (Fig. 2B). After a customized interval of 0.2 min, V1 was switched back to Pos-1 to connect the loop to the isocratic pump 2 (P2) and simultaneously V2 was switched to Pos-2 to carry the eluent from the chiral column to the HRMS detector and the one from the C_{18} column to the waste (Fig. 2C). Enantioseparation of carisoprodol on the second dimension was accomplished using the optimized conditions described in Section 2.2.2.

2.3. Incubation with pooled rat liver microsomes (RLM)

Carisoprodol was incubated with cryopreserved RLM as described in a previous work [19] using a validated protocol with minor changes where necessary [20]. Briefly, 26 μL of microsome suspension were gently mixed with 10 μL of carisoprodol (1 mg/mL stock solution) in phosphate buffer (864 μL) and cofactor NADPH 10 mM (100 μL) on an MTH-100 Thermo Shaker incubator (MIULAB, Hangzhou, China) at 37 °C (pH 7.4). Testosterone was used in the positive controls, while negative controls were obtaining by mixing all the reactants except for NADPH in accordance with the validated protocol described in Knights et al. [20]. At selected time points (0, 30, 60, 120, and 180 min), a 100-μL aliquot of incubates was quenched with 50 μL of ice-cold acetonitrile containing the racemic internal standard (IS) carisoprodol-d_7 (1 μg/mL) and centrifuged for 10 min at 13,000 × *g* with MiniSpin centrifuge (Eppendorf SE, Hamburg, Germany). Then, the supernatant was transferred into HPLC vials and a 5-μL sample volume was injected into the LC-HRMS system. Each incubation reaction was performed in triplicates.

2.4. Statistical analysis

The statistical analysis of raw data from microsome experiments was performed using GraphPad Prism 8.0.1. Specifically, the kinetics derived from the three replicates of each enantiomer were used as the basis for comparison between the two enantiomers. To this end, a *t*-test (k_{E1} vs. k_{E2}) was conducted to identify significant differences between their kinetics.

3. Results and discussion

3.1. Enantioseparation of carisoprodol

The chromatographic conditions for the resolution of carisoprodol enantiomers were optimized offline by screening various chiral stationary phases (CSPs), including Chiralcel OD, Chiralcel OJ-H, Chiralpak AD, Chiralcel OB-H, and Chiralpak AD-RH. A summary of the optimization results is presented in Fig. 3. The first column tested was Chiralpak AD-RH, an amylose-based phase (amylose tris(3,5-dimethylphenylcarbamate)), using an isocratic mobile phase composed of water and acetonitrile (ACN) in a 50:50 (v/v) ratio with 0.1 % formic acid. Initial analyses were performed at ambient column temperature. Although an acceptable resolution (R_S) was achieved, baseline separation was not obtained. Increasing the column temperature to 37 °C resulted in poorer separation, while lowering it to 8 °C led to distorted peak shapes without improving resolution.

A significant enhancement in separation, including baseline resolution, was achieved by reducing the ACN content to 40 % and maintaining the column temperature at 37 °C. Minor adjustments to the mass spectrometer settings (resolution, AGC target, and maximum injection time) resulted in only marginal performance differences. Although AGC does not affect chromatographic resolution, different settings were evaluated to assess their influence on signal quality and detectability of the enantiomers (Fig. 3). Additional CSPs were screened using the optimized or slightly modified conditions; however, no substantial improvements in peak shape or resolution were observed.

Under the optimal conditions, the first enantiomer (E1) eluted with a R_T of 6.63 min and an elution factor *k* of 0.93; the second (E2) eluted with a R_T of 7.33 min and an elution factor *k* of 1.13. The separation factor (α) and the resolution factor (R_S) were 1.22 and 1.98 respectively. No retention time shift was observed for carisoprodol-d_7 compared to non/labelled carisoprodol at the working flow rate.

3.2. Bidimensional heart-cut achiral-chiral LC-HRMS

A heart-cut bidimensional liquid chromatography approach was applied combining two methods: achiral analysis and chiral analysis. This strategy involved trapping a single peak from the achiral column in the first dimension and diverting the flow to a second chiral column with a post-column divert valve. The trapped compound could then be evaluated for its enantiomeric composition eliminating potential co-elution of ambiguous peaks that would otherwise be present with the direct analysis of the microsomal sample on the chiral column [21,22]. This approach justifies the use of the 2D achiral–chiral LC heart-cutting configuration, which enhances selectivity by removing matrix interferences prior to chiral analysis. By transferring only the analyte-containing fraction to the second dimension, this setup not only improves enantioseparation quality but also minimizes sample and solvent consumption, thereby increasing the overall efficiency of the

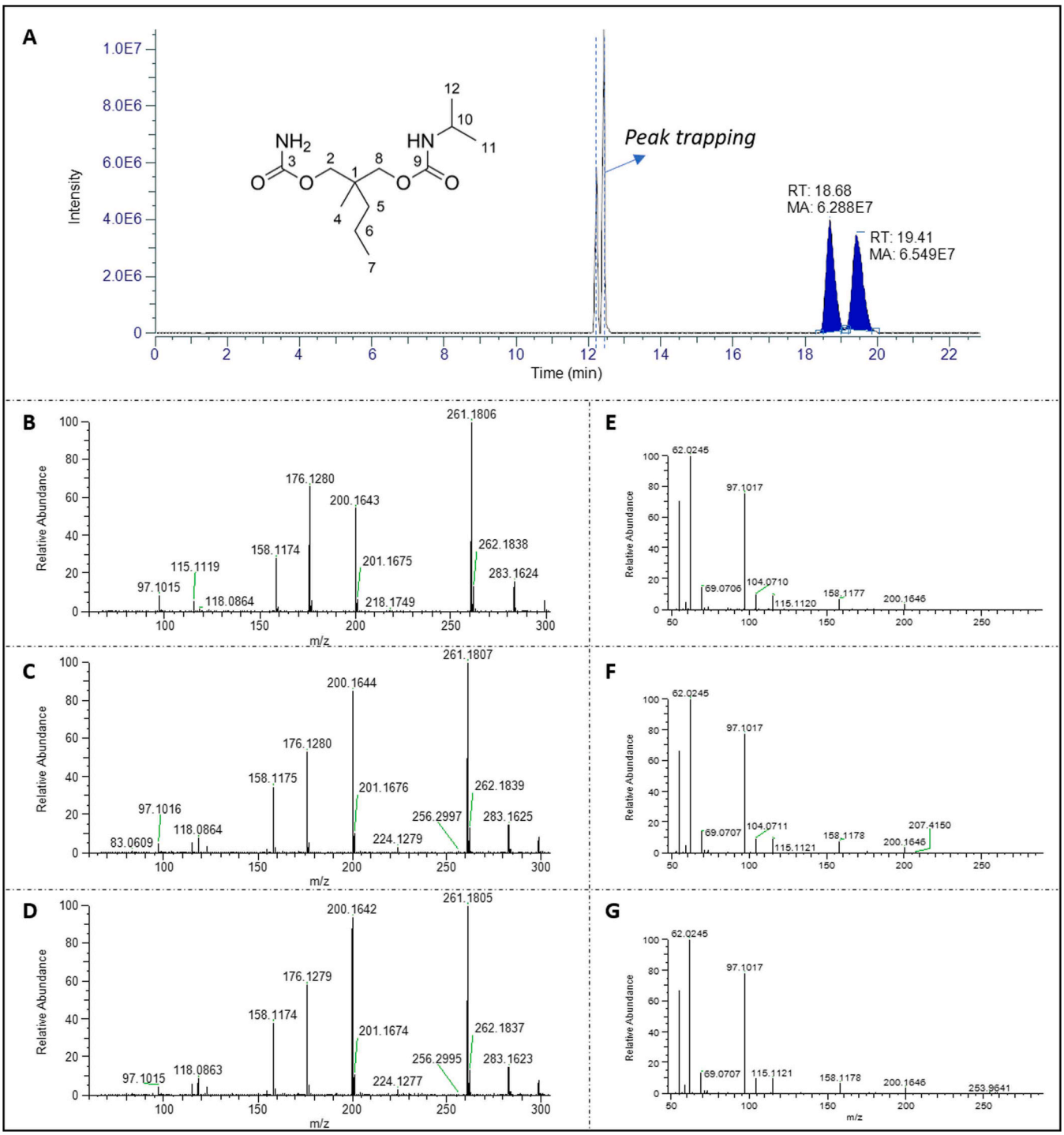

Fig. 4. Achiral-chiral LC-HRMS/MS analysis of carisoprodol. A) LC-HRMS chromatogram of carisoprodol with the bidimensional chromatographic method; B) MS^1 analysis of racemic carisoprodol; C) MS^1 analysis of E1; D) MS^1 analysis of E2; E) MS^2 analysis of racemic carisoprodol; F) MS^2 analysis of E1; G) MS^2 analysis of E2.

analytical process. Moreover, the use of reversed phase conditions in the chiral separation also ensured compatibility with HESI-HRMS detection. A scheme representative of the chromatographic settings employed is pictured in Fig. 2. Using the bidimensional chromatographic approach the peak of carisoprodol was trapped into the loop from the achiral column between 12.2 and 12.4 min and the two enantiomers eluted on the chiral column at 18.68 and 19.41 min as shown in Fig. 4A. The MS^1 analysis of carisoprodol on the first achiral dimension confirmed previously reported findings on the in-source fragmentation pattern of the molecule (Fig. 4B) [19]. Indeed, the MS^1 spectrum showed the protonated molecular $[M+H]^+$ ion at *m/z* 261.1806 and the corresponding sodium adduct at *m/z* 283.1624, along with four fragment ions: two of these fragments, at *m/z* 200.1643 and 158.1174, were attributed to the cleavage of either the C2-O or C8-O bonds, respectively. Additionally, an ion at *m/z* 176.1279 resulted from the cleavage of the carbonyl C3-O bond, while an ion at *m/z* 97.1015 was attributed to the simultaneous cleavage of both C2-O and C8-O bonds (data submitted). The MS^1 analyses of E1 and E2, shown in Figs. 4C and 4D respectively, exhibited a fragmentation pattern identical to that of the racemic compound. Similarly, the MS^2 spectra of the racemic compound

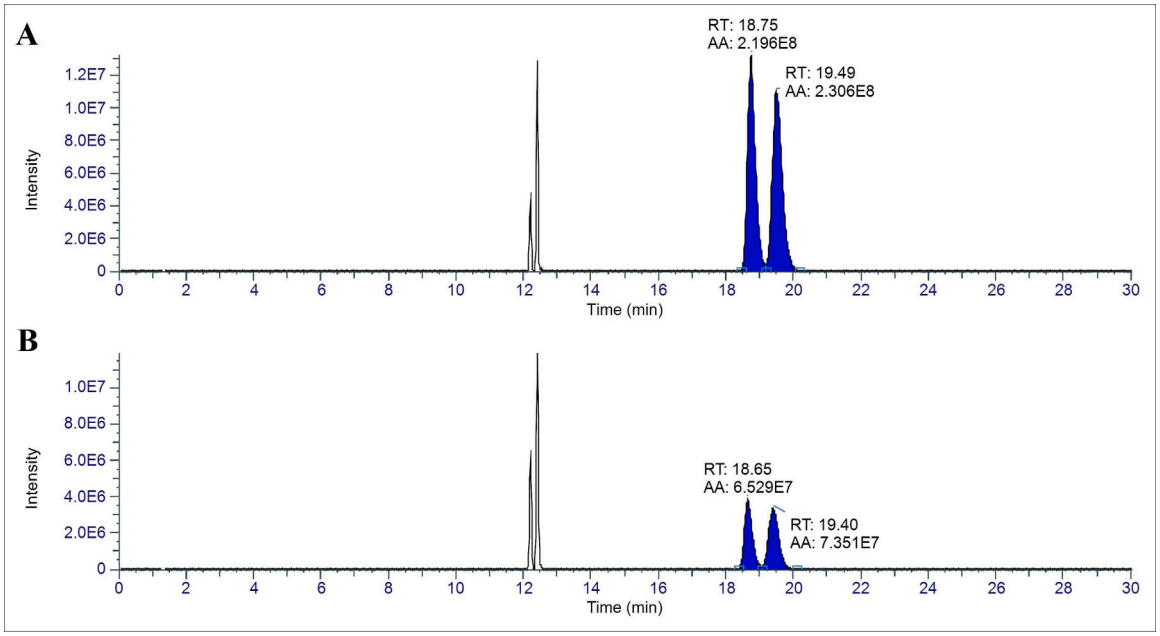

Fig. 5. Representative enantioselective chromatograms of carisoprodol after incubation with **RLM.** Chromatograms of carisoprodol enantiomers in rat liver microsome incubations at 0 min (upper) and 180 min (lower), acquired with the achiral–chiral 2D-LC-HRMS/MS method.

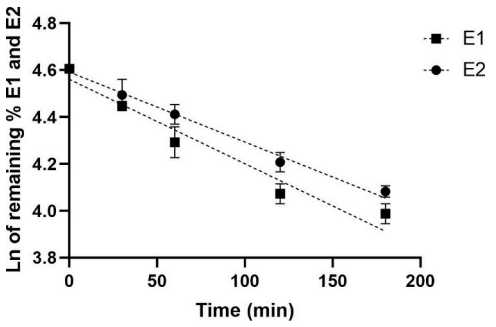

Fig. 6. Depletion kinetics of individual enantiomers of carisoprodol. Natural logarithm (ln) of the area depletion (%) of the individual enantiomers of carisoprodol vs. incubation time (min) in rat liver microsomes.

overlapped with those of the two enantiomers as shown in Fig. 4E, F, and G, respectively.

3.3. Stereoselective metabolism

To the best of the authors' knowledge, the chiral separation of carisoprodol has never been reported in the literature. Considering that enantiomers may exhibit different pharmacological activities and be metabolized in a stereoselective manner, it seemed appropriate to apply the developed bidimensional method to the evaluation of microsomal metabolism for each individual enantiomer. The method was specifically developed to achieve enantioseparation and to compare the relative degradation kinetics of carisoprodol enantiomers. No absolute quantification was performed.

By comparing the depletion of carisoprodol (normalized to the IS) in microsomal samples analyzed by bidimensional heart-cut achiral-chiral chromatography (Fig. 5), it was observed that the first-eluting enantiomer (E1, $R_T = 18.68$ min) underwent depletion at a slower rate than the second eluted enantiomer (E2, $R_T = 19.41$ min). The depletion kinetics of both enantiomers were compared in a graph plotting time (min) versus the logarithm of the percentage of carisoprodol depletion area and the two slope values ($k_{E1} = 0.0036$ and $k_{E2} = 0.0030$) differed significantly ($p < 0.0001$) (Fig. 6). These results suggested a stereoselective metabolism of carisoprodol driven by the CYP2C19 microsomal enzyme.

4. Conclusions

This study reports for the first time the enantioseparation of carisoprodol using a two-dimensional achiral-chiral LC-HRMS method, offering new insights into the chiral behavior of this clinically relevant yet controversial muscle relaxant. The developed method enabled accurate assessment of the enantiomeric composition after incubation in rat liver microsomes while minimizing matrix interferences from microsomal samples. Analysis of the incubates at different time points revealed a stereoselective metabolic profile, with the second-eluting enantiomer showing significantly slower depletion than its counterpart. Although the absolute configuration of each enantiomer has not yet been assigned, these findings highlight the importance of investigating the stereochemistry of carisoprodol and lay the groundwork for future pharmacokinetic, pharmacodynamic, and toxicological studies focused on its individual enantiomers.

CRediT authorship contribution statement

Cristian Caprari: Validation, Investigation, Formal analysis. **Elena Ferri:** Writing – original draft, Validation, Investigation, Formal analysis. **Marco Grasso:** Formal analysis, Data curation. **Loretta L. Del Mercato:** Funding acquisition. **Maria Angela Vandelli:** Resources, Data curation. **Giuseppe Cannazza:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Cinzia Citti:** Writing – original draft, Supervision, Project administration, Methodology, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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