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ScienceDirect
Journal of Chromatography B, 850 (2007) 507–514
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

A rapid method for the quantification of the enantiomers of Warfarin, Phenprocoumon and Acenocoumarol by two-dimensional-enantioselective liquid chromatography/electrospray tandem mass spectrometry

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Received 7 November 2006; accepted 22 December 2006
Available online 12 January 2007

Abstract

We describe a new fully validated enantioselective LC–MS/MS method for stereospecific quantification of both the racemic forms of Warfarin (WF), Phenprocoumon and Acenocoumarol in human plasma. Measurement specificity was assessed by using different blank donor plasma samples, where no interfering reagent peak appeared at the retention time (RT) of the targeted analytes. Response was linear for all analytes. Typical linear regression coefficients have >0.99. The recoveries ranged from 98% to 118%. Determinations in 10 normal healthy individuals revealed a high reproducibility of RTs. These findings confer to the method suitability for large population studies.

Keywords: Vitamin K-dependent factors; Venous thrombosis; Warfarin; Phenprocoumon; Acenocoumarol; enantiomers; LC–MS–MS

1. Introduction

Oral administration of anticoagulant drugs is one of the most employed therapies in clinical practice for prevention and treatment of patients with arterial and venous thrombosis [1].

Although 3 months time of oral anticoagulant therapy is sufficient to prevent recurrences, a lower rate of recurrent venous thromboembolisms has been observed in patients who were in treatment with oral anticoagulants for 6 months [2]. These findings suggested that a longer anticoagulation may be helpful [3], particularly in cases where venous thrombosis was not associated with a reversible risk factor [4]. Bleeding is by far the most important complication of the oral anticoagulation [5–9]. Much effort has been devoted to improve the safety of oral anticoagulation by adopting a series of measures (i.e. international normalized ratio [INR], lower anticoagulation levels, etc.), which have reduced major and minor haemorrhage rates. However, the bleeding risk remains significantly high [10]. Many studies have been carried out to identify high-risk patients [5–10]. The overall incidence of bleeding episodes was lower among individuals aged 70 years or less and in patients with lower intensity regimens of oral anticoagulation [9]. Life-threatening and fatal bleeding complications occurred more often in elderly patients [10].

In addition to narrow therapeutic interval and significant inter-individual variability in daily dose requirement, numerous drug–drug interactions often complicate treatment and over- or under anticoagulation frequently occur [11,12]. Careful monitoring of coagulation by measuring international normalized ratio of prothrombin time is necessary to tailor the treatment to individual patient, mainly due to complex pharmacokinetics.

Warfarin, Phenprocoumon and Acenocoumarol are the available oral anticoagulant drugs employed and required dose is variable, in particular between individuals but also within one individual, and depends on several factors, e.g. dietary intake (i.e. foods rich in vitamin K), variations in pharmaco-kinetics and -dynamics, compliance, etc. [13].

Beside acquired and environmental factors, it is well known that the response to oral anticoagulants is largely genetically

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doi:10.1016/j.jchromb.2006.12.050

determined [14]. Vitamin K epoxide reductase (VKORC1) recycles reduced vitamin K, which is essential for the post-translational gamma-carboxylation of vitamin K-dependent clotting factor II (prothrombin), VII, IX and X. Several rare mutations leading to amino acid changes in VKORC1 protein have been discovered in oral anticoagulants-resistant patients, suggesting that polymorphism in the VKRCO1 gene is associated with an inter-individual variability in the dose-anticoagulant effect of Warfarin [15–17].

Oral anticoagulants are clinically administered as a racemic mixture concentration of enantiomers that shows in blood marked difference in pharmaco-kinetics and pharmaco-dynamics because of stereoselective metabolism [18].

Several analytical methods for quantification in human plasma of racemic Warfarin, Phenprocoumon and Acenocoumarol have been reported. Some of them include high-performance liquid chromatography (HPLC) [19,20], coupled to either ultraviolet or fluorescence detection [20–23], or mass spectrometry [24,25]. Although these methods show a good sensitivity and specificity, nevertheless they require extraction procedures using solid phase extraction (SPE) with many laborious and time-consuming processes with a low suitability for routine analysis. Moreover, they were complicated by interfering endogenous compounds requiring several cleaning steps. Gas chromatography–mass spectrometry (GC–MS) method has also been used for detection and quantification of these anticoagulants [26,27]. These methods required a time-consuming derivatisation procedure [28,29]. None of these allow for the simultaneous analysis of Warfarin, Phenprocoumon and Acenocoumarol.

In hospital laboratories where large volumes of patient samples are daily analysed, it is often required that a method preferably has a high analytical performance and as few steps as possible.

In the present report, we describe a fully validated enantioselective on-line LC–MS/MS method for contemporary stereospecific quantification of both the racemic forms of Warfarin, Acenocoumarol and Phenprocoumon in human plasma.

2. Experimental

2.1. Chemicals and materials

R–S Warfarin (WF) was obtained from Bristol-Myers Squibb (Rome, Italia). R–S Phenprocoumon was obtained from Roche AG (GrenzachWyhlen, Germany). R–S Acenocoumarol was obtained from Novartis (Novartis Farma S.p.A. 21040 Origgio, Italy). Acetonitrile and methanol were of HPLC gradient grade and formic acid (FOA) of analytical reagent grade (all from Merck, Darmstadt, Germany). Double-distilled water from an in-house distillation system was used for chromatography and solutions preparation.

2.2. Study protocol

Human blank plasma was kindly supplied by the Blood Donation Centre, Hospital. Anticoagulated Sodium Citrate-plasma samples were collected from volunteers who were administered for 1 week with either 5 mg of Warfarin or 4 mg of Acenocoumarol.

2.3. Instrumentation

In developing and validating the method, we have used two different instrumental configurations. The seamless transportability between the two configurations has assessed the robustness of the presented approach.

One system was centred on a mass spectrometry API 2000 (Applied Biosystems-Sciex) connected to a Perkin-Elmer Series 200 LC-equipment (Micro pump and autosampler) plumbed for loading the sample of plasma on the column Poros and subsequently to make to pass it on the chiral column as depicted in Fig. 1 (insert A and B, respectively).

The second hardware configuration included an Integral 100Q HPLC system (Applied Biosystems USA) connected to a mass spectrometry API 3000 (Applied Biosystems-Sciex), plumbed as depicted in Fig. 2.

Both the configurations performed the same task. Mass spectrometers were run with Turbospray sources operating in positive ion mode. The quantification was performed using the Multiple Reaction Monitoring (MRM). Data were collected and processed with the Analyst 1.4 package.

Specifically for the API 3000, the optimized parameters were: spray voltage 5500 V for positive ion mode; curtain gas (nitrogen) setting at 12 (arbitrary units) with a declustering voltage of 75 V; “turbo” gas 8 L/min heated at 250 °C. For the MS/MS experiments, LINAC collision cell operated at a pressure regime of 8 m Torr and the chosen collision energy was 24 eV (lab frame) for all the analytes. For quantification, instrument was run in the MRM by exploiting the transitions m/z 309.3 > 163.1, 281.3 > 203.2 and 354.3 > 163.1 for Warfarin, Phenprocoumon and Acenocoumarol, respectively.

Concerning the bidimensional chromatographic operation, injected sample was on-line extracted by using a Perfusion column Poros R2/20 (2 mm × 30 mm) column (Applied Biosystems) while the chiral separation (second chromatographic dimension) was performed by a Chira-Grom-2 column (250 mm × 1 mm, 8 μm-Grom Analytik GmbH, Herrenberg, Germany), preceded by a 10 mm × 1 mm guard column filled with the same material. Chiral column was thermostated at 40 °C.

2.4. Stock and working solutions preparation

Stock solutions of racemic Warfarin, Phenprocoumon and Acenocoumarol, were prepared in methanol (1.0 mg/mL per racemate form) and stored, protected from light, at 4 °C for a period not exceeding 6 months. Working solutions of Warfarin and Acenocoumarol were prepared by diluting the stock solution with a 1:1 mixture of methanol/water. Phenprocoumon stock solution used as internal standard (IS) was further diluted with methanol to give a final solution at 100 ng/mL.

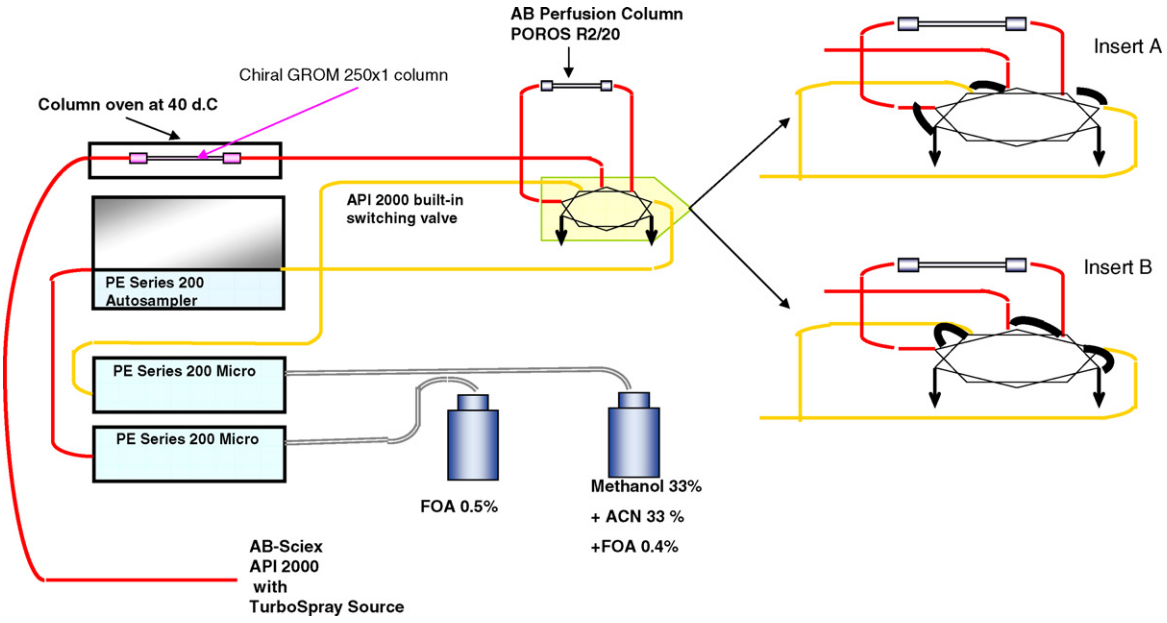

Fig. 1. Plumbing between the PE200 LC system and API2000 mass spectrometer. Insert A: valve in "load position". Loading of the plasma samples on the column "Poros R 2" and extraction (SPE, first chromatographic dimension). Insert B: valve in "inject position". The purified plasma samples pass on the chiral column for separation of the enantiomers (second dimension).

2.5. Calibrators

Stock solutions of Warfarin and Acenocoumarol were diluted with a methanol/water mixture to give working solution at concentrations of 0, 10, 30, 40, 50, 60, 70, 80, 90 and 100 μg/mL. For each point, 500 μL of working solution were added to 40 mL of blank non-pooled human plasma, which after mixing was subsequently portioned into 1.0 mL-aliquots. Resulting concentrations of calibration samples were thus 0, 125, 250, 375, 500, 625, 750, 875, 1000, and 1250 ng/mL, expressed as for each Warfarin and Acenocoumarol enantiomer.

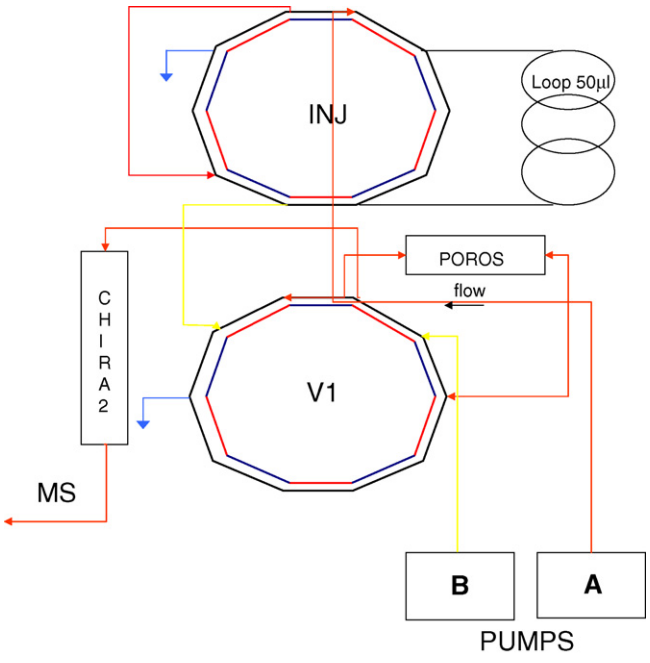

Fig. 2. Second hardware configuration included an Integral 100Q HPLC system (Applied Biosystems USA) connected to an API 3000 (Applied Biosystems-Sciex), position of the valve as described in Fig. 3.

2.6. Sample preparation step

To 250 μL of plasma sample or calibrator sample, 500 μL of methanolic IS solution were added. Samples were then mixed and centrifuged (10,000 g, 5 min, 4 °C). The supernatant was collected and 20 μL were injected into the HPLC system without further treatment.

2.7. Chromatographic conditions

Injected sample was processed by an on-line Solid Phase extraction (SPE, first chromatographic dimension), followed by a chiral separation (second dimension), following the protocol depicted in Fig. 3.

In the on-line SPE, injected sample was flushed through the perfusion Poros column with a high flow-rate (1.9 mL/min) of formic acid 0.5% (solvent A) for 1 min. In the meantime, the chiral column was flowed by 100 μL/min of an aqueous solution of Acetonitrile/methanol/formic acid (33/33/0.4—solvent B). Subsequently, with the valve switching, the analytes trapped by the Poros column are moved on the Chira column where the chiral separation was performed by the solvent B at a flow of 100 μL/min. After 9 min, valve was switched back and the Poros column was reconditioned with the solvent A at a high flow-rate subsequently degrading for a standby flow-rate setting of 300 μL/min.

```
General Settings: UV Detector Wavelength = 254 nM
    General Settings: Flow Rate = 2.000 ml/min
    General Settings: Turn UV Detector Lamp On
    Pump On
0.0          min  Method Start
0.0          min  [01] Equil Block
0.0   min         (A)  Step Segment
0.0   min                0.00 min  Set Solvent Blend 75.0% 1C:TFA 0.05% 25.0% 2C:ACN + TFA
0.0   min                0.00 min  Set Column 1 Inline*
0.0   min                0.00 min  Set Flow Rate = 0.400 ml/min*
0.0   min                0.00 min  Set AutoSampler Loop to Inject Position*
0.10  min                0.10 min  End Solvent Blend 75.0% 1C:TFA 0.05% 25.0% 2C:ACN + TFA
0.10  min
0.10  min  ____________________________________________________
0.10  min  [02] Load Block
0.10  min         (A)  AS Inject Segment
0.10  min                0.00 min  Load Sample through AutoSampler: 20.0µl of A 2: Warfarin
0.10  min                0.00 min  Set Column 1 Inline*
0.10  min                0.00 min  Set Flow Rate = 0.400ml/min*
1.12  min                1.02 min  Inject Sample through AutoSampler
1.12  min                1.02 min  Flush AutoSampler with 990µl of Flush solvent
1.12  min
1.12  min  ____________________________________________________
1.12  min  [03] Elute Block
1.12  min         (A)  Gradient Segment
1.12  min                0.00 min  Start Gradient 75.0% 1C :TFA 0.05% 25.0% 2C :ACN + TFA
1.12  min                0.00 min  Set Column 1 Inline*
1.22  min                0.10 min  Set Flow Rate = 2.000 ml/min*
1.22  min                0.10 min  End Gradient 95.0% 1C:TFA 0.05% 5.0% 2C:ACN + TFA
1.22  min
1.22  min  ____________________________________________________
1.22  min  [04] Elute Block
1.22  min         (A)  Step Segment
1.22  min               0.00 min  Set Solvent Blend 95.0% 1C:TFA 0.05% 5.0 % 2C:ACN + TFA
1.22  min               0.00 min  Set Column 1 Inline*
1.22  min               0.00 min  Set Flow Rate = 2.000 ml/min*
2.02  min               0.80 min  End Solvent Blend 95.0% 1C:TFA 0.05% 5.0% 2C:ACN + TFA
2.02  min
2.02  min  ____________________________________________________
2.02  min  [05] Elute Block
2.02  min         (A)  Gradient Segment
2.02  min               0.00 min  Start Gradient 95.0% 1C :TFA 0.05% 5.0% 2C :ACN + TFA
2.02  min               0.00 min  Set Column 1 Inline*
2.02  min               0.00 min  Set Flow Rate = 0.400 ml/min*
2.12  min               0.10 min  End Gradient 75.0% 1C :TFA 0.05% 25.0% 2C :ACN + TFA
2.12  min
2.12  min  ____________________________________________________
2.12  min  [06] Elute Block
2.12  min         (A)  Step Segment
2.12  min               0.00 min  Set Solvent Blend 75.0% 1C:TFA 0.05% 25.0% 2C:ACN + TFA
2.12  min               0.00 min  Set Column 1 Reversed*
2.12  min               0.00 min  Set Flow Rate = 0.400 ml/min*
11.12 min               9.00 min  End Solvent Blend 75.0% 1C:TFA 0.05% 25.0% 2C:ACN + TFA
11.12 min
11.12 min  ____________________________________________________
11.12 min  [07] Elute Block
11.12 min         (A)  Gradient Segment
11.12 min               0.00 min  Start Gradient 75.0% 1C :TFA 0.05% 25.0% 2C :ACN + TFA
11.12 min               0.00 min  Set Column 1 Inline*
11.22 min               0.10 min  Set Flow Rate = 2.00 ml/min*
11.22 min               0.10 min  End Gradient 95.0% 1C:TFA 0.05% 5.0% 2C:ACN + TFA
11.22 min
11.22 min  ____________________________________________________
11.22 min  [08] Elute Block
11.22 min         (A)  Gradient Segment
11.22 min               0.00 min  Start Gradient 95.0% 1C:TFA 0.05% 5.0% 2C:ACN + TFA
11.22 min               0.00 min  Set Flow Rate = 2.000 ml/min*
11.22 min               0.00 min  Set Column 1 Inline*
12.67 min               1.45 min  End Gradiet 95.0% 1C:TFA 0.05% 5.0% 2C:ACN + TFA
12.67 min
12.67 min         (B)  Gradient Segment
12.67 min               0.00 min  Start Gradient 95.0% 1C :TFA 0.05% 5.0% 2C :ACN + TFA
12.67 min               0.00 min  Set Flow Rate = 0.400 ml/min*
12.67 min               0.00 min  Set Column 1 Inline*
12.77 min               0.10 min  End Gradient 75.0% 1C:TFA 0.05% 25.0% 2C:ACN + TFA
12.77 min
12.77 min         (C)  Gradient Segment
12.77 min               0.00 min  Start Gradient 75.0% 1C :TFA 0.05% 25.0% 2C :ACN + TFA
12.77 min               0.00 min  Set Flow Rate = 0.400 ml/min*
12.77 min               0.00 min  Set Column 1 Inline*
14.12 min               1.35 min End Gradient 75.0% 1C:TFA 0.05% 25.0% 2C:ACN + TFA
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Fig. 3. Flow-chart of the automated protocol performed by the INTEGRAL system for on-line Solid Phase extraction (SPE, first chromatographic dimension), followed by a chiral separation (second dimension).

3. Results

3.1. Mass spectrometric characterization of the oral anticoagulant drugs

Mass spectrometer parameters were tuned for sensitivity optimization with the Turbospray ionization source operating in positive mode. This was accomplished by the direct infusion of standard solutions of racemic Warfarin, Phenprocoumon and Acenocoumarol (IS) delivered by a syringe pump at 10 µL/min. The analytes were identified on the basis of their retention times and mass spectra compared to standard solutions containing individual compounds.

MRM chromatogram of Warfarin, Phenprocoumon and Acenocoumarol are shown in Fig. 4. These spectra were analyzed and appropriate transitions were chosen for the subsequent MRM measurements. Resulting measurement specificity was assessed by using different blank donor plasma samples where no interfering reagent peak appeared at the retention time (RT) of the targeted analytes.

3.2. Matrix effects

To verify the "matrix" effect, we have added the same concentration of analyte to control plasma and water and, as depicted

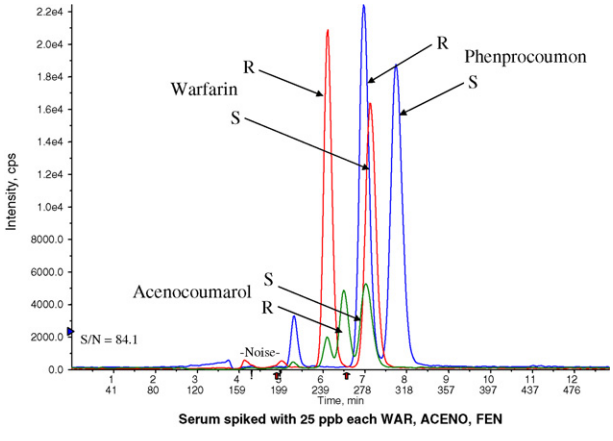

Fig. 4. MRM chromatogram for plasma spiked at 25 ppb of each enantiomer.

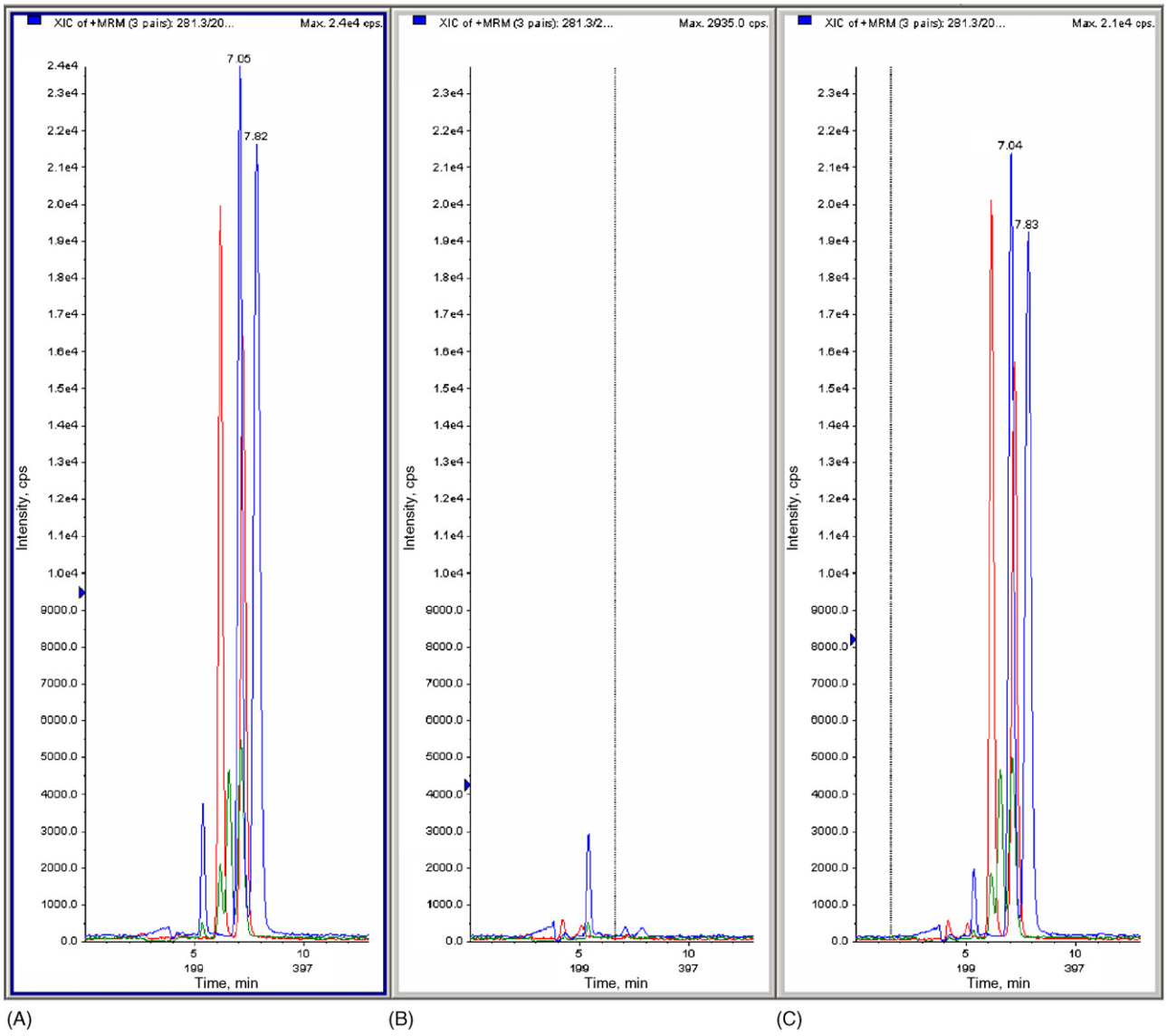

Fig. 5. MRM chromatograms of each enantiomer in aqueous standard (A) and control plasma (spiked plasma) (C) in which we have added the same concentration of analytes. (B) Blank. These compounds were analyzed by LC–MS–MS in the MRM mode by verifying the "matrix effect".

in Fig. 5, the times of retention and the heights of peaks are perfectly coincident.

3.3. Quantification performances

Measurement linearity of the assay is documented in Fig. 6 where over the range exceeding 1250 ng/mL responses was linear for both *R*- and *S*-Warfarin, *R*- and *S*-Phenprocoumon and *R*- and *S*-Acenocoumarol. Typical linear regression coefficients have been 0.9983 for *R*-Warfarin, 0.9935 for *S*-Warfarin, 0.9956 for *R*-Phenprocoumon, 0.9902 for *S*-Phenprocoumon, 0.9915 for *R*-Acenocoumarol and 0.9973 for *S*-Acenocoumarol.

3.4. Sensitivity

The limit of detection (LOD) in human plasma, defined as the concentration that could be determined with a signal-to-noise ratio of 3:1 was 0.5 ng/mL for each enantiomer. Limit of quantification (LOQ) defined as 10× LOD was calculated to 0.2 ng/mL for each enantiomer.

3.5. Recovery and precision of Warfarin, Phenprocoumon and Acenocoumarol enantiomers

Recovery was determined in triplicate at different concentrations by comparing spiked plasma with blank samples. When increasing amounts of oral anticoagulants were added to normal plasma samples and immediately processed according to the present method, a linear relation was observed between the peak area of the Phenprocoumon (IS) and the concentration of Warfarin or Acenocoumarol (μmol/L) added. A linear regression analysis yielded $y = 0.9033 + 0.0119x$ with a coefficient of 0.9979 for Warfarin and $y = 126.5133 + 0.7910x$ with a coefficient of 0.9968 for Acenocoumarol. The recoveries ranged

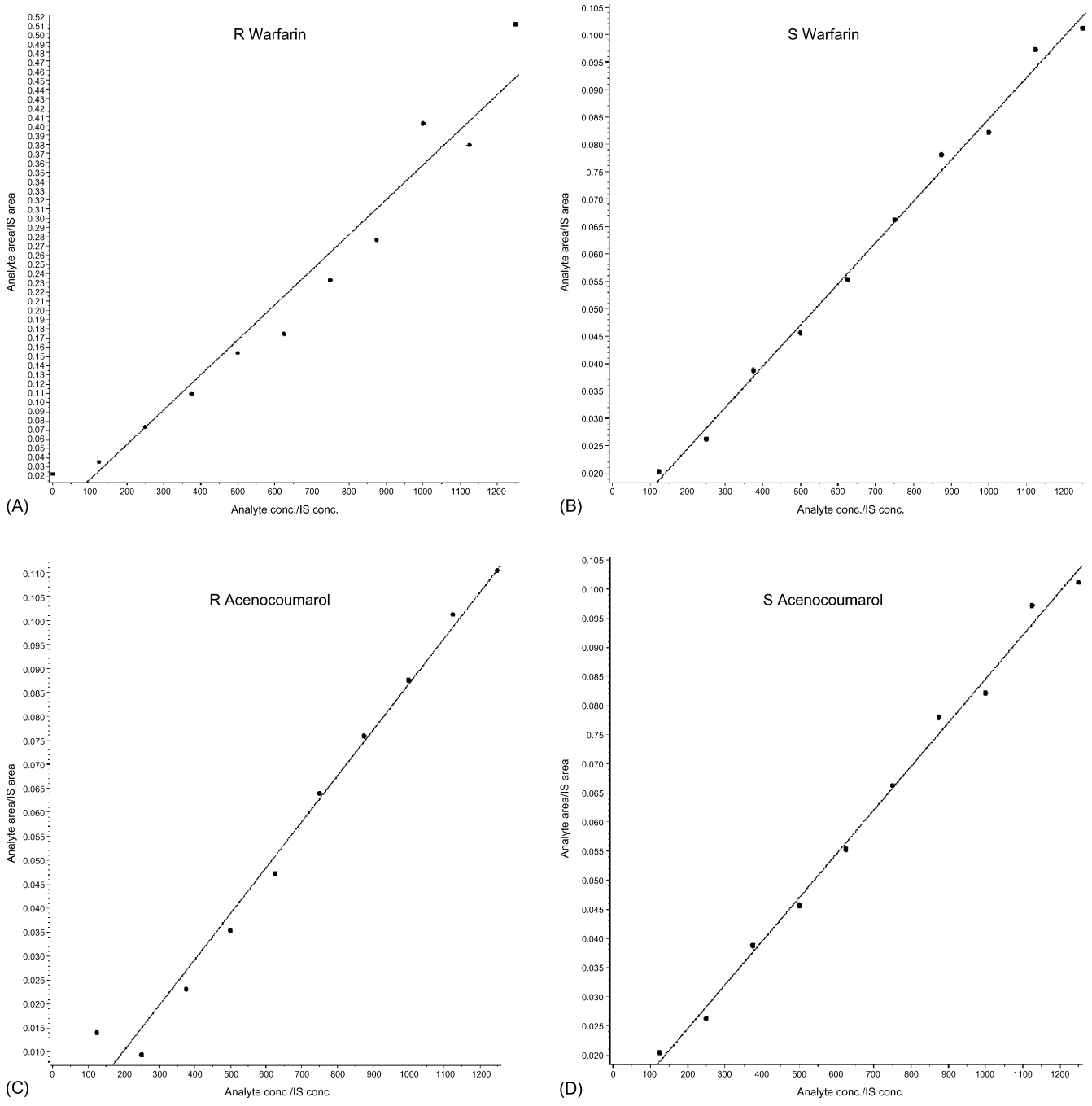

Fig. 6. Calibrations curves of peak area ratio analyte/IS concentrations (A: *R*-Warfarin; B: *S*-Warfarin; C: *R*-acenocumarol; D: *S*-acenocumarol).

Table 1
Recovery and precision of quantification of Warfarin and Acenocoumarol enantiomers

ng/mL added	Recovery of *R*-Warfarin (%)	Recovery of *S*-Warfarin (%)	Recovery of *R*-Acenocumarol (%)	Recovery of *S*-Acenocumarol (%)
125	100	99	102	105
375	102	98	106	99
625	112	108	118	109

Value calculated as a mean of three different experiments in triple.

from 98% to 118% for Warfarin and Acenocoumarol (Table 1). Determinations in 10 normal healthy individuals revealed a high reproducibility of RTs.

3.6. Intra- and inter-day accuracy and precision

Intra- and inter-day accuracy was tested by repeating each experiment in three replicates on the mass spectrometer and each sample was processed, as described above, three times in order to test the reproducibility of our method, each processed together with new calibration samples. The average precision for Warfarin and Acenocoumarol was around 6.1% and 5.3% for intraday and around 6.4% and 5.9% for interday reproducibility, respectively.

This data confirmed a good reproducibility of described method and renders the method suitable for large population studies.

4. Conclusion

The aim of this study was to develop a method for the sensitive stereospecific determination of the most commonly used oral anticoagulants in human plasma.

LC/MS/MS method was validated for the enantioselective and quantitative determination of Warfarin, Phenprocoumon and Acenocoumarol. In order to obtain a short time of analysis as well as properly separated peaks of the different enantiomers, a gradient system of mobile phase was chosen. The assay performance has been further improved both with respect to sensitivity and simplicity of execution. A selective on-line extraction step allowed to generate pure chromatograms free from interferences and good recovery of all analytes.

This method was used and validated for the analysis of Warfarin and Acenocoumarol, the only drugs used as anticoagulants in Italy; but it can also be applied for the stereoselective determination of Phenprocoumon using one of the other compounds as internal standard. Compared to previous assays, mainly targeting the specific quantification of either Warfarin or Phenprocoumon, the proposed methodology is an accurate, rapid, sensitive and reliable tool to independently resolve and quantify both the racemic forms of all enantiomers with a minimal sample preparation step.

In contrast to previously published protocols, the advantages of this method are a short time of analysis, a simple sample clean-up and the absence of a derivatisation step or special equipment [28,29].

Oral anticoagulants administration must be monitored by repeated analysis of INR. However, in several situations monitoring of the drugs itself is necessary for clinical decision. After starting treatment, in some patients a therapeutic INR is not reached even though the prescribed daily dose is increased or the INR value is unexpectedly low (or high) despite stables values for a long time. In similar cases, this method may be particularly helpful for phenotyping purposes by calculating metabolic ratios (parent compound/metabolite) that serve as a marker for the catalytic activity of hepatic CYP enzyme [30,31]. The results indicate that the presented method would be useful for clarifying the intra-patient variability.

Determination of individual enantiomers can give additional information leading to a best definition of pharmacokinetics of various drugs on absorption, metabolism and elimination [32].

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