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Short communication

A new RP-HPLC method as an auxiliary tool for optimization of sample preparation procedures for tracing of PPCPs of different hydrophilicities

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Recently, pharmaceutical and personal care products (PPCPs) have received considerable attention because of their increasing use. Analysis of PPCPs presents a significant analytical challenge, with high-performance liquid chromatography (HPLC) in reversed-phase mode, as the most widely used analytical technique. To facilitate the optimization of the procedures that are applied in the early stages of sample preparation, a simple and fast HPLC method is proposed in this work for the separation of some PPCPs with a wide range of hydrophilicity. Two columns were evaluated (Atlantis dC18 and Discovery HS F5); as for mobile phases: a formate buffer (40 mmol L^{-1}, pH 4) and methanol were tested in a gradient mode. The fluorinated column allowed better separation in a shorter time and better resolution for all analytes ($R_s > 1$). The proposed method delivered good performance for the tracing of PPCPs and is a suitable alternative to traditional C18-based HPLC methods.

Keywords: pharmaceutical and personal care products, reversed-phase high-performance liquid chromatography, ultraviolet detection

Pharmaceutical and personal care products (PPCPs) have received considerable attention in recent years in the scientific community because they are considered as emerging pollutants. These compounds are biologically active and widely used in a diverse range of common products such as fragrances and cosmetics, household cleaning products, foods, and prescription or over-the-counter pharmaceutical products (1). Given their increasing use in the world and their continuous introduction into the environment, the analysis of this heterogeneous group of compounds represents an important issue but it is also a significant analytical challenge (2). PPCPs have widely different physical and chemical properties and are present in very complex matrices, so analytical methods are usually tedious, time-consuming and expensive. Thus, the development of simple, fast and sensitive methods

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that can be used to analyze PPCPs of various classes within a single analytical procedure are needed (3). Moreover, given the typically low concentration levels of the analytes in environmental, food and biological samples, efficient cleanup and concentration procedures are required prior to instrumental analysis.

A number of gas and liquid chromatography methods have been proposed for the analysis of PPCPs. However, given that many of these compounds and their metabolites are polar and non-volatile, time-consuming derivatization procedures are required for gas chromatography, thereby limiting the application of this technique. Liquid chromatography is thus the most appropriate technique for the analysis of PPCPs (4). High-performance liquid chromatography (HPLC), mainly operated in the reversed-phase (RP) mode, is one of the most widely used analytical techniques. The most popular RP stationary phase is octadecylsilane (C18), although new stationary phases are continuously being developed to overcome the drawbacks of conventional RP stationary phases and to enable shorter analysis times, higher sensitivities, and better efficiencies (5). Interactions between the stationary phase and analytes are determined primarily by the properties of chemically bonded polar or nonpolar ligands in the support material, and also by residual silanol groups on the surface of silica particles or by silanol end-capping groups (6). On the other hand, the porosity of the stationary phase increases the surface area of the column, favoring a rapid mass transfer of analytes and results in good retention (7).

Liquid chromatography-mass spectrometry is the gold standard technique for the determination of PPCPs, and the use of a mass spectrometry detector has important advantages such as high selectivity, specificity and sensitivity (8). However, given that such instruments are very expensive and not commonly available in assay laboratories, the use of conventional HPLC with ultraviolet (UV) or photodiode array detectors dominates in many laboratory studies (9, 10).

The majority of methods currently available for the simultaneous determination of different PPCPs have been carried out either by analyzing different portions of the same sample under different experimental conditions (*i.e.*, using various analytical methods) (11, 12) or by using more complex and expensive instruments such as high-resolution mass spectrometers (13, 14). Although satisfactory results can be obtained, the use of any of these approaches is not an appropriate strategy during the development of new methods, and it is clear that there remains a need to find alternatives that allow the work in the laboratory to progress more efficiently. Therefore, the development of new methods for the analysis of biologically active compounds, such as PPCPs, in different matrices is still an important aspect of scientific research. In order to facilitate the first stages of optimization in the development of new sample preparation procedures for analysis of these compounds, in this work, a simple and fast HPLC method is presented for the separation of a mixture of PPCPs of markedly different hydrophilicities.

EXPERIMENTAL

Analytes, reagents and solvents

Benzophenone-3 (98 %) (BP-3), benzophenone-4 (≥ 97 %) (BP-4), caffeine (≥ 98.5 %) (CAF), diclofenac (95 %) (DIC), metformin (97 %) (MET), methylparaben (99 %) (MP),

naproxen (USP grade) (NAP), and propylparaben (≥ 99 %) (PP) were purchased from Sigma-Aldrich (USA). Methanol (HPLC grade) was from J. T. Baker (USA), formic acid (99 %) was obtained from Merck (Germany) and ammonium hydroxide (29 %) was purchased from Sigma-Aldrich. Deionized water was obtained with an Elga II system (Veolia, France).

Preparation of standards and reagent solutions

Stock solutions of PPCPs (200 μg mL^{-1}) were prepared in methanol and stored at –4 °C in the dark until use. Working solutions of target analytes (10 μg mL^{-1}) were prepared by diluting stock solutions in formate buffer/methanol (70:30, *V*/*V*) mixture. Formic acid/formate buffer was prepared by adjusting a formic acid aqueous solution (40 mmol L^{-1}) to pH 4.0 with ammonium hydroxide.

Chromatographic separation

Chromatography was carried out with an Agilent HP Series 1100 liquid chromatographic system (Hewlett Packard, USA), equipped with an online vacuum degasser model G1322A, quaternary pump model G1311A, autosampler model G1329A, thermostated column compartment model G1316A and a variable-wavelength UV detector model G1314A.

Optimization of the separation conditions was carried out with a mixture of target analytes (10 μg mL^{-1}) dissolved in formate buffer/methanol (70:30, *V*/*V*). All experiments were carried out in triplicate. The chromatographic columns evaluated in this study included an Atlantis dC18 (2.1 × 150.0 mm, 3 μm; Waters, USA) and a Discovery HS F5 (2.1 × 150.0 mm, 3 μm; Supelco, USA). As mobile phases, mixtures of formate buffer (40 mmol L^{-1}, pH 4) and methanol were tested. In all cases, the separation was carried out in a gradient mode with methanol content ranging from 0 to 95 %. Mobile phase flow rates from 0.15 to 0.20 mL min^{-1} were evaluated in accordance with column dimensions and mobile phase composition. Column temperatures between 35 and 45 °C and injection volumes of 3 and 5 μL were tested. Detection was carried out at 270 nm for all analytes with the exception of metformin, which was monitored at 233 nm. The HPLC system UV detector was programmed to make the change in the detection wavelength in the same analytical run, beginning at 233 nm and changing to 270 nm after 7 min.

Analytical validation

The proposed method was preliminarily validated by evaluating parameters such as linearity, the limit of detection (*LOD*) and the limit of quantification (*LOQ*), precision, and accuracy, considering the recommendations of the EURACHEM guidelines (15). To evaluate the linearity of the method, calibration curves were constructed by triplicate analysis of standard solutions of all target analytes at concentration levels of 2, 10, 20, 40, 80 and 100 μg mL^{-1} prepared in formate buffer/methanol (70:30). The association between variables was established by least-squares regression analysis for the responses of each analyte *vs.* concentration, the equation of the line was obtained and the correlation and determination coefficients (R^2) was calculated. The *LOD*s and *LOQ*s were calculated from the calibration curve data according to equations $LOD = 3.3\left(\frac{\sigma}{S}\right)$ and $LOQ = 10\left(\frac{\sigma}{S}\right)$, where σ is the standard deviation of the calibration line intercept and s is the slope of the calibration line.

Precision was evaluated by using the relative standard deviation percentage (RSD) values of the analyte response or peak areas at all concentration levels of the calibration curve (2, 10, 20, 40, 80 and 100 μg mL^{-1}) within of three consecutive analytical runs carried out. At the same time, the model accuracy was assessed by a back-calculated process for each standard of the calibration curve using the equation of the line. Correlation between nominal concentrations of the standards and calculated concentrations was evaluated by means of regression analysis and the deviation of the nominal value was calculated through recovery calculation.

RESULTS AND DISCUSSION

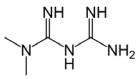
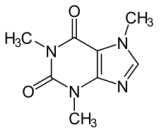
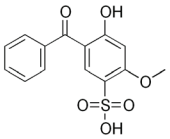
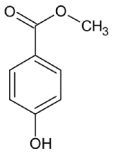
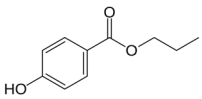
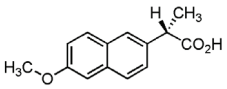
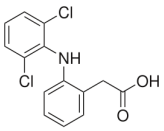
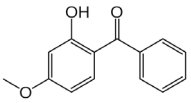
In this work, eight compounds pertaining to the PPCPs group were selected as model analytes: naproxen, caffeine, metformin, diclofenac, methylparaben, propylparaben, benzophenone-3 and benzophenone-4; all are commonly used in daily consumption products. As summarized in Table I, these compounds have different chemical structures and properties that cover a wide range of hydrophilicity or hydrophobicity (log *P* between –2.6 and 4.0) and acid dissociation constants (p*K* values from –2.4 to 13.9). These compounds were chosen in order to get a single method for the simultaneous analysis of molecules with differing polarities that co-exist in pharmaceutical and personal care products, environmental, food and clinical samples. This would greatly reduce laboratory work and the cost of analytical tests.

Optimization of chromatographic separation

Most HPLC and ultra-high performance liquid chromatography methods use C18 or C8 stationary phases (20). However, bearing in mind that some very hydrophilic compounds such as MET and CAF were included, an Atlantis dC18 column was tested because it can tolerate highly aqueous mobile phases. Fluorinated phases are nowadays considered a complementary option to classical alkyl-bonded reversed stationary phases because they offer different selectivity and retention because interactions such as hydrophobic, π-π interaction, dipole-dipole, and hydrogen bonding can be involved during the separation process (21–23). Thus, a column packed with pentafluorophenyl stationary phase (HS F5), was also evaluated. To keep the proposed method as simple as possible, only mixtures of methanol and a formic–formate buffer (pH 4, 40 mmol L^{-1}) were employed. As can be seen in Table I, in this work a mixture of ionizable molecules is involved. Hence, it was decided to work with a buffer in the mobile phase to control the ionization degree of molecules and, therefore, their relative hydrophobicity. In the same way, the buffer provided reproducibility and robustness to the system. Gradient elution to improve the separation and analysis time was necessary.

Several assays were carried out with each column, the retention of the investigated PPCPs decreased as the concentration of methanol in the mobile phase increased. The initial composition of methanol (1 and 5 %) was chosen so that the strength is appropriate to retain early eluting analytes or these with low log *P*. The experimental conditions with the best separation for the eight model analytes are presented in Table II. The chromatograms obtained under these conditions are collected in Fig. 1, and selected chromatographic parameters are summarized in Table III. Depending on the type of the stationary

Table I. Chemical structures, log $P_{o/w}$ *and* pK_a *of tested PPCPs*

Analyte	Structure	log $P_{o/w}$	pK_a	Reference
Metformin		–2.60	2.80/11.50	16
Caffeine		–0.07	13.90	17
Benzophenone-4		0.89	–2.40a/7.60a	18
Methylparaben		2.00	8.30	19
Propylparaben		2.90	8.20	19
Naproxen		3.18	4.30	17
Diclofenac		3.70	4.20	19
Benzophenone-3		4.00	7.60	19

[a] Values calculated with Chem Axon software from ChemSpider (Royal Society of Chemistry; Cambridge, UK).

phase and organic modifier content in the mobile phase, differences in the efficiencies of chromatographic systems were obtained. The order of elution resulting from the two tested stationary phases was very similar; a satisfactory correlation was observed between

retention and polarity in the Discovery HS F5 column. In Atlantis dC18 column, only MP and BP-4 showing an inverted elution order and showed the second-lowest resolution

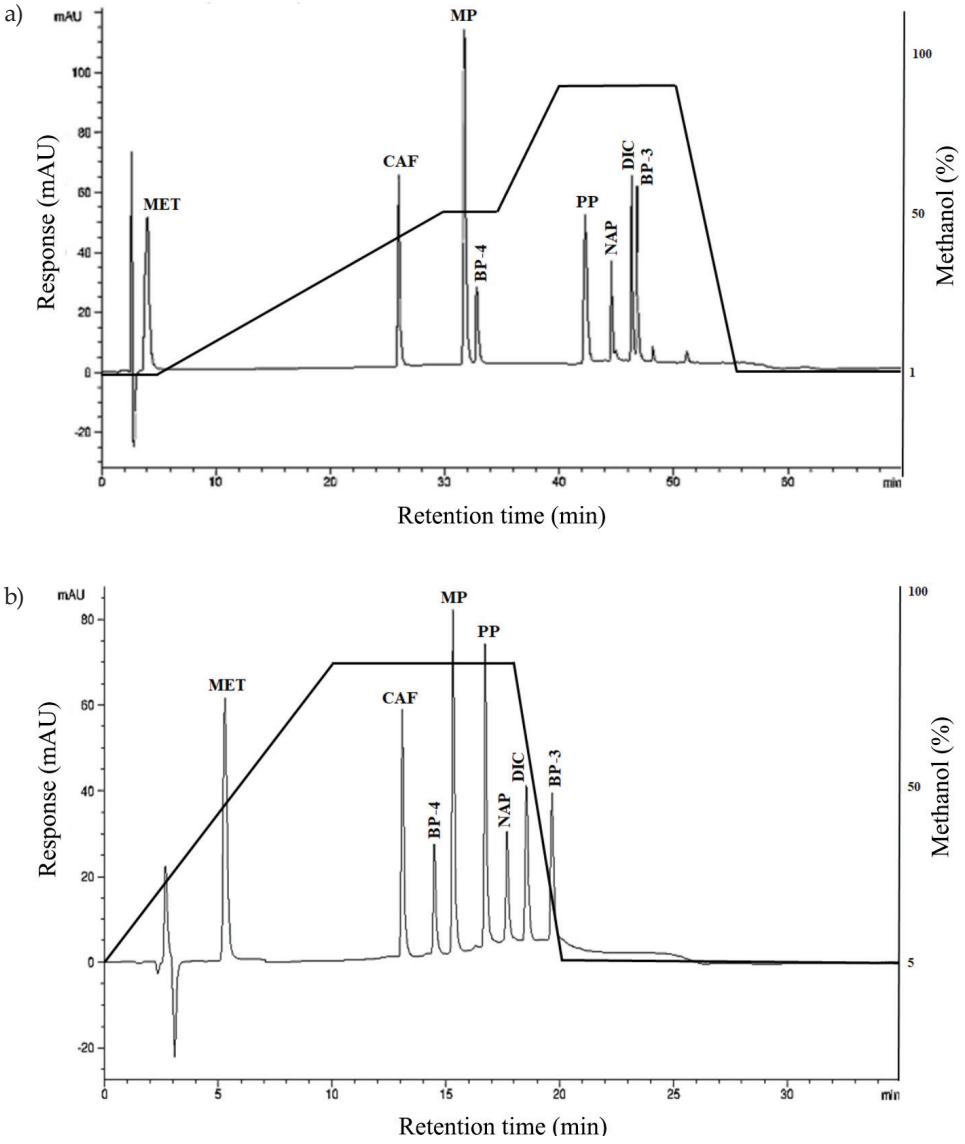


Fig. 1. Chromatograms obtained for PPCPs mixture with: a) Atlantis dC18 column and b) Discovery HS F5 column. The chromatographic conditions are described in Table II and the mobile phase gradient is shown with a thick line as a function of methanol portion. BP-3&BP-4 – benzophenone-3 and benzophenone-4, resp., CAF – caffeine, DIC – diclofenac, MET – metformin, MP – methylparaben, NAP – naproxen, PP – propylparaben.

Table II. Optimized separation conditions for the tested columns

Column	Atlantis dC18		Discovery HS F5	
Mobile phase	Formate buffer (A)/methanol (B)[a]			
Gradient program	*t* (min)	B (%)	*t* (min)	B (%)
	00	01	00	05
	05	01	10	85
	30	50	18	85
	35	50	20	05
	40	90	35	05
	50	90		
	55	01		
	70	01		
Flow (mL min^{-1})	0.20		0.20	
Column temperature (°C)	40		45	
Injection volume (μL)	5		3	
Wavelength program	*t* (min)	λ	*t* (min)	λ
	0	233	0	233
	7	233	7	233
	7.1	270	7.1	270
	70	270	35	270

[a] Formate-formic buffer (40 mmol L^{-1}), pH 4.

value (R_s = 1.36). The target molecule with the longest retention time in both columns was BP-3, due to the highest log $P_{o/w}$ of all tested molecules, but the retention time in the fluorinated column was two times shorter compared to Atlantis dC18 column. Furthermore, a good resolution was obtained with both columns for all compounds, except for DIC and BP-3 (R_s = 0.93) using the Atlantis dC18 column, as both analytes presenting $k' > 13$; this diminished resolution due to band-broadening effect. Although the two stationary phases showed similar elution behavior, expectedly, MET showed very low retention (k' = 0.27) in the Atlantis column. This can be explained since MET is a small, highly polar molecule. Some authors stated that the polar mobile phase was successfully applied for MET on the Diol-HILIC column and even the C18 column (24, 25). It must be noted that to achieve an acceptable separation for all the analytes with the Atlantis dC18 column, a slow solvent gradient was required, thus resulting in a long analysis time (ca. 1 h). Nevertheless, higher efficiency for most investigated PPCPs was obtained in this column, finding values of *N* greater than 200,000, except for MET (*N* = 3901). On the other hand, the fluorinated stationary phase showed a better retention capacity for MET (k' = 0.9), which can be attributed to its cationic exchange properties (26). In this column, the other seven analytes showed values of k' in the range 3.70–6.10, which is almost half the value obtained with Atlantis dC18

Table III. Chromatographic parameters for the separation of PPCPs using the Atlantis dC18 and Discovery HS F5 columns

Analyte	Atlantis dC18				Discovery HS F5			
	t_R (min)	*Rs*	*k´*	N	t_R (min)	*Rs*	*k´*	N
MET	4.057	–	0.27	3901	5.293	–	0.90	10823
CAF	26.057	23.91	7.14	237242	13.092	13.93	3.70	129850
BP-4	32.850	1.36	9.27	297063	14.497	2.82	4.20	143024
MP	31.790	7.21	8.93	259596	15.322	1.68	4.50	196112
PP	42.357	12.21	12.24	317969	16.723	2.80	5.00	250899
NAP	44.603	3.45	12.94	1067441	17.688	2.14	5.35	232566
DIC	46.380	4.15	13.49	1817733	18.544	1.91	5.66	203099
BP-3	46.810	0.93	13.63	1551771	19.669	2.39	6.10	242532

k´ – retention factor (capacity factor), *N* – number of theoretical plates, R_s – chromatographic resolution, t_R – retention time

Table IV. Validation parameters of the developed method for quantitative analysis of selected PPCPs

Analyte	Linearity		Precision	Accuracy			*LOD*
	equation[a]	R^2	RSD_{max} (%)		slope	%	(μg mL^{-1})
MET	$y = 73.5x + 12.247$	0.999	4.4	0.999	1.000	98.9–101.9	0.582
CAF	$y = 48.909x + 12.359$	0.999	3.6	0.999	0.999	98.5–102.3	0.316
PB-4	$y = 23.863x - 5.4822$	0.998	2.3	0.998	0.999	98.0–104.5	0.436
MP	$y = 61.571x + 41.426$	0.999	2.4	0.999	0.999	94.1–105.2	0.632
PP	$y = 55.807x + 15.324$	0.999	2.4	0.999	1.000	92.2–106.1	0.358
NAP	$y = 22.995x + 4.4284$	0.998	3.1	0.998	0.999	98.9–100.6	0.626
DIC	$y = 34.687x + 8.5097$	0.998	3.6	0.999	1.000	97.0–103.3	0.533
BP-3	$y = 32.636x + 10.138$	0.999	2.1	0.999	1.000	95.8–102.9	0.395

[a] Linearity range: 2–100 μg mL^{-1}.

LOD – limit of detection, *LOQ* – limit of quantitation: *LOQ* = 3x*LOD*

column (*k´* = 7.14–13.63). Taking *k´* values into account, it can be observed that the strongest retention was obtained with the Atlantis dC18 column, suggesting the strongest interactions between investigated PPCPs and the ligands on the surface of this column. Mean-

while, most of the molecules in the fluorinated column presented resolution values close to or greater than 2. It is also important to emphasize that in both evaluated columns CAF, PP and NAP showed the same elution order without problems in their retention and good separation from the adjacent analytes, showing *Rs* values close to or greater than 2. Given that the best separation with the shortest analysis time for all solutes was achieved with the fluorinated phase, the Discovery HS F5 column was considered the best option for the determination of the model PPCPs analytes.

Preliminary analytical validation

The performance of the proposed method was evaluated. Figures of merit included validation of parameters like selectivity, linearity, precision, accuracy and *LOD*/*LOQ*, and a summary of the results is given in Table IV. The method was found to be linear for all analytes, a good linear relationship was observed between the peak areas and the whole range of tested concentrations of PPCPs with R^2 values > 0.99. In precision, the RSD values of the analyte response were less than 5 % at all evaluated levels within analytical runs. As an approximation for the accuracy, correlation analysis was carried out between the concentration obtained for each calibration standard by using the linear equation from the regression analysis and the actual concentration. According to this model, the method presented acceptable accuracy since for all analytes values of $R^2 > 0.99$ with slope values close to 1 were obtained; for each back-calculated standard concentration, the percentages of deviation from the nominal value were lower than 10 %. The *LOD* values ranged from 0.316 to 0.632 μg mL^{-1}. Although, at first sight, these values can seem high, they can be considered adequate bearing in mind that the proposed method is intended for analysis of samples which, prior to HPLC analysis, have been subjected to some preparation procedure in order to reduce or eliminate matrix interferences, pre-concentrate the analyte and improve the analytical system performance.

CONCLUSIONS

As noted above, the aim of this work was to provide a simple and fast HPLC method for PPCPs, namely, as a tool during the development and optimization of new sample preparation procedures. In this work, two RP stationary phases bearing different functional groups (C18 and pentafluorophenyl) were evaluated for the separation of PPCPs featuring a wide range of hydrophilicity. In addition, according to our knowledge, it is the first time that the use of pentafluorophenyl stationary phase has been reported in the separation of these multiclass compounds. The Atlantis dC18 column offered longer analysis times and could not be used to separate the DIC/BP-3 pair. The best separation for the PPCPs included in the study was achieved with the Discovery HS F5 column (fluorinated phase), which exhibited better retention for more hydrophilic compounds, better resolution of the signals and shorter analysis time. This study demonstrated that pentafluorophenyl phase is a better option for the separation of molecules with widely different polarities. The proposed method is a good alternative to traditional C18-based HPLC methods for PPCPs during optimization of sample preparation procedures in pharmaceutical, environmental, food and biomedical analysis. Developed RP-HPLC method was preliminary validated showing to be selective, accurate and precise enough for quick screening tests.

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