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Short communication

High-throughput and reliable assessments of the ionization constant of monoprotic organic acids through an arginine based mixed mode HPLC

Giacomo Russo [a,*], Ardiana Kajtazi [b], Maura Vallaro [c], Pieter Surmont [b], Giuseppe Ermondi [c], Scott Anderson [d], Lucia Grumetto [e], Giulia Caron [c], Frédéric Lynen [b]

[a] School of Applied Sciences, Sighthill Campus, Edinburgh Napier University, 9 Sighthill Ct, EH11 4BN Edinburgh, United Kingdom
[b] Separation Science Group, Department of Organic and Macromolecular Chemistry, Ghent University, Krijgslaan 281 S4bis, B-9000 Ghent, Belgium
[c] CASSMedChem Research Group, Molecular Biotechnology and Health Sciences Department, University of Turin, piazza Nizza 44 bis 10126 Turin, Italy
[d] Regis Technologies Inc., 8210 Austin Ave, Morton Grove, IL 60053, USA
[e] Department of Pharmacy, School of Medicine and Surgery, University of Naples Federico II, Via D. Montesano, 49, I-80131 Naples, Italy

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ABSTRACT

The charge state of a molecule is the single most prominent attribute ruling out its interactions with the surrounding environment. In a previous study, the retention of acids on the new Celeris™ Arginine (ARG) column was found to be predominantly driven by electrostatics and, specifically, their charge state. Therefore, we analysed 41 compounds in liquid chromatography with ultraviolet detection to study possible relationships between the analytical retention on this phase and the pK_a of the acidic solutes. Highly significant relationships were observed indicating either a linear ($r^2 = 0.86$) or a quadratic ($r^2 = 0.89$) trend. To improve the throughput of the method, this was transferred to LC mass spectrometry, allowing the analysis of a molecule every 3 mins. The developed method was found to be fast, reliable, accurate, easily automatable and simple to set up. Finally, the analytical column's being industrially manufactured and commercially available offers broad applicability.

1. Introduction

Probably, the charge state of a molecule is the single most prominent attribute dictating its interactions with the surrounding environment [1,2].

Indeed, molecular charge state affects solubility, crystallinity, the partitioning in isotropic or anisotropic phases and can inform crucial decisions in (pre-) formulation. For molecules with pharmacological/toxicological properties, the charge state deeply influences the interaction with biological membranes (absorption), plasma proteins (distribution) and microsomal and non-microsomal enzymes (metabolism and elimination) [3].

The charge state of a molecule depends on its ionisation constant (pK_a) and on the pH of the medium [4]. For this reason, both the US Food and Drug Administration (FDA) and the Organizations for Economic Cooperation and Development (OECD) require that all new chemical entities (NCEs) have their pK_a measured as part of a new drug application [5].

pK_a does not hold its relevance in pharmaco- or toxico- kinetics (PK/TK) assessments, only. Indeed, the interaction of chemicals with both cytoplasmatic and nuclear receptors, which impact the pharmaco- or toxico- dynamics (PD/TD), takes place *via* an *ensemble* of recognition forces, which does include ionic bonds, whose contribution again is modulated by pK_a [6].

To date, potentiometry, exploiting the glass-membrane pH electrode and high-impedance pH meters, is universally regarded as the gold standard technique for accurately and precisely determining the pK_a of a molecule [7]. However, this method does suffer from some shortcomings, which can be identified in the need of having a sample of high purity and soluble at least at 10^{-4} M in water [8]. This restricts its applicability to lipophilic molecules, and although the use of co-solvent has been proposed to overcome this drawback, most experts argue that this approach affects the reliability of the measurements [9].

UV-Vis spectrophotometry can offer a viable alternative, though [10]. The main advantage is that this technique tends to be significantly more sensitive ($> 10^{-6}$ M for compounds with favourable molar absorption coefficients) than potentiometric titration is [11]. However, the most obvious limitation of this methodology is that the target compounds are required to support a UV-active chromophore close enough to the ionisation site of the molecule to afford pK_a measurement [12].

* Corresponding author.
E-mail address: g.russo@napier.ac.uk (G. Russo).

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Again, the solute of interest should be highly pure, or its impurity must not absorb in the UV-Vis range.

Conductometry can also provide some usefulness in determining the pK_a of solutes [13]. However, this technique is especially suited for acids with $1.9 \leq pk_a \leq 5.2$ but it is much less reliable for weaker electrolytes [13], whose pK_a determination is paradoxically even more critical in some stages of drug development, mainly – but not limited to – pre-formulation.

Separation science-based approaches are constantly gaining popularity as the common main trait of these endeavours is that samples impurities are not generally regarded as an issue and that universal detectors open up to the opportunity of measuring the pK_a of virtually any electrolyte. While capillary zone electrophoresis (CZE), in which the apparent electrophoretic mobility is dependent on the fraction of the compound in the charged form [14], is relatively new, high performance liquid chromatography (HPLC) based methodologies are instead better established [15]. This probably depends also on the aspect that, in CZE, the elution order is not dependent on charge to mass ratio but on the charge to degree of solvation radius, which makes the elution order much more difficult to predict and rationalize [16].

The value of reversed phase LC in the indirect determination of pK_a is historically based on Horvàth et al.'s solvophobic theory. In more recent iterations, several authors applied HPLC, mainly in reversed phase (RP), to derive pK_a of pharmaceutically relevant solutes [17,18]. The main disadvantage of these protocols lies in their being time-consuming and not universally applicable. Indeed, most of these analytical methods are based on ultraviolet (UV) detection, which means that only molecules supporting a UV-active chromophore can be measured and that each analyte must be analysed individually and not in a mixture. Furthermore, since the apparent pK_a depends, (also) on the percent of organic modifier in the mobile phase (MP), whose pH is changed according to a program during elution, a combination of organic solvent and pH gradient is needed to afford pK_a estimation.

For instance, Kaliszan and co-workers [17] set one pH gradient and one organic solvent gradient for the pK_a determination of a single compound. Moreover, the proposed elution program had to be adjusted to individual analytes, heavily discouraging implementation of this protocol in routine analysis.

The Celeris™ Arginine column (from now onwards called ARG phase) is a mixed-mode stationary phase based on a silica surface modified with the amino acid arginine, which has recently been launched on the market [19]. The ARG analytical column offers a mixed-mode selectivity and is amenable to both HPLC and supercritical fluid chromatography mode. The chromatographic behaviour of this column has been recently characterized by us [19] and we could ascertain how, for acids, analytical retention was predominantly dependent on the charge state of the molecules nearly at all percentages (from 10 to 80) of organic modifier (ACN).

For this reason, we decided to investigate the potential of this stationary phase to assist in robust and high-throughput assessments of monoprotic acids' pK_a by setting up an *ad hoc* analytical method based on LC coupled with electrospray ionization (ESI) time-of-flight (TOF) mass spectrometry (MS).

2. Materials and methods

2.1. LC/UV

2.1.1. Chemicals and sample preparation

The solutes were obtained from three commercial sources (Aldrich (www.sigmaaldrich.com, Darmstadt, Germany), VWR (www.vwr.com, Milano, Italy), Alfa Aesar (www.alfa.com, Kande, Germany), as reported in Table 1 and their purity was equal to or higher than 98 %. Acetonitrile (HPLC grade) was purchased from VWR, and Ammonium Acetate (reagent grade $\geq$ 98 %) was purchased from Alfa Aesar.

For LC/UV analysis, all the compounds were solubilised in the mobile phase, at a concentration range of 50–100 μg/mL.

Table 1
Names, experimental pK_a values and chromatographic retention coefficients *i.e.*, log $k^{ARG}_{30\%ACN}$ values, (along with their respective references) and supplier of the solutes included in the dataset.

Name	pK_a	log $k^{ARG}_{30\%ACN}$	Supplier
3-Amino Benzoic Acid	3.07 [5]	0.76	Sigma-Aldrich
4-Nitrophenol	7.15 [22]	-0.46	Sigma-Aldrich
4-Octyl Benzoic Acid	4.36 [22]	0.58	Alfa Aesar
Acetylsalicylic Acid	3.50 [5]	0.64	Sigma-Aldrich
Amobarbital	7.84 [22]	-0.74	Sigma-Aldrich
Atorvastatin	4.46 [22]	0.31	VWR
Benzoic Acid	3.98 [5]	0.42 [19]	VMR
Butobarbital	8.00 [22]	-0.65	Sigma-Aldrich
Captopril	3.70 [22]	0.42 [19]	Alfa Aesar
Cefazolin	3.60 [22]	0.58	VWR
Cefoxitin	2.75 [22]	0.62	Alfa Aesar
Chlorambucil	4.60 [5]	0.38 [19]	VMR
Cloxacillin	2.78 [22]	0.18 [19]	Alfa Aesar
Diclofenac	3.99 [5]	0.63 [19]	VWR
Ethosuximide	9.27 [5]	-0.80 [19]	Alfa Aesar
Flumequine	6.27 [5]	0.15 [19]	Alfa Aesar
Flurbiprofen	4.18 [5]	0.74	Sigma-Aldrich
Fluvastatin	4.31 [5]	0.33 [19]	Sigma-Aldrich
Hexobarbital	8.20 [22]	-0.93	Sigma-Aldrich
Ibuprofen	4.24 [5]	0.59 [19]	Alfa Aesar
Indomethacin	4.13 [5]	0.51 [19]	Alfa Aesar
Isonicotinic Acid	4.96 [22]	0.70	VWR
Ketoprofen	3.99 [5]	0.64 [19]	Alfa Aesar
Ketorolac	3.50 [5]	0.67	Alfa Aesar
Losartan	4.25 [5]	0.34 [19]	VWR
Nalidixic Acid	6.01 [5]	0.08 [19]	VWR
Naproxen	4.09 [5]	0.76 [19]	VWR
Nicotinic Acid	4.63 [5]	0.45 [19]	Sigma-Aldrich
Nitrofurantoin	7.05 [5]	-0.19 [19]	Sigma-Aldrich
Pentobarbital	8.18 [5]	-0.79	Sigma-Aldrich
Phenobarbital	7.41 [5]	-0.29	Sigma-Aldrich
Phenyl Acetic Acid	4.31 [22]	0.72	Sigma-Aldrich
Phenylbutazone	4.34 [5]	0.61	VWR
Phenytoin	8.28 [5]	-0.55 [19]	VWR
Piroxicam	4.96 [5]	0.67	VWR
Salicylic Acid	2.73 [5]	0.73 [19]	Sigma-Aldrich
Secobarbital	7.80 [22]	-0.82	Sigma-Aldrich
Sulfanilic Acid	3.25 [22]	0.70	VWR
Sulindac	4.70 [22]	0.50 [19]	VWR
Warfarin	4.82 [5]	0.37 [19]	VWR
Zidovudine	9.40 [5]	-0.90 [19]	VWR

2.1.2. Chromatographic hardware

An HPLC Varian ProStar chromatograph (Agilent, 5301 Stevens Creek Blv, Santa Clara, CA, USA) equipped with a 410 autosampler with a built-in thermostatable column compartment, a PDA 335 LC Detector and Galaxie Chromatography Data System Version 1.9.302.952 were used. The column was a Celeris™ Arginine 100 × 4.6 mm, 5 μm, 100 Å from Regis Technologies (Austin Avenue, Morton Grove, IL, USA).

2.1.3. Chromatographic conditions

All LC analyses were performed at 30 °C with a 70/30 (v/v) 20 mM ammonium acetate buffer pH 7.0/ACN. The pH was measured before the addition of organic modifier, with a pH meter calibrated using aqueous standard solutions. Flow rate was 1.0 mL min^{-1} and the injection volume was 10 μL. Retention factors results from the averages of at least three independent measurements.

2.1.4. Postprocessing of chromatographic signals

Retention factors on the ARG phase were accounted for by Eq. 1:

$$k = \frac{t_r - t_0}{t_0} \quad (1)$$

In which t_r is the retention time (min) of the analyte of interest and t_0 the dead time, determined by monitoring the baseline disturbance.

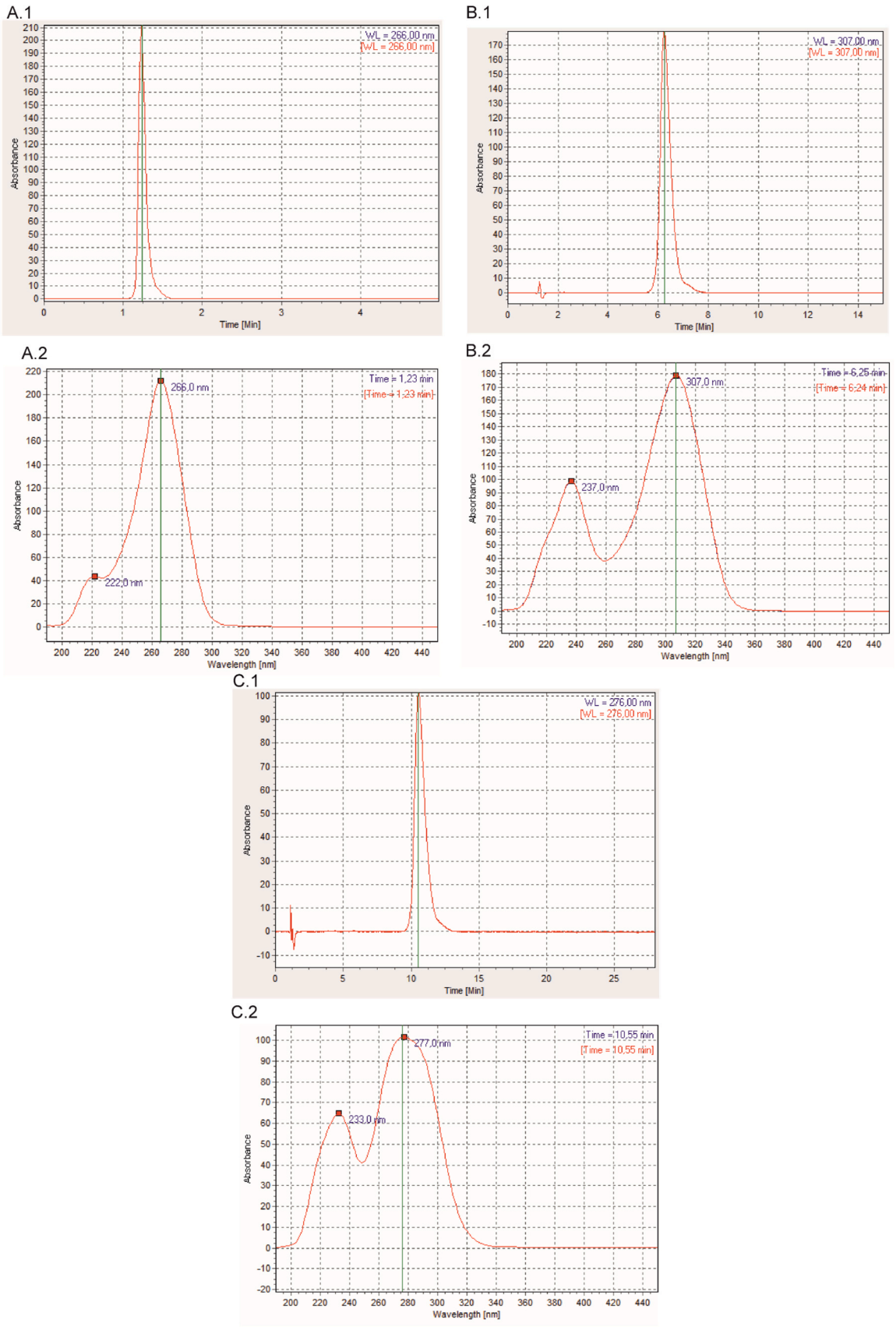

Fig. 1. HPLC UV chromatograms (.1) and spectra (.2) of Zidovudine (A), Warfarin (B) and Diclofenac (C).

Plotting and data analysis was done by Microsoft Excel for Office 365 v 16.0 at 64 bits.

2.2. LC/MS

2.2.1. Chemicals and reagents

LC-MS grade acetonitrile (ACN), methanol (MeOH), ammonium acetate (AA) and water (H_2O) were obtained from Sigma–Aldrich (Steinheim, Germany). Standards were obtained from the commercial sources reported in Table 1.

2.2.2. Sample preparation

Stock solutions of acids were prepared in concentrations from 3 to 18 mg/mL in ACN or MeOH, depending on their solubility. Once the stock solutions were prepared, they were stored in the freezer (−18 °C). Standard working solutions for LC/MS analysis were diluted to the concentration of 30–50 μg/mL in 50:50 (H_2O: Organic solvent) and prepared on the same day of analysis.

2.2.3. Instrumental conditions

Chromatographic separation was performed on a 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany). The system was constructed out of a 1100 quaternary pump equipped with a 1100 degasser, a 1100 auto injector, and a 1100 variable wavelength detector (VWD) equipped with a standard cell. RP-LC measurements were performed on a CELERIS™ Arginine column 5 μm, 150 × 2.1 mm (Regis Technologies, Inc., Morton Grove, IL 60053, USA). The eluents used in isocratic analysis were 70/30 (v/v) 20 mM AA at pH 7.0 / ACN with the flow rate 200 μL/min. The pH was measured before the addition of organic modifier, with a pH meter calibrated using aqueous standard solutions. Injection volume was 5 μL, the UV detection was recorded at 210 nm and the column temperature was kept at 30 °C during the analysis.

Mass detection was performed on Time-of-Flight Mass Spectrometer (TOF MS) 6230 system (Agilent Technologies, Waldbronn, Germany) equipped with electrospray ionization mass source with the mass range set from 50 to 1050 *m/z*. Gas temperature was set on 300 °C, gas flow 10.0 L/min, nebulizer 45 psi, sheath gas temperature 300 °C, sheath gas flow 10.0 L/min, Vcap 3500 V, nozzle voltage 500 V, and fragmentor 170 V. All compounds were measured in a negative ionization mode with the acquisition time of 30 min

For the MS analysis of chromatographic data, the OpenChrom [20] (freely available) software was used.

2.3. Dataset

To avoid any ambiguity, monoprotic acids only (2.0 – 9.5 pK_a values) were considered eligible. The dataset spans about 8 log P units (Sulfanilic acid, log P = −2.16 [21], 4-octyl benzoic acid, log P = 6.10 [21]). Experimentally determined pK_a values were collected from the scientific literature [5,22].

2.4. Model validation

Validation of the model was performed by the model validator of the software package VEGA ZZ (https://www.ddl.unimi.it/cms/index.php?Software_projects:VEGA_ZZ). This script allows the QSPR models to be validated by splitting randomly the whole dataset in a number of training and test set pairs. For each training set, the regression coefficients are calculated to evaluate the test set in terms of standard deviation of errors, angular coefficient, intercept and r^2 of the trend line of the chart of the predicted *vs.* experimental activities. Settings: test set size = 14, no of the iterations = 10, DepVar: Exp pKa values InDepVar is $\log k^{ARG}_{30\%ACN}$.

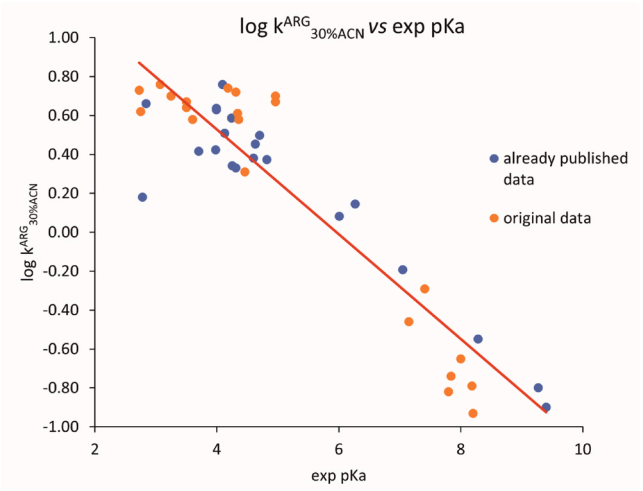

Fig. 2. Linear relationship between log k $^{ARG}_{30\%\ ACN}$ and experimentally determined pK_a values.

3. Results and discussions

3.1. General considerations

In our previous work [19], the ARG phase was found especially suited for molecules existing as anions at the experimental pH, while its affinity for bases, albeit covering a wide range of lipophilicity and pK_a values, was observed to be limited. In detail, the analytical retention of 26 pharmaceutically relevant acids was measured at percentages of organic modifier spanning from 10 % to 80 %.

As already mentioned in Introduction, at nearly all the MP compositions, the analytical retention of acids was found to be primarily driven by the molecule's electrostatics and, specifically, by its charge state. In modelling the chromatographic coefficients determined at 30/70 (v/v) ACN/ 20 mM ammonium acetate buffer pH 7.0, the charge state was found to account for over 85 % of the variance of the regression. This evidence motivated us to:

(i) Extend the dataset, analyse in HPLC UV, and derive log $k^{ARG}_{30\%ACN}$ values to ascertain whether this relationship was still verified.
(ii) Develop an analytical method based on LC/ESI TOF MS for the simultaneous determination of these analytical retention coefficients in a mixture and apply this to the dataset.

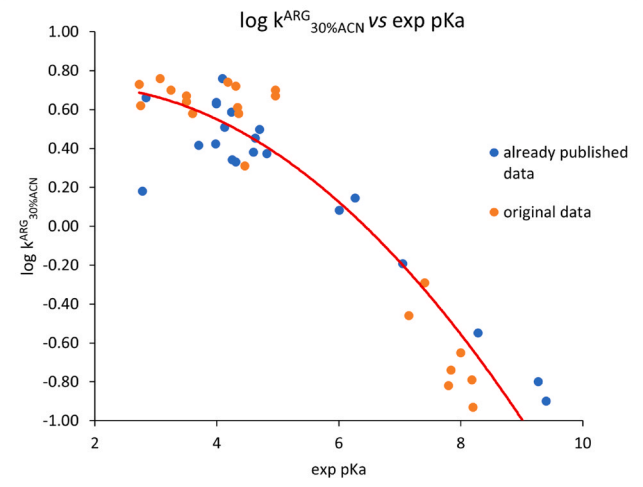

Fig. 3. Quadratic relationship between log k $^{ARG}_{30\%\ ACN}$ and experimentally determined pK_a values.

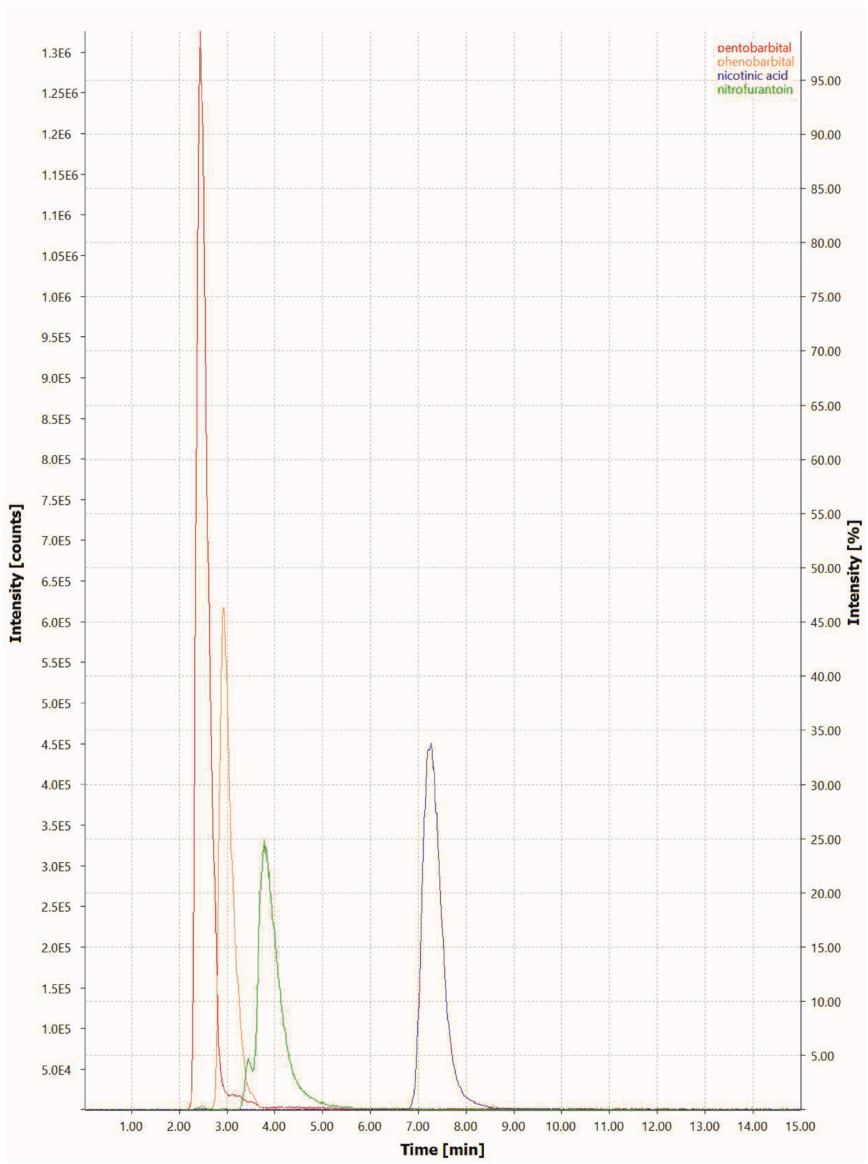

Fig. 4. Selected ion LC/MS chromatogram of nicotinic acid $[M\text{-}H]^-$ = 122.0248), nitrofurantoin ($[M\text{-}H]^-$ = 237.0265) pentobarbital ($[M\text{-}H]^-$ = 225.1245) and phenobarbital ($[M\text{-}H]^-$ = 231.0775) achieved on the ARG stationary phase. Methods are described in 2.2.

The dataset composition was chosen to include structurally unrelated, pharmaceutically relevant monoprotic acids spanning a wide range of pKa and lipophilicity.

3.2. LC/UV

The names, experimental pK_a values and chromatographic retention coefficients *i.e.*, log $k^{ARG}_{30\%ACN}$ values, and the supplier of the selected compounds are listed in Table 1. Exemplative UV chromatograms and spectra are reported in Fig. 1. The interpretation of the chromatographic signals was straightforward with symmetrical and well-resolved peaks. The studied analytes appeared well distributed in the elution window; 3-aminobenzoic acid was the compound retained for the longest time, while hexobarbital was the molecule exhibiting the least affinity for the ARG phase.

The log $k^{ARG}_{30\%ACN}$ values were studied *vs* the experimentally determined pK_a values. Highly significant relationships were observed hypothesizing either a linear (Fig. 2, r^2 = 0.86) or a quadratic (Fig. 3, r^2= 0.89) trend.

The equations, along with their statistical validation, are reported below:

$$\log k^{ARG}_{30\%ACN} = -0.2723(\pm 0.0173)pK_a + 1.626(\pm 0.0960) \quad (2)$$

n = 41 r^2 = 0·8634 q^2= 0·8599 SE = 0·211 $F_{1,41}$ = 246·4591 $F_{,1,41}$ α 0·001 = 15·56

and

$$\log k^{ARG}_{30\%ACN} = -0.0318(\pm 0.0097)pK_a{}^2 + 0.1052(\pm 0.1165)pK_a + 0.6394(\pm 0.3140) \quad (3)$$

n = 41 r^2 = 0·8933 q^2= 0·8877 SE = 0·1891 $F_{2,41}$ = 159·129 $F_{,2,41}$ α 0·001 = 8·21

In both equations, n is the sample size, r^2 is the square of the correlation coefficient, q^2 is the adjusted r^2, F is the Fisher coefficient along with its tabulated critical value calculated according to the dataset size and degree of freedom, at a 99 % confidence level.

A model validation of Eq. 2 is presented in Table S1. The high r^2 mean *i.e.,* 0.85, and its closeness to the q^2 mean *i.e.*, 0.83, accounting for

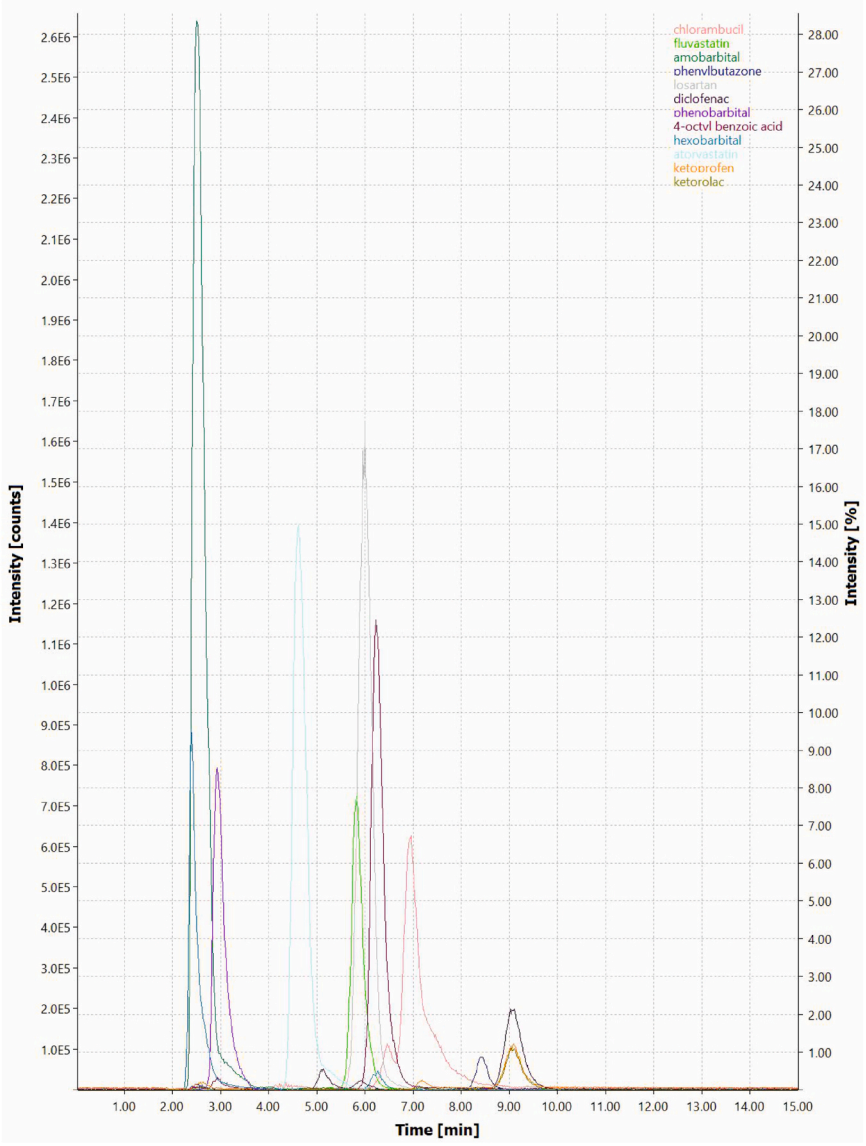

Fig. 5. Selected ion LC/MS chromatogram of 4-octyl benzoic acid ($[M-H]^-$ = 233.1547), amobarbital ($[M-H]^-$ = 225.1245), atorvastatin ($[M-H]^-$ = 557.2457), chlorambucil ($[M-H]^-$ = 302.072), diclofenac ($[M-H]^-$ = 294.0094), fluvastatin ($[M-H]^-$ = 410.1773), hexobarbital ($[M-H]^-$ = 235.1088), losartan ($[M-H]^-$ = 421.1549), ketoprofen ($[M-H]^-$ = 253.087), ketorolac ($[M-H]^-$ = 254.0823), phenylbutazone ($[M-H]^-$ = 307.1452) and phenobarbital ($[M-H]^-$ = 231.0775) achieved on the ARG stationary phase. Methods are described in 2.2.

all trials suggest the relationship between analytical retention and pKa is affected by the composition of the set of data to an only negligible extent.

Interestingly, the ARG stationary phase was previously characterised by us [19] by block relevance analysis, a powerful tool aimed at deconvoluting the individual contributions of intermolecular forces from the overall analytical retention. An interesting behaviour was observed for the size block, which refers to the molecular bulkiness. This was demonstrated to offer a positive contribution, therefore, to foster analytical retention, at %ACN $\leq$ 10 as it typically occurs in reversed phase (RP), whereas at %ACN $>$ 10 a sign change is observed for the size block, which becomes negative, as this contribution was demonstrated to hinder analytical retention, as expected in HILIC mode. This is very consistent with our results. Indeed, solutes with lower pK_a values, which are predominantly in anionic form at the experimental pH, are retained to a greater extent than compounds with higher pK_a values, which are instead prevalently in their uncharged form, so more hydrophobic.

log $k^{ARG}_{30\%ACN}$ values could be measured from only one isocratic run, without any need of column re-equilibration or of running multiple gradients. The technique demonstrated to hold potential as a high throughput tool for fast and reliable pK_a assessments, however, its main bottleneck lies in the low selectivity of the UV detection, which demands each compound to be analysed individually, greatly increasing the analysis time and the MP consumption. It should be noted that variation of the pH of aqueous buffer (ΔpH) with the addition of the organic modifier typically occur. For instance, ΔpH of 0.45 and 0.93 were recorded for phosphate buffers at pH 7 at 10 mM concentration with the addition of 10 % and 20 % acetonitrile, respectively [23].

3.3. LC/MS

For the development of the LC/MS method, a column format featuring lower internal diameter (2.1 mm) and greater length (150 mm), was chosen. This was done to (a) reduce the volume of MP that is delivered to the MS detector and therefore evaporated before ionization (b) widen the elution window and (c) increase the efficiency of the separation and, consequently, the resolution of the chromatographic signals. No peak was retained after 12 min. Exemplative LC/MS chromatographic runs of 4 and 12 compounds are provided in Figs. 4 and 5, respectively. While some minor tailing of few chromatographic signals can be observed, most signals were well-resolved and symmetrical, retention times were in all cases straightforward to assign and highly reproducible.

Our protocol afforded the estimation of pK_a of 12 acidic solutes in

only 36 min (in triplicate, 1 molecules/3 mins), consuming only 7.2 mL of mobile phase, of which 2.16 mL acetonitrile. Furthermore, this method can be applied for fulfilling more than one demand at the same time *e.g.*, stability assessment/dissolution studies, thus maximizing its throughput. Finally, the fact that the analytical column is industrially manufactured and commercially available offers broad applicability.

It is worth noting that being based on an isocratic elution program, this method is easily automatable, and no column re-equilibration is required. This means that on a daily work routine about 500 compounds can be screened for their dissociation constant, with less than 300 mL of MP consumed (less than 100 mL of acetonitrile). This is much higher than the methods developed by Wiczling and co-workers [24] on a dataset of same size, which albeit applicable indistinctly to acids and bases, offers a lower throughput of only 14 min per compound and an inferior accuracy (less than 0.5) than ours. The reason is that this is based on 9 runs of 36 mins at different pHs, which requires a superior human input and extra time for re-equilibration. The same research team validated their methodology on an ampler dataset ($n = 161$), however the throughput was unchanged [25]. Moreover, their technique was not applicable to very hydrophilic analytes (log P < 0.5).

4. Conclusions

Although preliminary, all in all this data suggest that the ARG phase can offer effectiveness, alone or with other well-established techniques, in drug discovery programmes. Indeed, this LC/MS method can be applied to mixture of drug candidates filtering out those with unfavourable dissociation constants. While the most obvious limitation of this method is its applicability to monoprotic acids only, the advantages lie in the fact that it is fast, reliable, accurate, easily automatable, and simple to set up. Moreover, no complex equations are required to assess the dissociation constants of the target analytes, but only some simple math operations. A further advantage is that since the pH of the MP is close to neutral, the method is applicable also to solutes supporting moieties that are not stable at the extreme pH values on which other LC methodologies are based.

CRediT authorship contribution statement

Giacomo Russo: Conceptualization, Project Administration, Writing – Original Draft, Writing – Review and Editing, **Ardiana Kajtazi:** Methodology, Investigation, Validation, Writing – Review and Editing **Maura Vallaro**: Investigation, Methodology **Pieter Surmont**: Methodology **Giuseppe Ermondi**: Conceptualization **Scott Anderson:** Resources **Lucia Grumetto:** Writing - Review & Editing **Giulia Caron**: Conceptualization, Writing - Review & Editing, Supervision **Frédéric Lynen**: Writing - Review & Editing, Project administration, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Scott Anderson was the Scientific Director of Regis Technologies Inc., that developed and manufactured the Celeris™ Arginine column, whose application is described in the present study.

Data availability

Data will be made available on request.

Acknowledgments

G.R. wishes to dedicate this work to the memory of his beloved father, S.R., (1940–2023).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115604.

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