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Characterization of acid–base properties of unstable drugs using a continuous-flow system with UV–vis spectrophotometric detection

Anna Argemí, Javier Saurina *

Department of Analytical Chemistry, University of Barcelona, Diagonal 647, Barcelona 08028, Spain

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Abstract

In this paper, we propose a continuous-flow system for the study of the acid–base characteristics of unstable drugs. 5-Azacytidine has been selected as a first model of unstable compound, which progressively decomposes in aqueous solutions. Besides, other compounds undergoing hydrolysis and oxidation side reactions have been also analyzed to explore the performance of the method. In comparison with conventional batch titrations, the drug decomposition can be minimized by the continuous renewal of the analyte solution. The composition of the buffer mixture is varied on-line during the process from successive changes in the flow rates of acid and basic stock solutions. As a result, the pH value of the test solution is varied in a controlled manner in the range of 1–13. Multivariate curve resolution based on alternating least squares has been used to extract relevant information concerning the acid–base properties of analytes. Results from the continuous-flow system have been compared with those obtained, using batch spectrophotometric titrations, and in the case of fast degradations, the performance of the proposed procedure has been superior.
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Keywords: Unstable drugs; 5-Azacytidine; Acid–base characterization; Continuous flow titration; Chemometric analysis

1. Introduction

Physicochemical properties of drugs, and especially acid–base constants, are of great importance in the pharmaceutical and clinical fields, as they influence the ADME profiles [1,2]. In the past decades, batch pH-metric and spectrophotometric titrations were considered as the reference methods for pK_a determinations [3]. Despite the passage of time, nowadays, these methods are being extensively applied to the calculation of dissociation constants. Some relevant features are the excellent precision and accuracy although certain solubility and stability problems may arise. Recent trends in determination of dissociation constants are based on predictive models established from correlations of chemical parameters of organic compounds [4,5], and chromatographic [6–9] and electrophoretic methods [10–17]. The use of separation techniques for pK_a determinations is based on the variation of the retention time or electrophoretic mobility as a function of pH. Apart for experimental methods, various computer programs such as PALLAS [18] and SPARC [19] have been developed for the estimation of dissociation constants from the chemical structure of the test compounds.

The lack of stability of the test solution during the measurement period may result in a significant drawback common to most of the experimental methods. In the case of unstable compounds, side reactions such as hydrolysis, oxidation, and precipitation may occur in parallel to the titration procedure so that the quality of the results may be affected. Flow methods based on a continuous renewal of the analyte solution during the experimental study have been proposed for solving this shortcoming. Besides, flow methods may offer additional advantages such as rapid analysis, excellent reproducibility, high degree of automation, and low expense of reagents [20,21]. Box et al. have proposed a flow system for the generation of a linear pH-gradient as a function of time from the mixture of acid and basic stock buffer solutions [22]. A computer-controlled pumping device is used to deliver solutions while providing the appropriate buffer composition. This system has been commercialized as the SGA-Profiler from Sirius for rapid determination of pK_a values of drugs with a sample throughput of 4 min per

* Corresponding author. Tel.: +34 93 402 12 77; fax: +34 93 402 12 33.
E-mail addresses: xavi@apolo.qui.ub.es, xavi.saurina@ub.edu (J. Saurina).

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assay (see www.sirius-analytical.com). Other papers describe the application of a pH-gradient flow-injection system for the estimation of acidity constants in which the whole titration is carried out in a few seconds. In this case, the pH gradient profile is first calibrated using suitable standards, and in a second stage, this pH profile is applied to calculate the pK_a values of test drugs [23,24].

The treatment of pH-metric and spectrophotometric data obtained in the acid–base studies has been carried out traditionally with computational methods such as SQUAD or SUPERQUAD as described in the Leggett's book [25]. These methods have not fallen into disuse but they have been updated with novel non-linear approaches for more stable and efficient modelling [26,27]. In last years, other treatments based on factor analysis have been proposed. Among them, target factor analysis (TFA) has been used extensively for the determination of acidity constants of organic compounds [28–31]. TFA estimates the abstract spectral and pH profiles from principal component analysis (PCA) and applies further rotation of factors to get meaningful contributions of the chemical species. TFA results in a simple chemometric method, which does not require special training, and can be easily automated. Another factor analysis method called multivariate curve resolution, based on alternating least squares (MRC-ALS) was proposed by Tauler et al. at the beginning of the nineties for the characterization of acid–base processes [32]. Since then, the method has been continuously improved with the implementation of natural constraints and model conditions for enhancing the analytical performance [33]. The simultaneous analysis of various processes, that is, various experimental data sets, has offered new possibilities for solving resolution ambiguities, and improving the precision and accuracy [34].

In this paper, 5-azacytidine has been selected as a model compound of unstable drugs. 5-Azacytidine is used as an anticancer agent belonging to nucleoside analogs [35–37]. The scheme of the deprotonation reactions is given in Fig. 1(a). Regarding stability, this drug decomposes progressively in aqueous solutions according to the reaction shown in Fig. 1(b) [38,39], this degradation being especially dramatic in basic media. For this reason, batch titrations are not suitable for evaluating the acidity constants due to its parallel hydrolysis in the titration vessel during the experimental procedure. Apart form 5-azacytidine, other unstable drugs undergoing hydrolysis, oxidation, or other side reactions, have been analyzed in this paper to check the performance of the proposed method in different circumstances.

The flow procedure proposed here consists of a continuous mixing of test solution with the titrant buffer solution using a two-pump manifold. The method has been adapted from a previous publication by Saurina et al. [40], which developed, and evaluated a continuous flow titration system for the spectrophotometric characterization of acid–base reactions. A similar approach was used in the study of the derivatization of amino acids with 1,2-naphthoquinone-4-sulfonate as a function of pH [41]. In these systems, the time of contact between analyte and buffer was short enough to avoid any decomposition, even at basic pH values. During the experimental procedure, the composition of the buffer solution (and thus the pH), was sequentially varied on-line from varying the ratio of the acid and basic stock solutions. Data generated in each run consisted of spectra registered at different pH values in the range of 1 to 13, approximately. MCR-ALS was used for data analysis in order to recover the acid–base profiles of species, and to calculate the corresponding acidity constants [42,43].

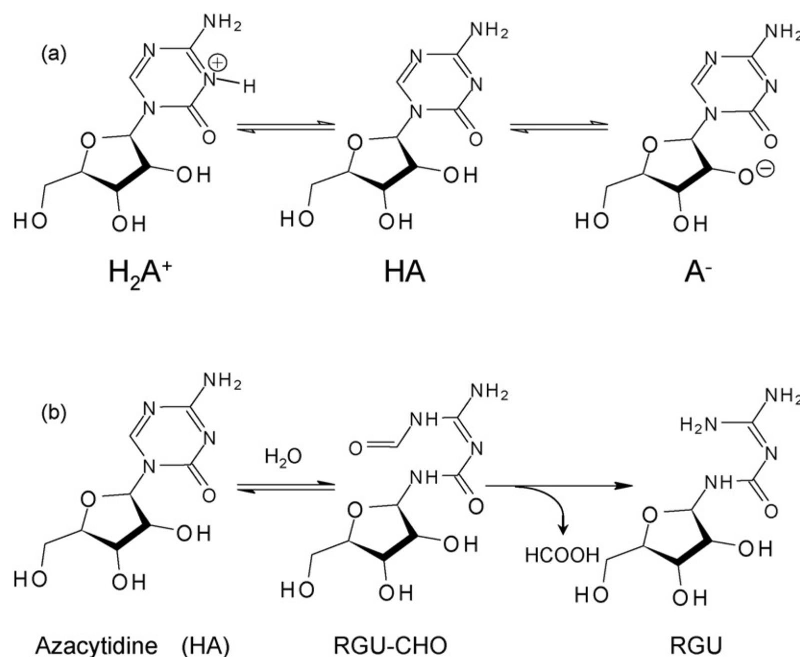

Fig. 1. Scheme of the protonation (a) and decomposition reactions (b) of 5-azacytidine. Species assignment: H_2A^+, protonated 5-azacytidine species; HA, neutral 5-azacytidine species; A^-, deprotonated 5-azacytidine species; RGU-CHO, N-(formylamidino)-N'-ß-D-ribofuranosylurea; RGU, 1-ß-D-ribofuranosyl-3-guanylurea.

2. Materials and methods

2.1. Reagents and solutions

Ultrapure water (Millipore, Milford, MA, USA) was used for the preparation of all solutions. Test compounds were 5-azacytidine, dopamine, didanosine, pyrocatechol, sodium naphthoquinone-4-sulfonate, thioguanine, tyramine, and tyrosine from Sigma-Aldrich (St. Louis, MO, USA), and triflusal (2-acetoxy-4-(trifluoromethyl) benzoic acid) from Uriach (Barcelona, Spain). Sodium acetate, boric acid potassium dihidrogenfostate, hydrochloric acid, and sodium hydroxide (all of them from Merck, Darmstadt, Germany), were used for the preparation of acid and basic stock solutions indicated below. Standard buffer solutions of pH 7.0 and 4.0 for the calibration of the glass electrode were purchased from Panreac (Barcelona, Spain).

The acid stock solution to be used in the flow system consisted of 0.05 M phosphoric acid + 0.05 M acetic acid + 0.05 M boric acid. The basic stock buffering solution was composed of 0.05 M phosphate + 0.05 M borate + 0.05 M acetate.

2.2. Apparatus

A Perkin-Elmer Lambda-19 spectrophotometer equipped with a Helma flow-cell of 10 mm path length and 60 μl volume was used for spectral measurements in the range of 220–300 nm. Spectroscopic data were acquired with a PC using the standard Perkin-Elmer software. The pH of the sample solution was measured in the waste solution emerging from the system with a CyberScan model 2500 pHmeter (precision of ±0.1 mV) using a combined pH electrode ORION 9103SC with an inner Ag/AgCl reference electrode.

2.3. Continuous flow procedure

The two-pump experimental set-up was adapted from the former system proposed by Saurina et al. for the study of acid–base reactions [40]. As shown in Fig. 2, the continuous flow manifold was composed of three channels for pumping sample, acid and basic stock solutions, and two peristaltic pumps (Watson Marlow 505DU) P1 and P2. The buffer (titrant) solution was obtained by mixing on-line these acid and basic stock solutions in a PTFE mixing coil (200 cm × 0.7 mm I.D.). During the procedure, the speed of P2 was varied sequentially which lead to a variation in the buffer composition, and thus, in the pH of the resulting solution. Subsequently, sample and buffer solution mixed in a PTFE reaction coil (35 cm × 1.1 mm I.D.), reached the detection flow cell in 9 s. After each modification of the P2 speed, the corresponding spectrum was registered under steady-state conditions and the pH was measured experimentally at the waste solution.

In more detail, the peristaltic pump P1 delivered sample and buffer solutions at a constant flow rate of 1.15 ml min^{-1} each channel. In the titration, the flow rate of the acid stock solution was varied from 1.9 to 0.0 ml min^{-1}, using the variable-speed pump P2. In parallel, the basic stock solution was aspired at a variable flow rate (from 0.4 to 2.3 ml min^{-1}), which resulted

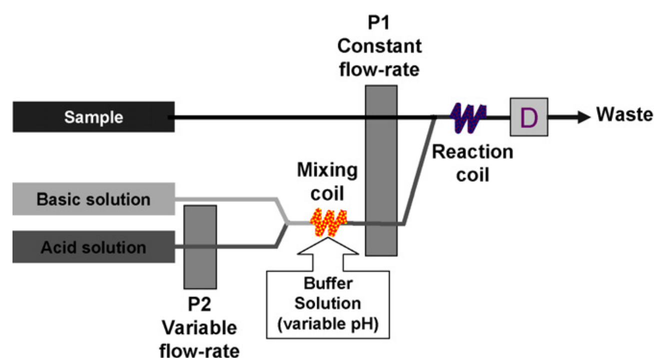

Fig. 2. Scheme of the continuous-flow system for the characterization of acid–base properties of unstable compounds. P1, Constant-speed peristaltic pump; P2, variable-speed peristaltic pump; D, spectrophotometer (spectral range: 220–300 nm); mixing coil = 200 cm × 0.7 mm I.D.; reaction coil = 35 cm × 1.1 mm I.D.; sample = 5×10^{-5} M 5-azacytidine solution; acid (stock) solution = 0.05 M phosphoric acid + 0.05 M acetic acid + 0.05 M boric acid; basic (stock) solution = 0.05 M phosphate + 0.05 M borate + 0.05 M acetate. Flow rates: sample channel = 1.15 ml min^{-1}; buffer channel = 1.15 ml min^{-1}; acid channel, variable from 1.9 to 0.0 ml min^{-1}; basic channel, variable from 0.4 to 2.3 ml min^{-1}.

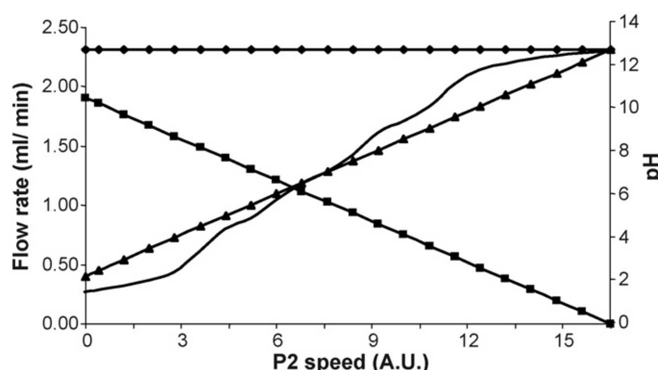

Fig. 3. Variation of the pH of the buffer solution as a function of the variation of the flow rates of acid and basic channels. –■–, Acid channel flow rate; solution; –▲–, basic channel flow rate; –♦–, titrant channel flow rate; —, pH profile.

from the difference between the flow rates of buffer and acid channels. Fig. 3 shows the variation of the flow rate of acid and basic stock solutions as a function of the speed of P2 (in arbitrary units). Complementarily, the corresponding variation of pH is also given.

3. Chemometric treatment

The spectroscopic data generated in each acid–base run were arranged in a data matrix **D**, in which each row represented a pH value, and each column a wavelength. Hence, the elements of the matrix consisted of absorbance values as a function of pH (through the pH domain) and wavelength (spectral domain). The experimental data were further analyzed with multivariate curve resolution based on alternating least squares (MCR-ALS method) to recover the concentration, **C** (i.e., distribution of species), and spectral profiles, $\mathbf{S}^T$, of species as shown in Fig. 4.

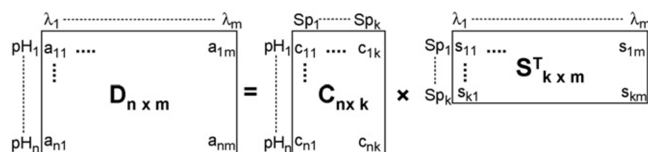

Fig. 4. Scheme of the resolution of the spectroscopic data matrix **D** into to the acid–base distribution profiles **C** and the spectral profiles $\mathbf{S}^T$ of species. Sp_i, acid–base species; a_{ij}, absorbance value at pH *i* and wavelength *j*; c_{ik}, concentration value of species *k* at pH *i*; s_{kj}, absorptivity of species *k* at wavelength *j*.

The principal steps of MCR-ALS are schematized in Fig. 5. For a more extensive description, see references [40–43]. Here, only a brief explanation of the procedure is given as follows.

3.1. Exploratory analysis

The first step consisted of a preliminary inspection of the corresponding data set using exploratory factor analysis tools such as singular value decomposition (SVD), PCA, and evolving factor analysis (EFA). This study provided relevant information concerning the number of chemical species present in the system as well as a first approximation to the analyte contribution profiles.

3.2. Initial estimations

They were the guesses of the spectral or pH profiles of species, that is, $\mathbf{S}^T$ or **C**, to be used in the optimization step. In this paper, spectral estimations were taken at the most representative pH values according to the predominance of 5-azacytidine species H_2A^+, HA, and A^-. Analogous considerations were followed for the other compounds under study.

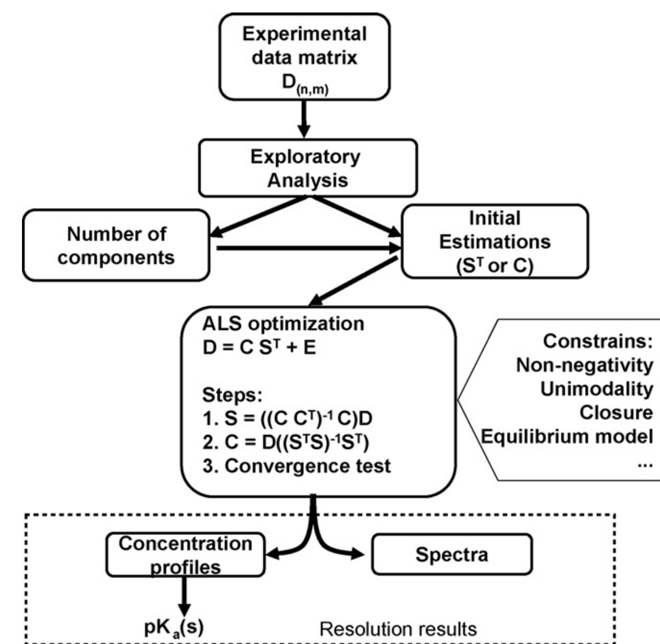

Fig. 5. Flow chart of the multivariate curve resolution method based on alternating least squares.

3.3. Optimization procedure

The least square optimization based on the compliance of the Beer's Law decomposed the experimental data matrix **D** into the distribution (concentration) profiles as a function of pH **C** and spectra $\mathbf{S}^T$. The general model can be written as: $\mathbf{D} = \mathbf{C} \times \mathbf{S}^T$.

At this stage, the initial spectral estimates were used as an input in the iterative optimization for the calculation of the concentration contributions as follows: $\mathbf{C} = \mathbf{D} \times (\mathbf{S}^T)^+$, where the superindex + refers to the generalized inverse.

Subsequently, $\mathbf{S}^T$ was updated using the expression: $\mathbf{S}^T = (\mathbf{C})^+ \times \mathbf{D}$, where $(\mathbf{C})^+$ is the generalized inverse of **C**.

In order to get better resolution results, various constrains were applied to restrict the mathematical solutions: non-negativity in the spectral and concentration profiles, unimodality, closure in the concentration profiles, and acid–base model.

The iterative calculations were repeated until obtaining the optimum **C** and $\mathbf{S}^T$ profiles. The stopping criteria were: (i) reaching a convergence fitting error defined beforehand, (ii) exciding a predefined number of iterations, and (iii) diverging in the fitting process 20 times consecutively.

The pK_a values of the test compounds were determined from the concentration profiles **C** at those points in which the concentrations of the acid and conjugate base were equal.

4. Results and discussion

4.1. Study of azacytidine processes

4.1.1. Optimization of the flow manifold

The rapid decomposition of 5-azacytidine in basic media was first evidenced in the study of spectroscopic data obtained from conventional acid–base titrations. The presence of a significant percentage of degradation occurring in parallel to the titration was demonstrated using chemometric tools. In particular, the SVD analysis showed the presence of, at least, five chemical species. Hence, apart from the three species involved in the deprotonation reactions, two additional components associated with degradation reactions were detected. This finding indicated that batch titrations were not suitable for the characterization of acid–base features of 5-azacytidine. In these circumstances, the performance of the continuous-flow assembly described in the experimental section was investigated as a way to overcome parallel degradation processes. Note that, 5-azacytidine was basified on-line, and the time of contact between the test drug and the buffer solution could be adjusted to a desired value in order to avoid decomposition.

Optimization studies were focused on the design of a setup to minimize the drug degradation. As commented, the key aspect in this optimisation, was decreasing the residence time, which depended on both reaction coil dimensions and flow rate. The effect of the reaction coil dimensions on the 5-azacytidine decomposition was evaluated using a constant flow-rate of 1.3 ml min^{-1} through the reactor. Various reactors of different lengths and internal diameters were checked. Table 1 summarizes the results obtained, suggesting that the residence

Table 1
Study of the degradation of the 5-azacitidine underwent in the continuous-flow manifold as a function of the residence time

Optimized variable	P1 flow rate (ml/min)	Reaction coil dimensions (cm × mm I.D.)	Residence time (s)	Degradation
Reaction coil	1.3	550 × 0.7	98	Very high
		95 × 0.8	22	Slightly
		35 × 1.1	15	Almost negligible
Flow rate	0.5	35 × 1.1	37	High
	1.0		20	Slightly
	1.5		13	Almost negligible
	2.3[a]		9	None[a]
	2.7[b]		7	None[b]

[a] Selected flow rate.
[b] High noise and bubble formation.

time should be, at least, shorter than 15 s to avoid degradation.

The influence of the flow-rate on the decomposition was studied using a reaction coil of 35 cm length × 1.1 mm I.D. Flow rates of sample and buffer channels were equal, and the overall flow-rate was varied from 0.5 to 2.7 ml min^{-1}. Accordingly, residence times varied from 37 to 7 s, approximately. As indicated in Table 1, the degradation at long residence times was noticeable, while it was almost negligible for times shorter than 10 s. However, it should be mentioned that the use of high flow-rates produced undesired effect such as an increase in the level of noise and risk of formation of bubbles. The optimum conditions corresponded to an overall flow-rate of 2.3 ml min^{-1} and a 35 cm length × 1.1 mm I.D.

4.1.2. Resolution of 5-azacytidine spectroscopic data

As an example, Fig. 6(a) shows the spectroscopic data obtained in the range of pH from 1 to 13 for the analysis of a 5×10^{-5} M 5-azacytidine solution, using the proposed flow-method. Spectral changes were noticeable around pH 3 and 11, approximately, so the corresponding pK_a(s) should be close to these values. As shown in the protonation scheme of Fig. 1(a), the first deprotonation of 5-azacytidine (H_2A^+) occurred in N-3 of the triazin-2-one ring. The pK_2 was associated to the deprotonation of the hydroxyl group in position three of the ribofuranosyl ring.

The MCR-ALS method was applied to the analysis of the spectroscopic data for the resolution of spectra and distribution profiles of drug species. The SVD analysis indicated the presence of three significant chemical species and no evidence of further degradation. Spectral initial estimations of H_2A^+, HA, and A^- were chosen at pH 1, 7, and 13 as they predominate around these pH values.

The ALS algorithm was applied to extract the 5-azacytidine profiles. The following constrains were used in order to improve the quality of the resolution: (i) Unimodality in the concentration profiles, (ii) non-negativity in both spectral and concentration profiles, (iii) physicochemical model based on the compliance of the mass-action law, (iv) closure in the concentration profiles.

Fig. 6 shows the results recovered from the MCR-ALS analysis. In the case of the concentration profiles (Fig. 6(b)), the crossing points corresponded to the pK_a values of 5-azacytidine. Spectra of H_2A^+ and HA species were quite similar (with peak maxima around 255 and 245 nm, respectively), while A^- species displayed higher molar absorptivity.

This type of experimental studies was carried out in triplicate, so that analogous data were obtained from the other runs. As summarized in Table 2, results from the MCR-ALS were similar in the three analyses. The average pK_a values of 5-azacytidine were 2.67 ± 0.03 and 12.26 ± 0.02.

4.2. Study of other unstable drugs

Additional examples of other unstable compounds were analyzed according to the proposed method, in order to evaluate its performance for different model compounds. In particular, the following compounds were selected: Triflusal (2-acetoxy-4-(trifluoromethyl) benzoic acid) as a model of ester compound related to acetylsalicylic acid, which is being commercialized as a platelet aggregation inhibitor. The ester group undergoes a rapid hydrolysis in basic media. Didanosine and thioguanine were chosen as antiretroviral and anticancer nucleoside analogues suffering hydrolysis reactions in basic media. Apart from hydrolysis reactions, other compounds such as tyrosine, tyramine, and dopamine were selected as examples of oxidizable phenolic derivatives. More complex side reactions were expected in the case of pyrocatechol, which was analyzed as an example of polyphenol compound undergoing oxidation and condensation reactions. Finally, naphthoquinone-4-sulfonic acid was chosen as a model of substances with oxidation and hydrolysis problems.

These compounds were studied in a similar manner to 5-azacytidine case detailed in the previous section. As a com-

Table 2
Values of pK_a of 5-azacitidine calculated using the proposed procedure

Titrations	Experimental pH range	pK_{a_1}	pK_{a_2}
T1	1.40–12.65	2.66	12.25
T2	1.40–12.61	2.70	12.25
T3	1.63–12.54	2.64	12.29
Average		2.67 ± 0.03	12.26 ± 0.02

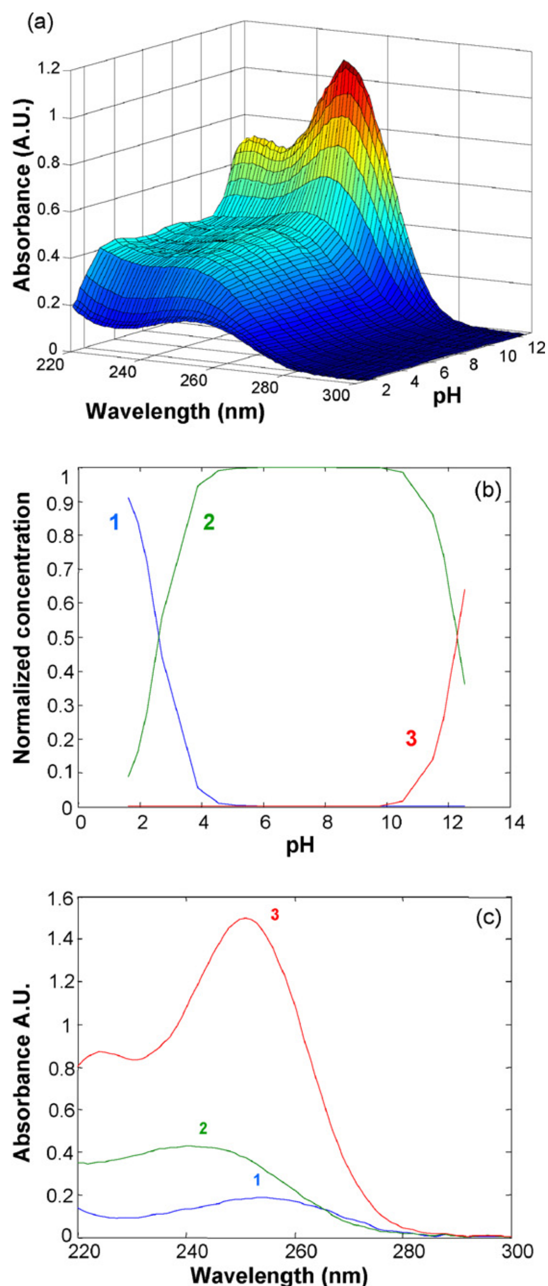

Fig. 6. Determination of the pK_a values of 5-azacytidine. (a) Spectroscopic data obtained for a 5×10^{-5} M 5-azacytidine; (b) concentration (distribution) profiles recovered using the MCR-ALS and (c) spectral profiles recovered using the MCR-ALS. Species assignment: 1, H_2A^+; 2, HA; 3, A^-, defined according to Fig. 1a.

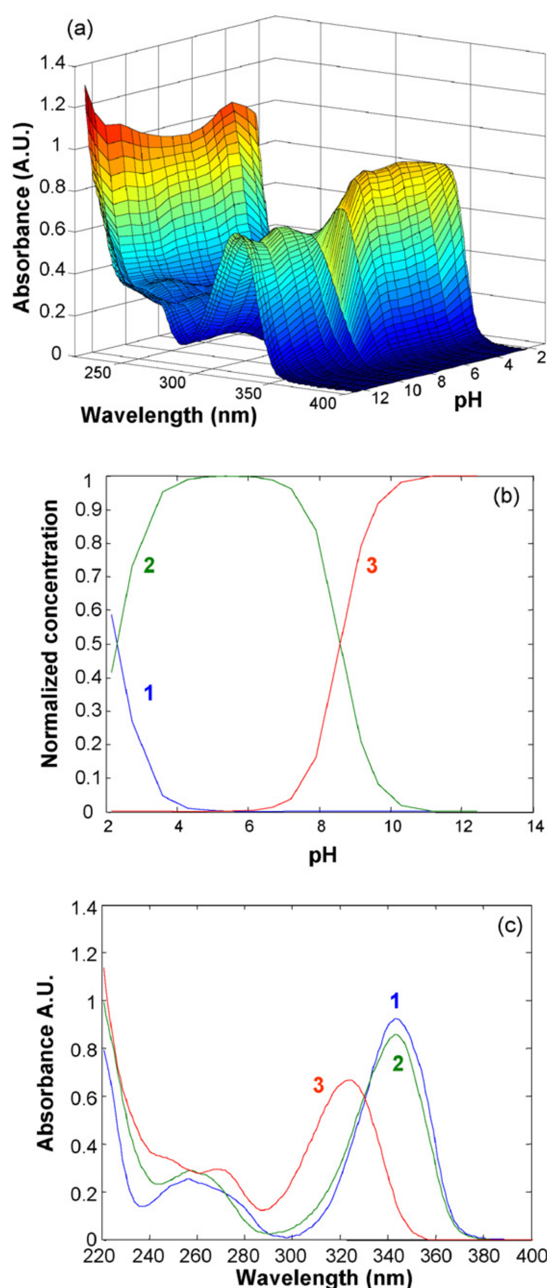

Fig. 7. Determination of the pK_a values of thioguanine. (a) Spectroscopic data obtained for a 5×10^{-5} M thioguanine; (b) concentration (distribution) profiles recovered using the MCR-ALS and (c) spectral profiles recovered using the MCR-ALS.

plementary example, the characterization of thioguanine has been illustrated with figures depicting the experimental data set as well as the resolved spectral and distribution profiles (see Fig. 7).

The pK_a values calculated according to the proposed procedure were compared with those available in the scientific literature (see Table 3). As indicated, in the case of didanosine and thioguanine, dissociation constants were obtained from a pH-gradient flow-injection method, which was able to minimize extension of degradation processes. In the absence of suitable published values as a reference, pK_a values were estimated from the calculation programs (PALLAS and SPARC) based on the structure of the molecules. The lack of some experimental values was attributed to the existence of degradation processes, which may hinder the calculation of pK_a values. As shown in Table 3, a satisfactory concordance was found in all cases so that the accuracy and efficiency of the proposed procedure was demonstrated.

Table 3'
Determination of pK_a values of some model compounds using the proposed method

Compound	Proposed method	Reference method		
	Experimental pK_a	Reference pK_a	Comments	Reference
Didanosine	9.50	9.35	pH-gradient FIA	[24]
Dopamine	8.73	8.87	Potentiometry	[44]
1,2-Naphthoquinone-4-sulfonic acid	9.08	8.86	PALLAS estimation	[18]
	10.90	11.68		
Pyrocatechol	9.56	9.45	Potentiometry	[3]
6-Thioguanina	2.30	–	pH-gradient FIA	[24]
	8.45	8.25		
		2.30	SPARC estimation	[19]
		8.60		
Triflusal	2.97	3.04	PALLAS estimation	[18]
Tyramine	9.58	9.53	Potentiometry	[44]
Tyrosine	9.24	9.21	Potentiometry	[3]

5. Conclusions

The continuous flow method resulted in a highly attractive and efficient approach for a rapid characterization of acid–base properties of drugs. The performance of this method was demonstrated in the case of unstable compounds in which batch procedures may fail. The time of contact between analyte and buffer solution was modulated from the reaction coil dimensions and total flow-rate to minimize the degradation process. For 5-azacytidine, the degradation in basic media was noticeable in less than 1 min, so that shorter residence times were required. The method was used with no modification to the analysis of other unstable compounds such as didanosine, naphthoquinones sulfonate, etc. The system could be even adapted for dealing with faster degradations through re-optimization of the manifold variables for getting shorter residence times. Multivariate curve resolution based on alternating least squares was used to recover the contributions associated to the chemical species from the treatment of the experimental data sets. The number of species detected mathematically was consistent with the acid–base reactions so that possible parallel decompositions were avoided. In comparison with commercial instruments, the system proposed here can be easily assembled from apparatus and pieces commonly present in the majority of pharmaceutical and analytical laboratories with no extra cost, and it can be used for both eventual and routine determinations. The versatility of the method can be extended through the use of other detectors of interest when test drugs display poor spectrophotometric features.

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