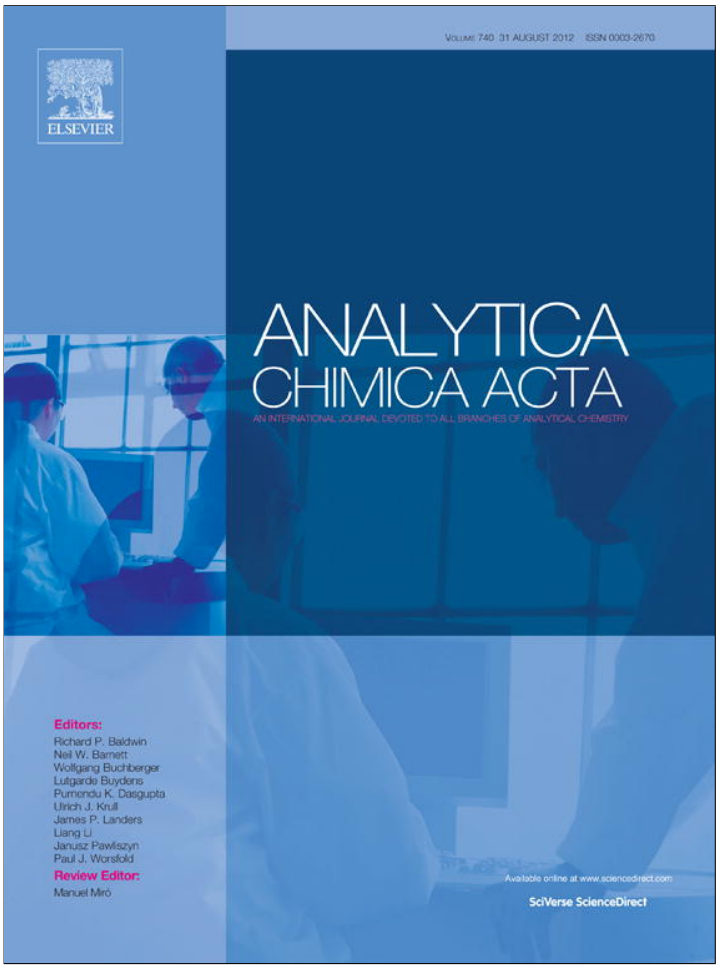

Analytica Chimica Acta 740 (2012) 27–35

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

High-performance liquid chromatography with fast-scanning fluorescence detection and multivariate curve resolution for the efficient determination of galantamine and its main metabolites in serum

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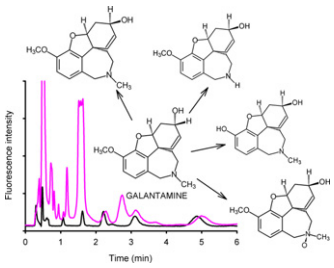
HIGHLIGHTS

- A simple and direct analytical method for galantamine and its serum metabolites is developed.
- Low detection limits are rapidly achieved, with minimal organic reagents and sample amounts.
- Quantification is accomplished using green-chemistry principles.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 May 2012
Received in revised form 15 June 2012
Accepted 19 June 2012
Available online 27 June 2012

Keywords:
Chromatography
Spectrofluorimetry
Multivariate calibration
Galantamine
Metabolites

ABSTRACT

Based on green analytical chemistry principles, an efficient approach was applied for the simultaneous determination of galantamine, a widely used cholinesterase inhibitor for the treatment of Alzheimer's disease, and its major metabolites in serum samples. After a simple serum deproteinization step, second-order data were rapidly obtained (less than 6 min) with a chromatographic system operating in the isocratic regime using ammonium acetate/acetonitrile (94:6) as mobile phase. Detection was made with a fast-scanning spectrofluorimeter, which allowed the efficient collection of data to obtain matrices of fluorescence intensity as a function of retention time and emission wavelength. Successful resolution was achieved in the presence of matrix interferences in serum samples using multivariate curve resolution-alternating least-squares (MCR-ALS). The developed approach allows the quantification of the analytes at levels found in treated patients, without the need of applying either preconcentration or extraction steps. Limits of detection in the range between 8 and 11 ng mL^{-1}, relative prediction errors from 7 to 12% and coefficients of variation from 4 to 7% were achieved.

1. Introduction

Alzheimer's disease (AD) is a progressive illness caused by the degeneration of neurons in several areas of the brain, and represents the most common form of dementia affecting a significant percentage of the population above 80 years [1]. Although there are no drug treatments available for a cure, both cholinesterase inhibitors and *N*-methyl *d*-aspartate receptor antagonists are recommended aiming at improving the patients quality of life [2]. Galantamine (GAL, Fig. 1) is a competitive acetylcholinesterase inhibitor and a nicotinic allosteric modulator, effective for a broad spectrum of AD symptoms [3], and is the most recently approved drug by the US-FDA (United State Food Drug Administration) for AD treatment [4].

Several methods, based on chromatography, spectrophotometry, capillary electrophoresis and spectrofluorimetry, have been developed for the determination of this alkaloid in samples such as

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http://dx.doi.org/10.1016/j.aca.2012.06.034

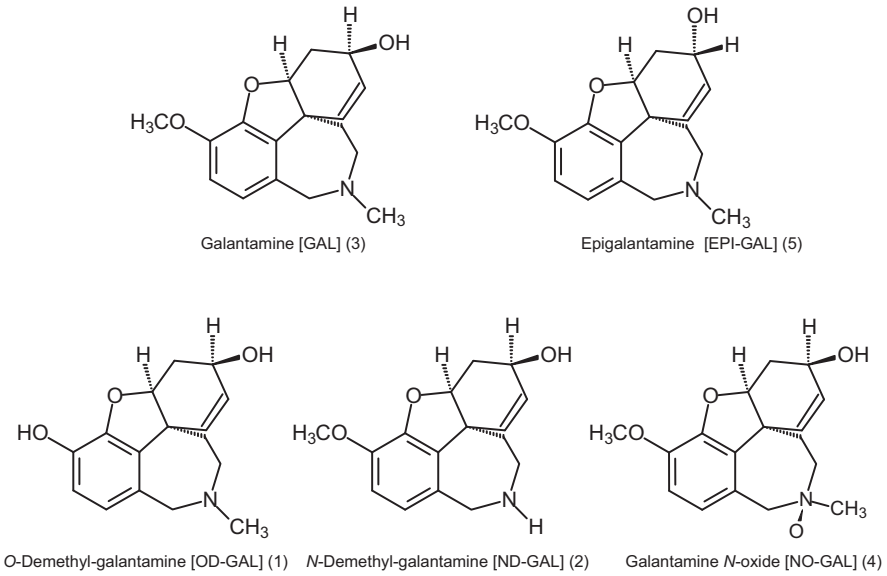

Fig. 1. Structures of galantamine (GAL) and of its main serum metabolites. Numbers correspond to the chromatographic elution order under the applied working conditions (see Fig. 3).

pharmaceuticals, natural products, environmental waters, tissues, serum/plasma and urine, among others [5–19]. Most GAL analyses in biological or complex matrices need sample preparation involving either liquid–liquid extraction with organic solvents (*e.g.* dichloromethane, chloroform, toluene, diethylether) or solid-phase extraction using significant amounts of methanol to both rinse the cartridge and elute the analyte [4].

As part of a program devoted to the development of high performance methods within the framework of green analytical chemistry [20,21], recently GAL was determined in natural waters using spectrofluorimetry in aqueous organized media [15]. As a continuation of such work that follows the green chemistry principles, further efforts were made to extend the spectrofluorimetric method to the quantification of GAL in human serum. However, the spectrofluorimetric analysis in serum samples brings two main issues which must be considered. The first one is the presence of serum components which can interfere in the determination, and the second one is the presence of GAL metabolites with similar fluorescence properties to the target analyte. Among the main serum GAL metabolites (Fig. 1), *O*-demethylgalantamine (OD-GAL) proved to be even more potent than GAL [22], while the remaining ones are pharmacologically less active [23]. Regardless of their activity, the evaluation of these metabolites is important for both pharmacokinetic and biodistribution studies and thus they should all be quantified.

Indeed, preliminary studies showed that GAL excitation and emission fluorescence spectra is very similar to the ones of its main metabolites and, therefore, a direct spectrofluorimetric method could not be applied, even with the aid of chemometric tools. High-performance liquid chromatography (HPLC) coupled to fast scanning fluorescence spectrometry (FSFS) [24] was proposed as a useful approach for rapid and sensitive detection. However, because of the strong overlapping of the chromatographic peaks of the studied compounds with those corresponding to endogenous components of serum, a multivariate calibration strategy was proposed, which allows the determination of analytes in the presence of foreign components [25]. This property is called the "second-order advantage" and avoids the requirement of physically removing interferences (either the matrix itself or some unexpected compound which can be present in real samples) [26]. Such approach simplifies the conditions to be applied for the chromatographic separation.

The presently studied second-order chromatographic data with multivariate spectral detection may impose some challenges to data processing algorithms. First, component profiles in the time mode may change in shape and/or position from sample to sample, and second, fluorescence emission spectra of analytes, which are structurally close, may be very similar and chromatographically unresolved. Multivariate curve resolution-alternating least-squares (MCR-ALS) is a very efficient algorithm which has been previously employed for coping with the mentioned limitations [27–29]. In the so-called extended MCR-ALS mode [30] these difficulties are minimized by building an augmented data matrix, placing calibration and test data matrices adjacent to each other or on top of each other. The augmentation mode is selected so that the augmented matrix has a chemical rank (number of responsive chemical components) equal to the mathematical rank (number of bilinear components needed to model the data in the absence of noise, or pseudorank). However, both of the above mentioned limitations cannot be simultaneously accounted for. See Section 4.4 for a discussion on how these problems were solved in the present report. In Ref. [24], the investigated analytes (ten polycyclic aromatic hydrocarbons) present distinctive fluorescence spectra. Although they are strongly overlapped, satisfactory results were obtained working with the full chromatographic data matrix and MCR-ALS. In that case, the augmentation was implemented in the time direction, because of the presence of retention time shifts between different chromatographic runs.

In the present work, a strategy was applied based on: (1) division of the retention time axis in windows to avoid having spectrally similar analytes in each window, and (2) performing augmentation in the time mode to allow for modeling of varying chromatographic profiles from run to run.

The comparison of the performance of the method with other ones reported in the literature for the determination of GAL in serum or plasma samples was made, showing the clear advantages of the proposed approach.

2. Materials and methods

2.1. Reagents and solutions

All reagents were of high-purity grade and used as received. Galantamine hydrobromide (GAL) was purchased from Sigma–Aldrich (Milwaukee, WI, USA). Galantamine-*N*-oxide (NO-GAL), epi-galantamine (EPI-GAL), *N*-demethylgalantamine (ND-GAL) and *O*-demethylgalantamine (OD-GAL) were acquired from Toronto Research Chemicals Inc. (North York, ON, Canada).

Methanol (MeOH) and acetonitrile (ACN) were obtained from J.T. Baker (Deventer, The Netherlands). Ammonium acetate, monobasic sodium phosphate, hydrochloric acid and sodium hydroxide were purchased from Ciccarelli (San Lorenzo, Argentina) while trichloroacetic acid (TCA) was obtained from Anedra (San Fernando, Argentina). Water was purified using a MilliQ system (Millipore, Bedford, USA).

Stock solutions containing 208 μg mL^{-1} GAL, 93 μg mL^{-1} EPI-GAL, 83 μg mL^{-1} ND-GAL, 500 μg mL^{-1} NO-GAL and 114 μg mL^{-1} OD-GAL were prepared in methanol and stored in dark flasks at 4 °C. In these conditions, solutions were stable for at least two months.

Two buffer solutions were prepared: 10 mmol L^{-1} ammonium acetate/hydrochloric acid (pH 5.0) and 1 mol L^{-1} sodium phosphate monobasic/sodium hydroxide (pH 7.0).

Fresh human serum samples were immediately frozen at −18 °C before analysis.

2.2. Instrumentation and procedure

A Perkin Elmer (Llantrisant, United Kingdom) LS 55 luminescence spectrometer equipped with a xenon discharge lamp was used to measure excitation and emission fluorescence spectra.

Chromatographic measurements were carried out on an Agilent 1100 Series instrument (Agilent Technologies, Waldbronn, Germany) equipped with degasser, quaternary pump, autosampler, column oven compartment, fast-scanning fluorescence detector, and the ChemStation software package for instrument control, data acquisition and data analysis. LC separations were performed on a Zorbax Eclipse XDB-C18 4.6 × 75 mm (3.5 μm particle size) analytical column (Agilent Technologies).

The mobile phase consisted of a mixture of ammonium acetate buffer (pH 5.0) and ACN (94:6, v/v) flowing at 2.2 mL min^{-1}. The flow rate was selected in order to separate the five analytes in the shortest possible time, but maintaining the pressure of the column below 200 bar.

Chromatographic elution was made under isocratic regime. The column temperature was set at 25 °C.

The pH of solutions was measured with an Orion (MA, United States) 410A potentiometer equipped with a Boeco (Hamburg, Germany) BA 17 combined glass electrode. The samples were mixed with a Scientific Industries vortex (Bohemia, NY, USA) and centrifuged in a Sigma 1–14 laboratory centrifuge (Osterode, Germany).

2.3. Calibration and test samples

A calibration set of 14 samples containing all studied analytes in the range from 20 to 150 ng mL^{-1} was prepared. Twelve samples of the set corresponded to the concentrations provided by a Plackett-Burman design. The remaining samples were a blank solution (with no addition of any of the five analytes) and a mixture of all studied analytes at an intermediate concentration (*e.g.* 85 ng mL^{-1}). Each sample was prepared by transferring appropriate aliquots of stock solutions of GAL, EPI-GAL, ND-GAL, NO-GAL and OD-GAL to a calibrated tube and completing to 200 μL with milliQ water.

A set of eleven serum samples taken from two different pools and fortified with GAL and its metabolites at concentrations ranging from 0 to 140 ng mL^{-1} was analyzed. These concentrations were selected considering the levels usually found in serum of treated patients (see below).

Before the chromatographic analysis, both calibration and serum samples were treated as follows: after adding 20 μL of TCA 20% (w/v) each sample was shaken on a vortex mixer during 30 s and ultracentrifuged at 14,000 rpm for 10 min. Then, 100 μL of the supernatant were transferred to a vial containing a 300 μL fixed insert, and 20 μL of phosphate buffer (pH 7.00) were added. Finally, 100 μL were injected into the chromatographic system.

2.4. Data generation and software

Retention time-emission fluorescence matrices (TEMs) were collected exciting at 230 nm and measuring the intensity of the emission spectra, from 290 to 440 nm each 1 nm in the retention time range from 0 to 8.5 min, recorded each 0.72 s. Thus, TEMs were of size 708 × 151, and contained the chromatograms in the columns and the spectra in the rows.

All employed algorithms were implemented in MATLAB 7.6 [31]. Those tools for applying MCR-ALS are available in the Internet at http://www.mcrals.info/. Homemade routines based on the Eilers algorithm were applied to perform the second-order data baseline correction [32].

3. Theory

3.1. Baseline correction adapted to second order data

Baseline correction was performed through the asymmetric least-squares methodology proposed by Eilers [32], adapted to second-order data [33]. This method consists in the minimization of the following cost function:

$$Q = \sum_i v_i (y_i - f_i)^2 + \lambda \sum_i (\Delta^2 f_i)^2 \quad (1)$$

where y is the experimental signal, f is a smooth trend based on the use of splines (the baseline approximation), and v is a prior weight. The advantages of using splines in Eq. (1) have been discussed in Ref. [32]. The elements of v are 1 in all places where y is observed or allowed to influence f. In all other places, the elements of v are 0. The positive regularization parameter λ sets the second term weight. It acts as a roughness penalty: the larger λ, the smoother f will be. Δ denotes the derivative of f.

Considering the following choice of asymmetric weights: $v_{JK} = p$ if $y_{JK} > f_{JK}$ and $v_{JK} = 1 - p$ if $y_{JK} \leq f_{JK}$ with $0 < p < 1$, positive deviation from the trend will get weights different from negative residuals. Experience shows that starting from $v \cong 1$ and iterating between the two computations, quickly and reliably leads to a solution in about 10 iterations.

3.2. MCR-ALS

MCR-ALS is an algorithm capable of handling data sets deviating from trilinearity, *e.g.* data in which retention time shifts or peak shape changes occur for analytes from sample to sample. This can be done due to the strategy of augmenting matrices along the mode, which is suspected of breaking the trilinear structure, *i.e.* if matrix-to-matrix variation of profiles occurs along the column direction, a column-wise augmented matrix is created. The bilinear decomposition of the augmented matrix **D** is performed according to the expression:

$$\mathbf{D} = \mathbf{C} \times \mathbf{S}^{\mathrm{T}} + \mathbf{E} \quad (2)$$

in which the rows of **D** contain the fluorescence emission spectra (K wavelengths) and the columns contain all the experimentally registered successive time profiles (J times each) of each of the sub-matrices contained in **D**, the columns of **C** contain the time profiles of the N compounds involved in the process in all samples, the columns of **S** their related spectra, and **E** is a matrix of residuals not fitted by the model. Decomposition of **D** is achieved by iterative least-squares minimization of $||\mathbf{E}||$, under suitable constraining conditions, *i.e.* non-negativity in the spectral profiles, unimodality and non-negativity in the time profiles. In the case of samples containing uncalibrated interferents, a useful additional restriction is the so-called correspondence among species and samples. The latter one provides information as to the presence or absence of each analyte in each sample (for example, uncalibrated interferents are present in the unknown samples, but absent in the calibration samples).

Usually **D** contains calibration sub-matrices and each of the test data matrices. The pure spectrum of each compound should be the same in all experiments, but the time profiles in the different **C** sub-matrices need not share a common shape. This is the reason why chromatographic runs can be analyzed together, even in the presence of retention time shifts from sample to sample. Successful resolution also requires that the spectral mode be selective (see below).

It is necessary to point out that MCR-ALS requires initialization with system parameters as close as possible to the final results. In the case of the column-wise augmentation mode, the analyte spectra are required. In this work, the latter were obtained by the selection of the purest spectra based on SIMPLISMA (simple interactive self-modeling mixture analysis) [34]. Column-wise augmentation was selected because this allowed for successful resolution in the presence of chromatographic retention time shifts (see Section 4.4).

After MCR-ALS decomposition of **D**, concentration information contained in **C** can be used for quantitative predictions, by first defining the analyte concentration score as the area under the profile for the ith sample:

$$a(i,n) = \sum_{j=1+(i-1)J}^{iJ} C(j,n) \quad (3)$$

where $a(i, n)$ is the score for analyte n in sample I, and $C(j, n)$ is the element of the analyte profile in the augmented time mode. It should be noted that the area under each of the resolved chromatograms corresponds to the total sum of the profile intensities at all data points. Scores are employed to build a pseudo-univariate calibration graph against the analyte concentrations, predicting the concentration in the test samples by interpolation of the test sample score.

4. Results and discussion

4.1. General considerations

The simultaneous quantification of GAL and its metabolites using conventional spectroscopic techniques is difficult, even when coupled to chemometric tools, because they have very similar UV [14] and fluorescent spectra (Fig. 2). During exploratory experiments, several organized media (*e.g.* micelles and cyclodextrins) were added to aqueous analyte mixtures, aiming to achieve spectral selectivity. However, no significant selective changes in either the excitation or emission were achieved.

An important issue to be considered is the sensitivity of the method that should allow quantification of the analyte to levels usually found in the analyzed matrix. Pharmacokinetic studies of GAL in humans reveals maximum plasma/serum concentrations in the range of 30–180 ng mL^{-1}, depending on the sex, administered doses, route of drug administration, and the health of the subject [35–38], although higher concentrations (1000–3200 ng mL^{-1}) have been reported by other authors [6,39]. In addition, the interference produced by endogenous and exogenous compounds in serum samples represent an additional problem which should be considered. In principle, second-order multivariate calibration achieving the second-order advantage may remove the contribution of serum interferents. However, because of the strong spectral overlapping due to the structural similarity of the analytes, this strategy is also inefficient. Therefore, with the purpose of overcoming the drawbacks indicated above, a strategy combining liquid chromatography with fluorimetric detection and multivariate calibration was proposed.

Table 1
Instrumental and chemical parameters.

	Values/reagents
Mobile phase	0.01 mol L^{-1} ammonium acetate (pH 5.0)/acetonitrile (94:6, v/v)
Column	Zorbax Eclipse XDB-C18, 4.6 × 75 mm, 3.5 μm
Volumetric flow-rate (mL min^{-1})	2.20
Scanning speed (nm s^{-1})	180
Response time (s)	4
Temperature (°C)	25
pH of sample	7 (1 mol L^{-1} phosphate buffer)
Injection volume (μL)	100
Time range (min)	From 0 to 8.5
Emission range (nm)	From 290 to 440
Excitation wavelength (nm)	230
Excitation/emission slits (nm)	20
Photomultiplier gain	18
Calibration range (ng mL^{-1})	From 0 to 150

4.2. Selection of optimal experimental conditions

In Table 1 instrumental and chemical parameters used on the proposed method are summarized. These parameters were employed to evaluate the corresponding figures of merit.

In exploratory experiments, the conditions suggested by Verhaeghe et al. [13] for the determination of GAL alone in human plasma were applied. Then, these conditions were gradually modified in order to achieve a separation of GAL and its metabolites in the shortest time possible and using the least amount of organic solvent. It is important to remark that the use of a column packed with particles of 3.5 μm average diameter (rather than 5 μm) is crucial for the success of such analysis.

Some preliminary tests were performed with untreated serum samples. Although in principle the subsequent chemometric data analysis allows to overcome the spectroscopic interference problem, with the purpose of preserving the useful life of the chromatographic column, a simple deproteinization step with TCA was used.

4.3. Chromatographic analysis

First, a model system of the five analytes prepared in a buffer solution was tested using the selected working conditions, and all analytes peaks were resolved in five min using isocratic elution (Fig. 3, black line), the elution order being OD-GAL, ND-GAL, GAL, NO-GAL and EPI-GAL.

Fig. 3 also shows the obtained chromatogram corresponding to a serum sample containing the studies analytes (pink line). In this case, the large peaks from serum components bands produce a significant interference, precluding the quantification through the

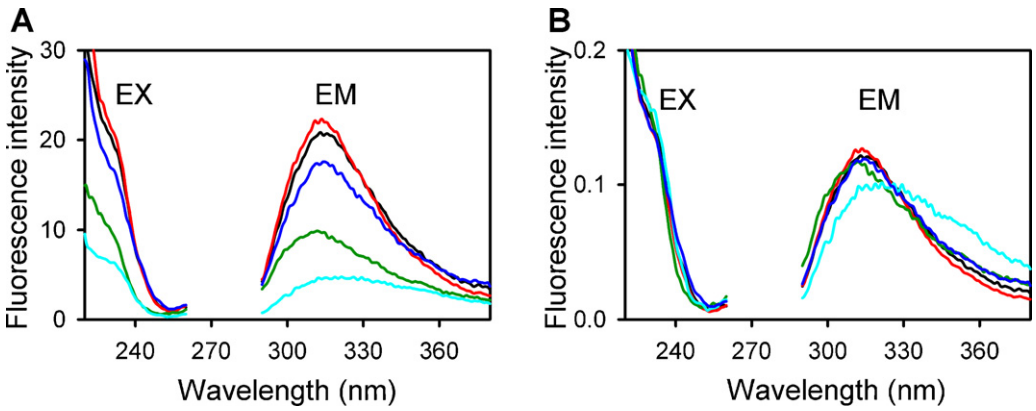

Fig. 2. (A) Excitation and emission spectra in aqueous solution of epi-galantamine (EPI-GAL, red line), galantamine (GAL, black line), galantamine-*N*-oxide (NO-GAL, blue line), *N*-demethylgalantamine (ND-GAL, green line), and *O*-demethylgalantamine (OD-GAL, cyan line). (B) Same spectra as in (A) after normalization to unit length. Concentration of each analyte = 500 ng mL^{-1}; $\lambda_{exc}/\lambda_{em}$ = 230/310 nm; excitation and emission slit widths = 5 nm; photomultiplier tube sensitivity = 650 V. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

classical zeroth-order calibration. Moreover, in addition to the lack of repeatability in the retention times between successive runs, the analyte chromatographic bands contained in serum samples are shifted in relation to those corresponding to analyte samples prepared in buffer solution. Some of them suffer a marked shift, as in the case of OD-GAL, whose retention time in calibration samples significantly differs from a typical serum sample (see inset of Fig. 3).

The high complexity of the analytical problem under study can also be seen in Fig. 4, which shows two contour plots of the complete landscape of fluorescence intensity as a function of emission intensity and retention time for a calibration sample (Fig. 4A) and for a serum sample (Fig. 4B).

MCR-ALS was selected for data processing because this algorithm achieves the second-order advantage and has the additional advantage of not requiring that a given component show the same chromatographic profile in each experimental run [24].

4.4. MCR-ALS analysis

Removal of chromatographic baseline facilitates data processing and improves the quality of the analytical results. Therefore, with the aim of subtracting the background present in both calibration and test samples, the methodology proposed by Eilers was applied by using ten spline basis functions and a single regularization parameter whose value was 1.

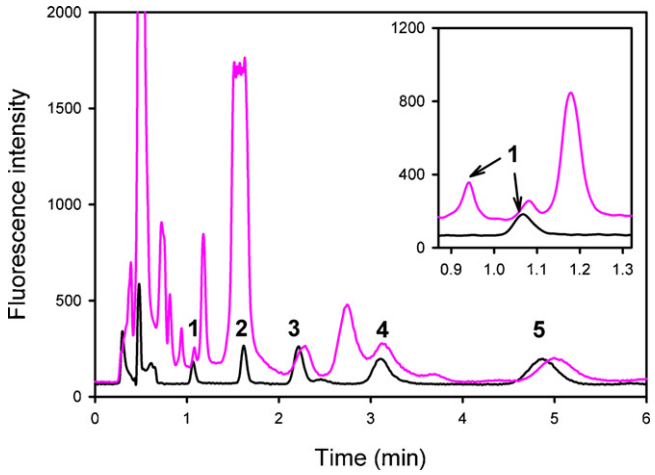

Fig. 3. Chromatograms of a typical calibration sample (black line) and a serum sample (pink line) containing OD-GAL (1), ND-GAL (2), GAL (3), NO-GAL (4) and EPI-GAL (5). The inset shows a selected range displaying a significant retention time shift of OD-GAL. Concentration of each analyte = 85.0 ng mL^{-1}; $\lambda_{exc}/\lambda_{em}$ = 230/310 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Due to the significant spectral overlapping of the studied analytes (see Fig. 2), it was not possible to perform MCR-ALS analysis with matrix augmentation in the temporal direction when working with the full chromatogram (*e.g.* involving the complete retention time range). On the other hand, because of the retention time shifts and band shape changes among the calibration and serum samples, matrix augmentation in the spectral direction was also inconvenient. Therefore, this algorithm was applied to the chromatographic data matrices, dividing the retention time axis in 5 time regions, each one including a single analyte (Table 2 and Fig. 5). In each of these regions, interferences occur, with spectra which are overlapped with the analytes, and with only partially resolved chromatographic profiles.

In each of these time regions, if matrix augmentation were applied row-wise, *i.e.* in the spectral direction, the chemical rank (total number of analytes and interferents) would be lower than the mathematical pseudorank, because a large number of bilinear components would be required to model the variation in retention time profiles in each data matrix. Conversely, in the column-wise augmentation mode, *i.e.* in the time direction, the chemical rank would be equal to the mathematical pseudorank, because the component spectra do not change from sample to sample.

Therefore, data processing comprised the building of augmented column-wise **D** data matrices containing, for each time region, the serum sample data and the calibration data matrices. Before starting resolution, the determination of the number of spectrally active components in each **D** data matrix was carried out by applying singular value decomposition (SVD). Typically, the plot of singular values as a function of principal component number is visually inspected, locating a number for which the plot stabilizes. This number is initially employed for MCR-ALS analysis, and is afterwards refined (increased or decreased) until an appropriate

Table 2
Selected chromatographic/spectral ranges used for MCR-ALS data processing.

Analyte	Region	Time (min)	Emission wavelength (nm)
OD-GAL	R1	0.90–1.04	290–349
ND-GAL	R2	1.57–1.67	290–349
GAL	R3	2.02–2.45	290–349
NO-GAL	R4	2.80–3.64	290–339
EPI-GAL	R5	4.34–5.61	290–369

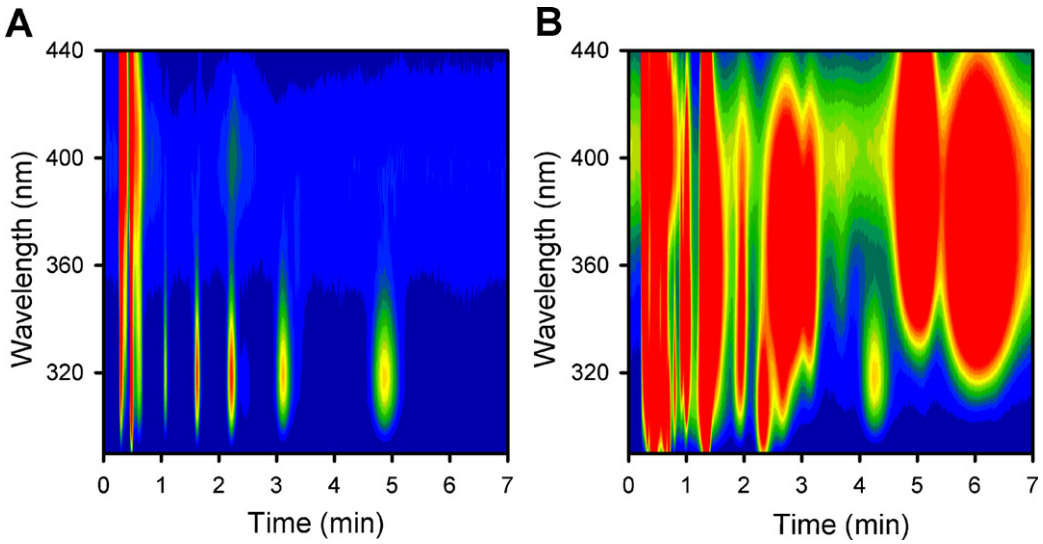

Fig. 4. Two-dimensional contour plots of time-retention/fluorescence emission matrices for a typical calibration sample (A), and that corresponding to a serum sample spiked with the studied analytes (B). Concentration of each analyte = 85.0 ng mL^{-1}.

solution is found, with a reasonable least-squares fit and physically recognizable profiles.

Given the number of responsive components, their spectra were then obtained from the analysis of the so-called "purest" spectra, based on the SIMPLISMA methodology, a multivariate curve resolution algorithm which extracts the purest spectra of the mixture from a series of spectra of mixtures of varying composition [34]. The spectra provided by SIMPLISMA were suitable to perform the resolution and, therefore, it was not necessary to include reference spectra for the analytes or to employ chromatograms as initial estimates for MCR-ALS.

With the purpose of driving the iterative procedure to chemically interpretable solutions, several constraints were applied, *i.e.* correspondence restriction, non-negativity in both modes and unimodality in the temporal mode. It was not necessary to restrict the analyte spectra to be equal to the pure spectra, because the profiles retrieved by MCR were highly satisfactory. The selected MCR convergence criterion was 0.1% (relative change in fit for successive iterations) and the maximum number of iterations was 50. Convergence was achieved after less than 20 iterations in most of the evaluated samples.

Fig. 5A shows the chromatograms obtained at multiple emission wavelengths and Fig. 5B the profiles retrieved by MCR-ALS in the temporal mode for a typical serum sample spiked with GAL and its metabolites. As can be seen, although the overlapping is significant, the chromatographic bands are recognized as belonging to the analytes or interferences contained in the serum. Further, the quality of the MCR-ALS recovered spectral profiles was evaluated using the criterion of similarity which involves a comparison, through the correlation coefficient (R) between the reference and evaluated spectrum [40]. The value of R found for OD-GAL, ND-GAL, GAL and NO-GAL was 0.9999, and for EPI-GAL it was 0.9996, corroborating the excellent quality of the MCR-ALS obtained results.

Table 3 shows the prediction results corresponding to the application of MCR-ALS to a set of 11 serum samples with analyte concentrations randomly selected within the studied range, and

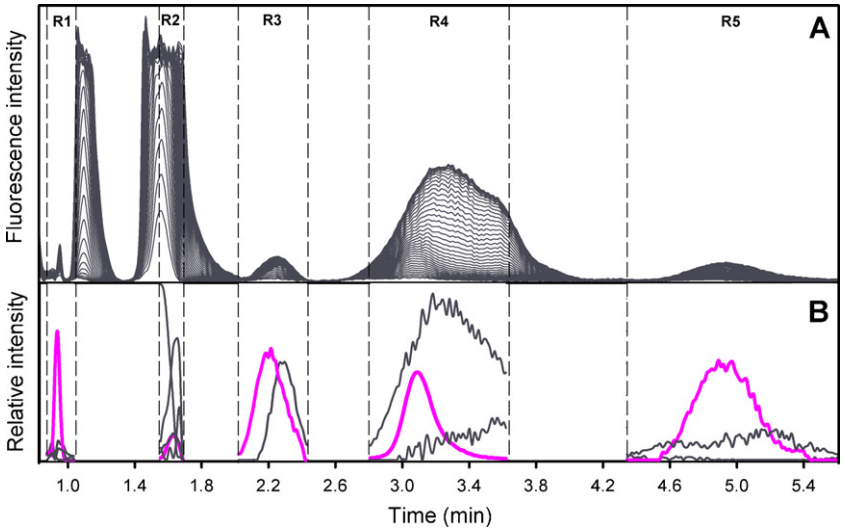

Fig. 5. (A) Baseline corrected chromatograms with fluorescence emission detection, at wavelengths between 290 and 440 nm for a typical serum sample spiked with the studied analytes. (B) Time profiles retrieved by MCR-ALS for each selected region (bottom). The pink line corresponds to the analyte of interest and the gray lines belong to the interferents. R1, R2, R3, R4 and R5 are the temporal regions used to perform the MCR-ALS analysis for OD-GAL, ND-GAL, GAL, NO-GAL and EPI-GAL, respectively. Concentration of each analyte = 85.0 ng mL^{-1}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Recovery study for GAL and its main metabolites in spiked serum samples using MCR-ALS.[a]

	GAL		EPI-GAL		ND-GAL		NO-GAL		OD-GAL	
	Taken	Found	Taken	Found	Taken	Found	Taken	Found	Taken	Found
1	0.0	ND[b]	0.0	9	0.0	ND[b]	0.0	ND[b]	0.0	ND[b]
2	30.0	24 (80)	140	152 (108)	30	34 (113)	30	24 (80)	140	127 (91)
3	85.0	86 (101)	85.0	86 (101)	85.0	81 (95)	85.0	73 (86)	85.0	78 (92)
4	140	141 (101)	30.0	26 (87)	140	139 (99)	140	131 (94)	30.0	28 (92)
5	50.0	54 (108)	50.0	51 (102)	50.0	66 (132)	50.0	41 (82)	50.0	46 (93)
6	118	117 (99)	118	121 (103)	118	95 (81)	118	120 (102)	118	110 (93)
7	85.0	83 (98)	85.0	80 (94)	85.0	83 (98)	85.0	86 (101)	85.0	86 (101)
8	85.0	81 (95)	85.0	80 (94)	85.0	87 (102)	85.0	81 (95)	85.0	86 (101)
9	40.0	49 (122)	60.0	55 (92)	60.0	69 (115)	40.0	41 (102)	60.0	65 (108)
10	60.0	69 (115)	40.0	41 (102)	40.0	40 (100)	60.0	58 (97)	40.0	38 (95)
11	85.0	90 (106)	85.0	92 (108)	85.0	87 (102)	85.0	80 (94)	85.0	74 (87)

[a] Concentrations are given in ng mL^{-1} and recoveries (between parentheses) are given in percentage.
[b] ND, not detected.

Fig. 6 illustrates the recovery results in addition to the elliptical joint confidence region (EJCR, [41]) test for the slope and intercept of the plot corresponding to each analyte. All ellipses include the theoretically expected values of (1,0) for slope and intercept, respectively, indicating the accuracy of the used methodology for these compounds.

The good recoveries obtained after the application of MCR-ALS suggest that matrix effects, which could be present in chromatographic analysis of biological matrices [42], are not significant and, therefore, the use of external calibration was an adequate option.

The statistical results are complemented with the values shown in Table 4. The relative errors of prediction and the coefficient of variations (all below 15%) indicate good precision [43]. The largest relative error of prediction for ND-GAL (12%) is related to its high degree of overlapping with serum components (see Fig. 5).

Both the limits of detection (LODs) and limits of quantification (LOQs) obtained in the serum samples are acceptable, taking into account that a very simple methodology is applied to a complex multicomponent system. Considering the typical values which can be found in real samples (see above), the proposed method could be directly applied without need a pre-concentration step.

Table 4
MCR-ALS statistical results for serum samples.

	GAL	EPI-GAL	ND-GAL	NO-GAL	OD-GAL
RMSE (ng mL^{-1})[a]	5	6	10	6	5
REP (%)[b]	7	8	12	8	7
CV (%)[c]	5	7	4	7	7
LOD (ng mL^{-1})[d]	11	8	9	12	10
LOQ (ng mL^{-1})[e]	34	24	27	36	30

[a] RMSE (root mean square error) $= \left[\sum_1^I (c_{act} - c_{pred})^2 / I\right]^{1/2}$, where $I = 11$.
[b] REP (relative error of prediction) $= 100 \times \text{RMSE}/\bar{c}$, where $\bar{c}$ is the mean calibration concentration.
[c] CV, coefficient of variation calculated from samples of concentrations 85 ng mL^{-1} for each analyte ($n = 4$).
[d] LOD, limit of detection calculated according to Ref. [44].
[e] LOQ, limit of quantification calculated as LOD × (10/3.3).

In comparison with the performances of selected methods for GAL (Table 5), limits of detection from 0.25 to 50 ng mL^{-1} and limits of quantification from 0.5 to 23 ng mL^{-1} have been found using

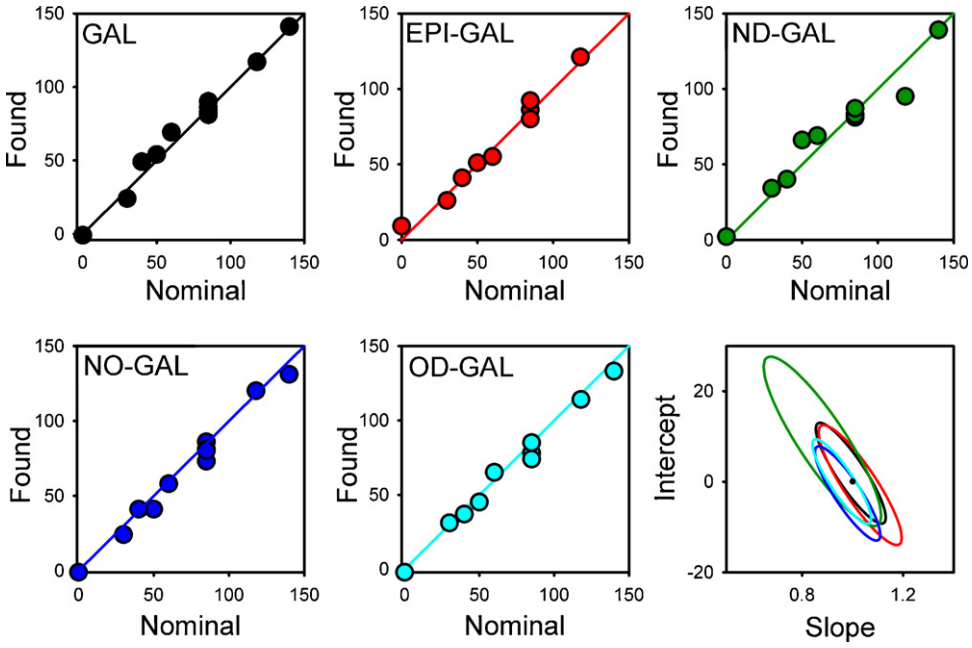

Fig. 6. Plots of GAL (black), EPI-GAL (red), ND-GAL (green), NO-GAL (blue) and OD-GAL (cyan) predicted concentrations as a function of the nominal values in spiked serum samples (as indicated), and elliptical joint regions (at 95% confidence level) for the slopes and intercepts of the regressions for the corresponding predictions. The black dot in the elliptical plots marks the theoretical (intercept = 0, slope = 1) point. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
Comparison of the analytical performance of selected methods reported for the determination of GAL in serum/plasma samples.

Methodology	Metabolites	Volume (mL)	Sample preparation	Range[a]	LOD[a]	LOQ[a]	CV (%)[b]	Ref.
HPLC (IM: DCM/*n*-hexane/ethanolamine [50:50:0.25]), UV	Not determined	2	PP (TCA)/LLE (DCM)	10–100	5		37.8 [5]	[5]
HPLC (IM: metanol/water [40:60] [5 mM dibutylamine]), UV	EPI-GAL, galantaminone	2	PP (TCA) and LLE (chloroform)	100–10,000	50		4.52 [500]	[6]
CE (BGE: tetraborate buffer), UV	Not determined	2	$MgCl_2$, PP (ACN)/SPE		6[c] 35[d]		1.17	[9]
HPLC (GM with linear steps: from 100% 0.1 M ammonium acetate (A) to 75% A and 25% 1 M ammonium acetate/methanol/ACN (10:10:80) (B) 40 min). Then, 1 min 100% B. Radioactivity and F	ND-GAL, OD-GAL, NO-GAL, EPI-GAL, ND and OD-EPI-GAL, glucuron-ides of GAL, OD-GAL, OD-EPI-GAL, OD-GAL sulfate	1	PP (ACN)	~1.2–15		~1.2[e]		[11]
HPLC (IM: ACN/0.01 M ammonium acetate [15:86]), MS–MS	Not determined	0.2	LLE (toluene)	1.0–500		1	2.1 [50]	[13]
HPLC (GM: ACN/5 nM ammonium acetate [15:85] increasing to 25% ACN in 4 min, held for 26 min), F	OD-GAL, ND-GAL, NO-GAL, EPI-GAL	0.1[f]	SPE (mixed mode sorbent)	23–5635	8.6	23	6.7 [43]	[14]
HPLC (IM: 0.03% formic acid/ACN [20:80]), MS–MS	Not determined	0.2	LLE (toluene)	0.5–100		0.5	7.3 [40]	[15]
MECK (BGE: 25 mM Tris buffer/160 mM SOS/20% ACN/0.01% PVP), UV	Not determined[g]	0.1	LLE (diethylether)	1.0–120	0.25	1	3.4 [30]	[16]
HPLC (GM: ammonium acetate pH 9.3/ACN), MS	Not determined[h]	0.50	SPE (mixed-mode sorbent)	2–300		2	1.1–4.9	[18]
UPLC (GM: ammonium acetate buffer/acetonitrile), MS–MS	Not determined[h]	0.25	PP (ACN)	1–300		1	3 [50]	[19]
HPLC–FSFS (IM: ammonium acetate pH 5.0/ACN [94:6]), F, MCR-ALS for data processing	OD-GAL, ND-GAL, NO-GAL, EPI-GAL	0.2	PP (TCA)	31–150[i]	11	31		This work

Abbreviations (others defined in the text): ACN, acetonitrile; BGE, background electrolyte; CE, capillary electrophoresis; DCM, dichloromethane; F, fluorescence detection; GM, gradient mode; IM, isocratic mode; LLE, liquid–liquid-extraction; MECK, micellar electrokinetic capillary chromatography; MS–MS, tandem mass spectrometry; PP, protein precipitation; PVP, poly vinyl pirrolydone; SOS, sodium octanesulfonate; SPE, solid-phase extraction; TCA, trichloroacetic acid; UPLC, ultra-performance liquid chromatography; UV, ultraviolet detection.

[a] Working range, limit of detection (LOD) and limit of quantification (LOQ) in ng mL^{-1}.

[b] Coefficient of variation (CV = standard deviation/mean × 100) measured at the concentration (in ng mL^{-1}) between brackets.

[c] Estimated value after SPE.

[d] Obtained value without SPE.

[e] Minimum evaluated plasma concentration.

[f] Rat plasma.

[g] Rivastigmine and its metabolite were also determined.

[h] Donepezil, memantine, rivastigmine and its metabolite were also determined.

[i] Maximum evaluated concentration.

different strategies. It should be noticed that most of the reported methods in Table 5 involve sample preparation steps for clean-up and/or pre-concentration, and also that many of them do not determine GAL metabolites.

In the present case, satisfactory LODs are achieved using a simple analytical approach, without applying pre-concentration steps and significantly decreasing the use of organic solvents. Additionally, a sampling rate of about seven samples per hour (excluding deproteinization, which can be carried out simultaneously on many samples) makes the method very advantageous.

5. Conclusions

The use of MCR-ALS combined with high-performance liquid chromatography-fast scanning fluorescence spectroscopic detection demonstrated the capability for the sensitive and selective determination of galantamine and its main serum metabolites. Analyses were accomplished in a significant short time, with a minimum operator effort and using small amounts of organic solvents. The good quality of the obtained results suggests that the developed technique is appropriate for the rapid quantification of galantamine and its metabolites in serum samples.

Acknowledgments

Universidad Nacional de Rosario and CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Project PIP 1950) are gratefully acknowledged for financial support.

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