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Determination of enantiomeric composition of ibuprofen in pharmaceutical formulations by partial least-squares regression of strongly overlapped chromatographic profiles☆

Jaiver Osorio Grisales[a], Juan A. Arancibia[b], Cecilia B. Castells[a], Alejandro C. Olivieri[b,*]

[a] Laboratorio de Separaciones Analíticas, División Química Analítica, Universidad Nacional de La Plata, 47 y 115 (1900) La Plata, Argentina
[b] Departamento de Química Analítica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Instituto de Química de Rosario (IQUIR-CONICET), Suipacha 531, Rosario S2002LRK, Argentina

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ABSTRACT

In this report, we demonstrate how chiral liquid chromatography combined with multivariate chemometric techniques, specifically unfolded-partial least-squares regression (U-PLS), provides a powerful analytical methodology. Using U-PLS, strongly overlapped enantiomer profiles in a sample could be successfully processed and enantiomeric purity could be accurately determined without requiring baseline enantioresolution between peaks. The samples were partially enantioseparated with a permethyl-β-cyclodextrin chiral column under reversed-phase conditions. Signals detected with a diode-array detector within a wavelength range from 198 to 241 nm were recorded, and the data were processed by a second-order multivariate algorithm to decrease detection limits. The R-(−)-enantiomer of ibuprofen in tablet formulation samples could be determined at the level of 0.5 mg L^{-1} in the presence of 99.9% of the S-(+)-enantiomorph with relative prediction error within ±3%.

1. Introduction

Many drug molecules are chiral and most of them have the desired therapeutic action in only one single enantiomer. Therefore, the efficient synthesis of the pure enantiomer of a target drug should be the goal in any chiral pharmaceutical project, which ultimately demands for the development of improved methods for the assessment of enantiomeric purity of these synthesized stereoisomer compounds. Similarly, in pharmacological research and drug discovery, chiral methods are also needed for disclosing the potential of an enantiopure drug to undergo rotational inversion during storage or in metabolic processes. These chiral analytical methods should facilitate the routine analysis of racemates, enantiomeric mixtures of any composition and also, intermediate asymmetric products within a manufacturing process.

The measurement of the optical rotation using a polarimeter is the classical method to estimate enantiomeric excess (ee), although it has already lost its role over the more sensitive nuclear magnetic resonance (NMR) and chromatography [1]. The latter methods, with the use of chiral stationary phases (CSP), became the gold standard of accuracy against which all new strategies are compared due to their relative simplicity as compared to NMR [2]. A large number of chiral compounds can be resolved by some form of chromatography. Gas chromatography using a CSP is useful for volatile compounds of relatively low molecular weight, whereas liquid chromatographic (HPLC) methods are suitable for more polar non-volatile compounds, with a relatively high molecular weight such as pharmaceutical compounds.

Any chromatographic strategy requires the use of a chiral auxiliary for the discrimination between both enantiomers. Although enantiodiscrimination can be possible, the optimization of the analytical procedure to achieve this goal for a given molecule often requires expensive and time-consuming testing of different columns and chromatographic conditions. Finally, the most usual scenario is to hardly get baseline resolution but with relatively low enantioseparation factors, which is reasonable for racemic mixtures. However, for checking enantiomeric purity, the enantiomer of interest (minor peak) has to be usually quantitatively analyzed at levels down to 1% of the main enantiomer. As one is injecting larger amounts of sample to increase sensitivity for the minor peak, the other component may become broader and tailed as a result of mass overload. Thus, higher accuracy in detection and quantitation of a minor signal close to the major peak in the chromatogram can be obtained as larger the enantioresolution is, being the aim to get truly baseline separated these very unequal profiles. These large enantioresolution factors are not so easily achieved in chiral

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* Corresponding author.
E-mail address: olivieri@iquir-conicet.gov.ar (A.C. Olivieri).

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chromatography at a reasonably analysis time [3], and it is very often observed a partial overlapping between both enantiomer profiles, causing loss in the quantitation accuracy.

Chemometric analysis of multivariate chromatographic data for quantitative purposes is becoming popular, particularly for the study of samples of complex composition, as has been recently discussed in several review articles [4–7].The expressions 'chromametrics' [8] and 'chro*math*ography' [9] have been coined to describe the combination of chromatography and chemometrics/mathematics. However, previous studies in chiral analysis by regression modeling of data involving the combination of enantioselective guest–host interactions with spectroscopic measurements are scarce. In 1996, MacDonald and Hieftje [10] demonstrated that the enantiomers of α-pinene could be differentiated in a media containing β-cyclodextrin by applying principal component analysis of the first-derivative NIR spectral data. In 2003, Busch et al. determined enantiomeric composition of 2-phenylglycine and other amino acid samples using chemometric analysis of UV–visible spectra also using β-cyclodextrin as a chiral auxiliary [11]. Using a partial least-squares (PLS) algorithm, the authors could predict the enantiomeric composition of the phenylglycine samples over a range of mole fractions from 0.5 to 0.9 of (R)-2-phenylglycine with errors within ±3%.

In this study, we explored the role of chemometric methods, such as multivariate regression analysis, as a means of extracting information about the enantiomeric composition of samples from chromatographic-spectral matrix data obtained after partial resolution of peaks in a typical chiral chromatographic separation followed by diode-array detection. In order to study the potential of chemometric techniques, a separation which is relevant to pharmaceutical analysis was chosen: the enantiomeric analysis of ibuprofen in single tablet formulations.

Ibuprofen is used as a non-steroidal anti-inflammatory analgesic in several disorders, especially in case of rheumatic disease. Although, it is commonly administered as a racemate, the S-(+)-enantiomer has the pharmacological activity. Furthermore, ibuprofen is metabolized enantioselectively in the human body [12], and thus an excess of S-(+)-ibuprofen is found in biological fluids. Similarly, it has been found that bacteria and algae communities are involved in enantioselective transformation of this molecule, present in the waste-water plant, with a kinetic favorable to S-(+)-ibuprofen [13]. The pure S-enantiomer is commercialized in the local market, being the R-enantiomer an impurity of this drug.

2. Theoretical

2.1. Partial least-squares regression

In the absence of interferents in the unknown samples, both first-order (i.e., chromatograms for single wavelength detection) and second-order (chromatograms at multiple wavelengths) may be employed for analyte quantitation. The latter data are intrinsically more sensitive, and were thus selected for the present determinations.

A number of algorithms are available for processing second-order data. Popular methodologies are parallel factor analysis (PARAFAC) [14], multivariate curve resolution coupled to alternating least-squares (MCR-ALS) [15] and partial least-squares regression [16,17]. In the case of data of chromatographic origin, a potential problem may arise because of experimental changes occurring from run to run in retention times and/or peak shapes. The PARAFAC model, for example, requires that all chromatographic profiles are adequately synchronized both in time position and shape [9]. MCR-ALS and PARAFAC2 [18] (a variant of PARAFAC) are more flexible in this regard, and allow for changes from sample to sample in chromatographic profiles [9]. Another alternative is to employ latent structured methods, such as unfolded partial least-squares (U-PLS) [16] or multi-way PLS (N-PLS) [17], which are intrinsically flexible towards profile changes, provided they are modeled during the calibration phase. We found that both U-PLS and N-PLS were successful in quantitating the analytes in the present case, and hence the simpler U-PLS variant is discussed.

In the U-PLS method, the calibration data matrices are unfolded (i.e., transformed into vectors), and then a usual PLS model is built using these data together with the vector of calibration concentrations [16]. This provides a set of loadings, weight loadings and, most importantly, regression coefficients for prediction in new samples [16]. See details in Supplementary material.

The number of loadings or latent variables to be included in the PLS model can be selected by techniques such as leave-one-out cross-validation [19]. Details are provided in Supplementary material.

After calibrating the model, the regression vector is employed to estimate the concentration of a given analyte.

Figures of merit can be readily estimated using known expressions, such as sensitivity, uncertainty in predicted concentration and limit of detection (LOD) [19,20]. The expression for estimating the LOD incorporates the latest IUPAC recommendations based on both type I and II errors for estimating detection capabilities [20,21].

It should be noticed that the above scheme is repeated for each analyte to be quantitated. Since all U-PLS parameters are concentration-dependent, because the calibration concentrations for each analyte are different, the analytical figures of merit are specific for each analyte as well.

3. Experimental

3.1. Reagents and solutions

Ibuprofen and (S)-(+)-ibuprofen were obtained from Sigma–Aldrich (St. Louis, MO), HPLC-grade methanol was purchased from Baker, water was purified by means of a Milli-Q Purification System (Simplicity, Millipore, MA, USA). Mobile phases consisted in mixtures of methanol:buffer 0.1% TEAA (triethylammonium acetate) pH = 4.0 (measured in pure water). TEAA buffer was generated by mixing 300 μL of TEA (triethylamine) and 700 μL of glacial acetic acid in 1 l of Milli-Q water. TEA and glacial acetic acid were supplied from Baker (Mallinckrodt, Baker, US).

Standard solutions were prepared by mixing three stock solutions of racemic ibuprofen (760, 896, 1084 mg L^{-1}) and S-(+)-ibuprofen (808, 912, 988 mg L^{-1}) in different ratios, in order to obtain three concentrated solutions with R:S compositions of 0.5:99.5, 1:99 and 5:95, each one at six concentration levels within the range from 0 to 1000 mg L^{-1}. These solutions were prepared in triplicate, and their concentrations are reported in Table 1.

Samples of S-ibuprofen tablets (Cefalex, Bagó, Argentina) were treated as follows. Ten pills were crushed in a mortar and dissolved in methanol to yield a 1.040 mg mL^{-1} stock solution (considering the nominal content declared by the manufacturer). Adequate dilutions were prepared from the latter solution in (70:30 V/V) methanol/water, and the resulting solutions were filtered through a 0.45 μm nylon membrane before injection.

3.2. Instrumentation

Chromatographic studies were performed on an HP 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with vacuum degasser, binary pump, autosampler, thermostated column device, photodiode array detector (DAD) and

Table 1
Solutions with their concentration and compositions rates.[a]

R:S 5:95		R:S 1:99		R:S 0.5:99.5		S 100
S-(+)	R-(−)	S-(+)	R-(−)	S-(+)	R-(−)	S-(+)
0.0	0.0	0.0	0.0	0.0	0.0	0.0
42.8	2.7	174.4	1.8	108.6	0.5	1.0
46.7	3.3	230.1	2.2	122.9	0.6	2.9
75.7	4.6	243.1	2.6	159.3	0.8	5.4
85.6	5.4	348.8	3.6	217.2	1.1	8.4
93.4	6.5	460.3	4.3	245.8	1.3	29.4
151.3	9.1	436.0	4.6	318.6	1.5	67.2
171.3	10.8	486.2	5.2	434.4	2.1	108.0
186.9	13.0	575.4	5.4	491.6	2.5	147.0
378.3	22.8	690.4	6.5	543.0	2.7	336.0
428.2	26.9	607.7	6.5	637.2	3.0	540.0
467.2	32.5	653.9	6.8	614.5	3.1	840.0
567.5	34.2	729.3	7.8	796.5	3.8	1080.0
642.2	40.3	863.0	8.1	814.5	4.0	
756.6	45.6	871.9	9.1	921.7	4.7	
700.9	48.8	911.6	9.8	1086.0	5.3	
856.3	53.8	1150.7	10.8	1194.8	5.7	
934.5	65.0	1215.4	13.0	1228.9	6.3	

[a] Units at mg L^{-1}.

computer-based HP Chemstation. The chiral column was a Nucleodex-β-PM (200 mm × 4.0 mm, particles of 5 μm) from Macherey-Nagel (Düren, Germany). The mobile phase composition was 70:30 methanol:buffer TEAA, the flow-rate was set to 0.8 ml/min and temperature to 25 °C. Output signals, detected between 190 and 290 nm every 1 nm and with a frequency of 2.5 Hz, were acquired. The injection volume was 10 μL.

The obtained chromatograms, in the retention time range from 6 to 13 min, were exported as matrices and analyzed with routines in MatLab 7.0. These matrices were firstly aligned in the time dimension with respect to one of the calibration matrices used as reference. Then, all matrices were vectorized in order to be analyzed by U-PLS. A representative group of samples were chosen in order to be used as calibration data, and all others were used as test data.

4. Results

4.1. Chromatographic considerations

An enantioresolution factor of 1.37 was achieved with a 60:40 methanol/buffer mobile phase at 25 °C after 40 min, which is extremely slow. We could have slightly increased the resolution by changing the eluotropic strength (decreasing the methanol content), but the analysis time had been even longer. During the preliminary assays seeking for more robust conditions, we tested mobile phases without TEAA, but the obtained peak profiles were tailed. We also tested acetonitrile instead of methanol, without getting better results. The column temperature, on the other hand, was always kept relatively low due to two main reasons: (1) to avoid a potential racemization reaction which would impair the measurements, and (2) because enantioselectivity factors usually decrease as temperature is increased in most chiral separations. Enantioresolution factors can exhibit a maximum at intermediate temperatures, due to a compromise between enantioselectivity and efficiency. The latter normally decreases due to slow mass transfer as temperature decreases. In all studied conditions, the elution order corresponds to the S-(+) followed by the R-(−) enantiomer, which complicates even more the analysis, as the ratio (R)/(S) decreases and the S-isomer becomes in considerable excess.

Fig. 1A shows the chromatogram of the racemic drug eluted by using a stronger mobile phase composition with an enantioresolution factor of 0.87. These chromatographic conditions were used for the following experiments. Fig. 1B corresponds to the chromatogram obtained for samples containing an (S)/(R) ratio of 99.5/0.5. It is clear that, under these conditions, the determination of the R-impurity can not be performed satisfactorily from univariate data analysis.

A very interesting finding was to observe an unusual increase in peak width, which was independent from the enantiomeric ratio. The separated enantiomers of the racemic mixture had peak widths significantly broader than those obtained after injecting the pure S-(+)-enantiomer. Previously, unusual peak-broadening phenomenon in inclusion GC has been related to overload conditions. In order to determine column overload as a possible source of the observed peak width, varying amounts of racemic and enriched (S)-enantiomorph were injected. Broader peak profiles already occurred under non-overloaded analytical conditions. These profiles might be attributed to slow kinetics of diastereomeric equilibration, as previously suggested in supercritical fluid chromatography [22]. However, this anomalous broadening effect deserves a thorough and systematic study that will be published elsewhere.

4.2. Retention time alignment

Changes in retention times with different runs were detected in the chromatograms, as it is shown in Fig. 2. A number of algorithms is available for performing time alignment and correcting for this experimental time shifts; one of the most flexible ones in this

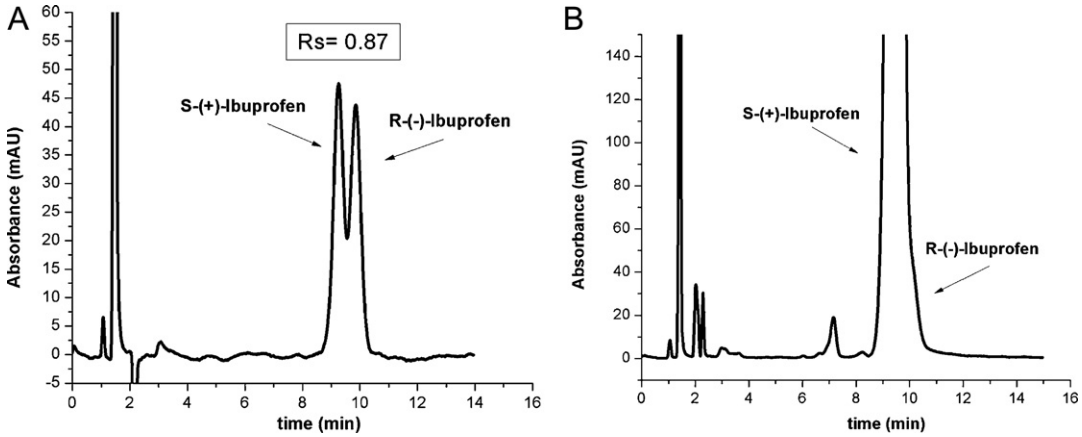

Fig. 1. Partial resolution of ibuprofen eluted from a permethyl-β-cyclodextrin chiral column (Nucleodex β-PM, Macherey-Nagel). Mobile phase: (70:30) methanol/0.1% triethylamine acetate buffer (pH = 4), flow-rate: 0.8 mL/min. Column temperature: 25 °C. Detection at 220 nm. (A) racemic mixture; (B) enantiomeric ratio: (R)/(S) = 0.5/99.5.

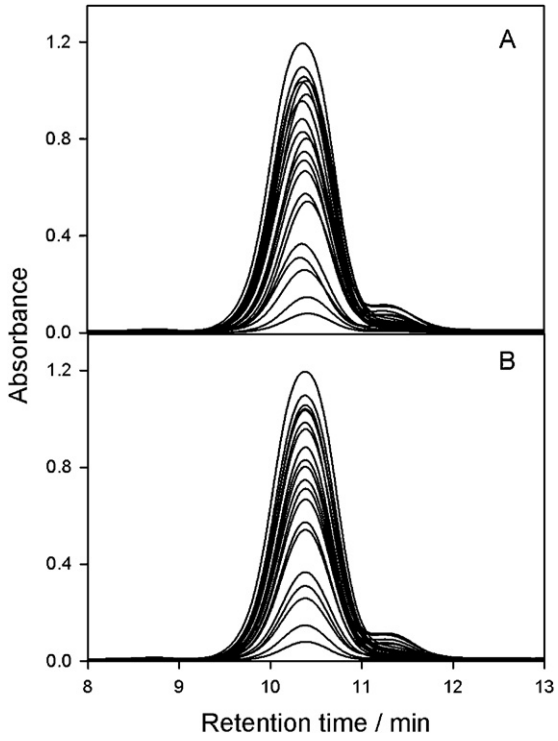

Fig. 2. (A) Raw chromatograms, plotted in a selected retention time range, at the absorption wavelength of 220 nm. They were taken from the complete data matrices employed for calibrating the U-PLS model for quantitating the R isomer. (B) Aligned chromatograms so that the position of the maximum for the S-enantiomer match.

regard is called correlation optimized warping (COW) [23]. However, the latter methodology could not be successfully applied to the present case, because of the presence of a very small peak due to one the analytes (the R-enantiomer), which in some data matrices was completely masked by the major peak due to the S isomer. We decided to align all chromatograms in the time dimension by selecting one of the recorded data matrices as reference, digitally moving all the remaining matrices in the time dimension until the maximum peak for the major component was aligned with the one in the reference matrix. This simple procedure was shown to be adequate, on account of the small time window were the alignment was performed. No significant changes in baseline were detected in this time region.

4.3. Multivariate analysis

As explained above, the retention time-wavelength matrices were aligned by digitally moving all spectra in the time dimension. This procedure was carried out for all calibration and test data matrices. Then adequate time and wavelength regions were selected from the full data matrices. The spectral range was set for both analytes from 198 to 241 nm. The time ranges were from 9.0 to 10.34 min (1.34 min) for the S isomer and from 10.34 to 11.35 min (1.01 min) for the R isomer. These final data matrices (44 × 201 data points for the S isomer and 44 × 151 data points for the R isomer) were unfolded and subjected to U-PLS analysis.

Notice that the number of calibration samples was different for each model. When calibration was planned for the S-enantiomer, the maximum concentration was set at ca. 500 mg L^{-1}, because beyond this limit the linearity in signal-concentration is lost. However, for the calibration model involving the R-enantiomer, higher concentrations of the S-enantiomer were employed (up to 1150 mg L^{-1}) in order to cover larger values of the S/R ratio. The absolute concentrations of both analytes in each calibration set are given in Table 2.

Leave-one-out cross validation allowed to estimate that two and three latent variables were appropriate for modeling the calibration data for quantitating the S and R isomer respectively (see Table 3). In the case of the R-enantiomer, although two analytes are present in the samples, changes in shape occurred in the time dimension, which led the U-PLS model to need an extra factor for modeling the variance in calibration data.

The calibrated models were then applied to the sets of test samples, not included in calibration. The results are collected in Table 4.

Table 2
Calibration samples and their concentrations for each analyte.[a]

Sample	Analyte S-(+)-ibuprofen (mg L^{-1})		Analyte R-(−)-ibuprofen (mg L^{-1})	
	S-(+)-ibuprofen	R-(−)-ibuprofen	S-(+)-ibuprofen	R-(−)-ibuprofen
1	230	2.2	230	2.2
2	349	3.6	349	3.6
3	436	4.6	436	4.6
4	47	3.3	523	5.5
5	85.6	5.4	608	6.5
6	151	9.1	729	7.8
7	187	13.0	863	8.1
8	378	22.8	872	9.1
9	467	32.5	912	9.8
10			1151	10.8
11			47	3.3
12			85.6	5.4
13			151	9.1
14			187	13.0
15			378	22.8
16			467	32.5
17			567	34.2
18			642	40.3
19			757	45.6
20			856	53.8
21			8.4	0
22			67	0
23			108	0

[a] Units at mg L^{-1}.

Table 3
Leave-one out cross validation results for both analytes.[a]

Latent variables	S-ibuprofen			R-ibuprofen		
	PRESS	*F*	*p*	PRESS	*F*	*p*
1	36,566	42.1	0.999	1496	237	0.999
2	**1130**	**1.3**	**0.718**	54.4	8.6	0.999
3	1417	1.6	0.858	**6.3**	**1**	**0.5**
4	1491	1.7	0.882	11.4	–	–
5	1195	1.4	0.758	16.4	–	–
6	870	1	0.5	18.4	–	–
7	1140	–	–	11.0	–	–
8	1222	–	–	11.1	–	–

[a] Optimum values in boldface.

Table 4
Statistical results for the test samples and analytical figures of merit.

Parameter	S-ibuprofen	R-ibuprofen
Concentration range (mg L^{-1})	0–467	0–53.8
Wavelength range (nm)	198–241	198–241
Retention time range (min)	9.0–10.34	10.34–11.35
No. of test samples	70	120
RMSEP[a]	5.5	0.43
REP[b]	3.0	3.0
R^2	0.9980	0.9993
Sensitivity (AU L mg^{-1})	0.010	0.015
LOD (mg L^{-1})	5	0.5

[a] Root mean square error of prediction (mg L^{-1}).
[b] Relative error of prediction (%).

For quantitation of the R-eantiomer, the mean calibration concentration of the S-enantiomer is ca. 500 mg L^{-1}, thus we estimate that the R-enantiomer can be detected in these samples in a proportion of 0.5:500, i.e., about 1:1000, which means that the ratio of enantiomers can be traced down to 0.1% of the R-enantiomer (99.9% of the S-enantiomer). The relative standard error of prediction was ±3%. Notice in Table 4 that the minor R-enantiomer shows a smaller LOD than the S-enantiomer, because the calibration range for the former has been narrowed down very small concentrations, in order to be able to detect traces of R- in samples containing the S-enantiomer as the main component. Since S- and R-ibuprofen have been calibrated in different concentration ranges, it is natural to expect different detection capabilities.

Fig. 3 shows the three-dimensional plots of absorbance as a function of retention time and wavelength. This plot corresponds to the chromatogram of the mixture (99/1) (R/S) (Fig. 3B has been enlarged to show the R-enantiomer profile.

There is no specific regulation for the thresholds about enantiomer impurities in chiral drugs produced as a single enantiomer [24,25]. It is only stated that in the new drug substance as well as in the new drug product, the other enantiomer has to be considered as an impurity. Although the same documents state that the "technical limitations to measure enantiomeric purity may preclude the same limits of quantification or qualification". In this study, the threshold for the R-enantiomer was taken according to the conventional criteria for degradation products in new drug products, i.e., 0.1% for a dose below 1 g for total daily intake [26]. Concerning the commercial tablets studied and using the above criteria, our results did not show evidence for the R-enantiomer detected in these samples.

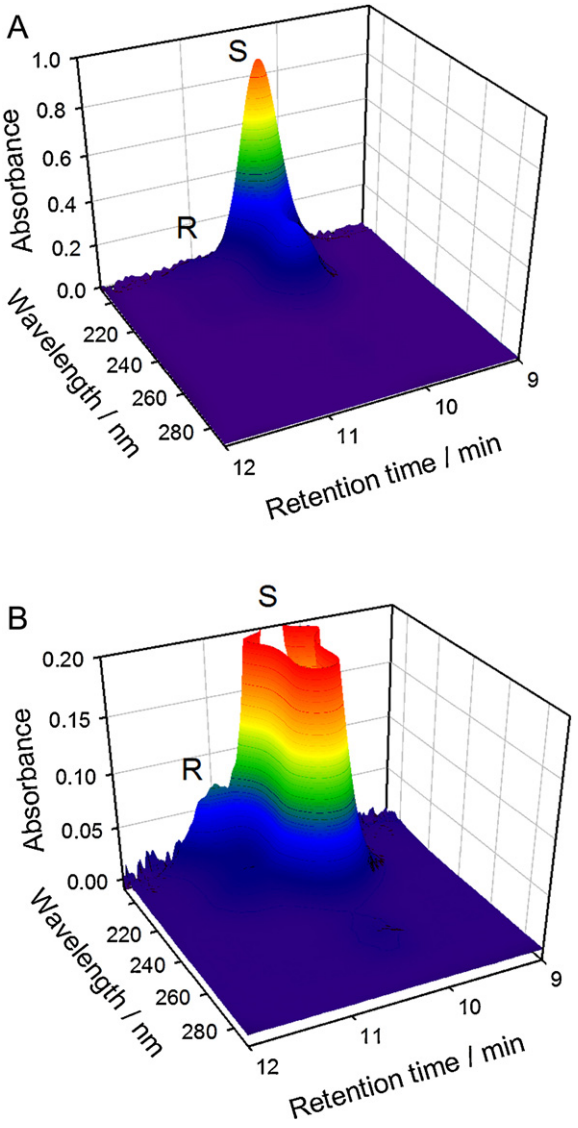

Fig. 3. (A) Three-dimensional surface showing the absorbance as a function of retention time and wavelength for a typical sample showing the small peak due to the R-enantiomer. (B) Vertical scale expanded for better appreciation of the small R-peak.

4.4. Validation and accreditation

If the presently described analytical procedure is intended to be adopted in a routine laboratory, some issues have to be taken into consideration, besides those normally associated with liquid chromatographic analysis of pharmaceutical samples. They include the need of having operators with some skill in computer operations, although software for multivariate calibration can be easily adapted to non-experienced users. The availability of algorithms should also be taken into account: software for partial least-squares analysis is freely available on the web [27], but can also be easily programmed from a very simple set of basic instructions, such as those detailed in Ref. [19]. Data storage should not be particularly troublesome, as the calibration data to be stored, i.e., chromatographic-spectral data matrices and concentrations, regression coefficients, loadings and weight loadings (see Supplementary material) should not represent a problem for modern computer means.

As regards the validation process, although it is achieved through specialized chemometric parameters, the latter can still be related to the fundamental validation characteristics required for any analytical method, as has been established in official documents for pharmaceutical analysis based on near infrared (NIR) spectroscopy and PLS-based calibrations [28]. This is similar to the provisions adopted for the well-established NIR/PLS methodologies in food analysis [29].

In general terms, validation protocols should describe how calibrations are generated by constructing a mathematical model relating the response to sample properties. This can be done using a suitable chemometric algorithm which should be clearly defined in an exact mathematical expression. Calibration parameters such as regression coefficients should then be properly stored and uniquely identified, checked for possible corruption, and be adequately transferred among instruments. On the other hand, the documentation should include a detailed operating manual and a description of the legally relevant software, the requirements of system configuration and resources, the security means of the operating system, the sealing methods (physical, software encryption), the type of computer, network, user interface, menus and dialogues, data sets stored or transmitted, the possibility of fault detections, etc. [29].

5. Conclusions

In this study we demonstrated the potential of obtaining quantitative information about enantiomeric purity low down 0.1% levels of strongly overlapped peaks with data acquired from a diode-array detector after elution from a chiral column by using indirect calibration of both peaks by U-PLS.

The combination of any of several chemometric tools along with chromatographic–spectroscopic data will allow the analysis of chiral sample components for which is it very troublesome to obtain an appropriate resolution. It is also envisaged that this combination would be very powerful in the following circumstances: (1) to reduce analysis time by using stronger mobile phases to elute earlier all the interesting peaks and (2) to circumvent the usual lower enantioresolution factors achieved in reversed-phase chiral systems as compared to the larger normal phase enantioselectivities and, therefore, to reduce analysis costs.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.04.018.

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