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RESEARCH ARTICLE

Bioanalytical method optimization for simultaneous quantification of structurally related probe drugs in a phenotyping cocktail using liquid chromatography-tandem mass spectrometry

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Abstract

The physicochemical diversity of the structurally related aromatic probe drugs, used together in a drug cocktail to assess metabolic and transport phenotypes, require optimized analytical procedures for simultaneous quantification. The analytical conditions can greatly influence the analyte selectivity, retention, stability, and ultimately the robustness of the method. The aim of this study was to assess the selectivity of the structurally related ionizable analytes between the commonly used C18 column chemistry and an alternative biphenyl column chemistry as well as the influence of changes in the analytical conditions on method robustness using liquid chromatography-tandem mass spectrometry. A repeated measure two-factor analysis of variance with Geisser-Greenhouse correction was used to determine statistical significance. The results showed that a biphenyl stationary phase in combination with methanol as the organic eluent, could provide improved resolution and analyte selectivity. Changes in analytical conditions caused statistically significant variation in the retention behavior, selectivity, column efficiency, and sensitivity of the analytes of interest The robustness experiment confirmed the importance of controlling analytical conditions to ensure the reproducibility and reliability of the quantitative method.

KEYWORDS
analyte selectivity, bioanalytical method optimization, ionization efficiency, liquid chromatography-tandem mass spectrometry, retention factor

Article Related Abbreviations: CYP450, Cytochrome P450 enzymes; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; k', Retention factor; MRM, Multiple reaction monitoring; m/z, Massto charge ratio; tR, Averageretention time; XIC, Extracted ion chromatogram.

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1 | INTRODUCTION

Poor therapeutic response to medication has been attributed to inter-individual and interethnic variability in cytochrome P450 (CYP450)-dependent metabolism and altered drug absorption via expressed transport channels such as P-glycoprotein. A single time point, non-invasive capillary sampling, combined with a low-dose probe drug phenotyping cocktail, would enhance the feasibility and cost-effectiveness of routine phenotyping in resource-constrained countries to guide personalized prescribing [1]. The rationale for choosing the Geneva phenotyping cocktail [2], for the purpose of simultaneously quantifying in vivo drug and metabolite concentrations in a genetically diverse population, has been described elsewhere [3]. The phenotyping cocktail consists of structurally related probe drugs with different physicochemical properties. The Log *P*, pKa, proton acceptor, and donor counts of the Geneva cocktail probe drugs and their metabolites are given in Table S1. The lipophilic pharmaceutical probe drugs and their more polar metabolites, present in the same biological matrix, present a challenge in the development of a single extraction and bioanalytical method for analysis with liquid chromatography-tandem mass spectrometry (LC-MS/MS). In this cocktail omeprazole degrades at acidic pH levels [4], bupropion in its free base form undergoes first-order catalysis by the hydroxide ions present in an aqueous solution at pH above 5 [5] and midazolam undergoes ring closure rendering it more lipophilic at pH above 4 [6]. This will have an influence on the sample storage conditions, sample preparation, and LC-MS/MS conditions.

During the development and optimization of a new method for quantitation both the choice of organic eluent (type and composition) and stationary phase on analyte selectivity and retention must be considered to ensure the robustness of the method [7, 8]. The most effective tool for changing and optimizing resolution is by altering the selectivity factor since it is dependent on many different factors, that is, analyte chemistry, choice of both stationary and mobile phase, solvent pH (applicable to ionizable analytes), elution strength and composition and column temperature [9]. If changes in analytical conditions cause statistically significant variation in the retention behavior, selectivity, efficiency, and sensitivity of the analytes of interest, they should be suitably controlled to yield reliable, reproducible quantitative results.

When amphoteric ionizable analytes are present, the pH of the mobile phase needs to be controlled to ensure reproducible retention times and to improve peak shape [10]. However, when combining a number of analytes with markedly different physicochemical properties this becomes more difficult. To optimize the separation selectivity in a complex analyte mixture of ionizable analytes, retention factors at different mobile phase pH conditions may be plotted during method development [10]. Where pK_a values differ, the retention versus pH plots will vary from one analyte in the mixture to the next, and significant changes in selectivity and retention might be possible with a small change in the pH of the mobile phase, particularly if the pH is within 1 unit of the analyte pK_a [11]. Ionization efficiency in the electrospray ionization (ESI) source could be increased by pairing appropriate mobile phase additives at the molar concentrations that optimize the aqueous pH so that analytes are already charged in a solvent before LC separation [12]. The retention of ionizable analytes will change as a function of the pH of the mobile phase and the analyte pK_a [13]. Alternative column chemistries may be explored to better define the differences between analytes and change their selectivity [14]. This is done by plotting the logarithm of the retention factors, obtained under the same conditions, obtained on an alternative reverse phase column against the reference column [15], which is C18 for the previously validated Geneva method [16]. The degree of scatter around the regression line as well as the slope of the regression line demonstrate orthogonality between the two different column chemistries with a high degree of scatter indicative of greater orthogonality. It should be conceded that the pH of the mobile phase and the pK_a of the analyte will change with the addition of the organic phase [17] and that the stability of the stationary phase will also influence the selectivity of the analytes at different conditions [18]. For the purpose of bioanalytical method development, however, the focus was placed on assessing the mass spectrometric response at varying mobile phase compositions and aqueous pH in combination with an alternative stationary phase.

Therefore, given the analytical challenges previously described with this phenotyping cocktail [4–6], the objectives of this experiment were to optimize an LC-MS/MS method, using an alternative biphenyl stationary phase, by comparing analyte selectivity to that of a C18 stationary phase used traditionally in phenotyping assessments and to evaluate the influence of different solvents, different mobile-phase compositions and aqueous pH on analyte ionization during separation.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Reference standards caffeine (batch # BCBR6677V), bupropion as hydrochloric salt (batch # 063M4707V), flurbiprofen (batch # SLBD4598V), hydroxy-omeprazole (batch # BCBS0382V), dextromethorphan (batch #

SLBQ0513V) and dextrorphan (batch # 065K3257) were purchased from Sigma Aldrich (Pty) Ltd., paraxanthine (batch # FN11121501), hydroxy-bupropion (batch # FN0213150Z), omeprazole (batch # FN02201501) and α-hydroxy-midazolam (batch # FN02041502) from Cerilliant (Pty) Ltd. supplied by Sigma-Aldrich and 4-hydroxy-flurbiprofen (batch # CRC-0151-048-F) and fexofenadine (batch # S-FF-516) from Clearsynth (Pty) Ltd. Midazolam (batch # F1058F03, Roche) was obtained as Dormicum 15 mg^3/mL ampoules from a local hospital pharmacy. Internal standards (ISs) imipramine (batch # 107K0697) for positive mode was supplied by Sigma Aldrich (Pty) Ltd. and probenecid as European Pharmacopoeia standard by Cayman Chemicals.

All solvents used during sample preparation and chromatography were HPLC grade. Acetonitrile and methanol (Romil purity > 99.9%) and Romil HPLC-water were purchased from Microsep (Pty) Ltd. Analytical grade formic acid (purity ≥ 98%), ammonium formate (batch # MKCF2569), ammonium acetate (batch # 15398/4773), and ammonium bicarbonate (batch # 060M0177V) were obtained from Sigma Aldrich (Pty) Ltd. In-house double deionized pyrogen-free water (> 18 MΩ and < 5 ppm TOC), used during sample preparation, was produced using an ELGA Genetics water purification unit (ELGA) housed in the Department of Pharmacology. Kinetex C18 and Kinetex Biphenyl 100 mm × 2.1 mm, 2.6-μm columns were purchased from Phenomenex.

2.2 | Instrumentation

The LC-MS/MS system consisted of an Agilent 1100/1200 combined series HPLC system (Agilent Technologies), coupled to an ABSciex 4000 triple quadrupole mass spectrometer, equipped with a Turbo-V ESI source (Sciex). Analyst Software, version 1.5.2 (Sciex), was used to operate the system. The triple quadrupole LC-MS/MS system is housed at the Department of Pharmacology at the University of Pretoria.

2.3 | Optimization of MS detection parameters

Targeted multiple reaction monitoring (MRM) was used for quantitation. Each of the seven probe drugs present in the Geneva cocktail, six corresponding CYP450 metabolites, and structural internal standards imipramine and probenecid were individually tuned to determine their optimal detection parameters (Table S2) using the manual tuning function on Analyst 1.5.2 software. Approximately 100 ng/mL were infused directly into the ESI source at a constant flow rate of 10 or 20 μL/min using a Harvard syringe pump (Harvard Apparatus).

2.4 | Preparation of stock and working standard solutions for bioanalytical method assessment

An analyte mixture in methanol was prepared from 1 mg/mL (m/v) stock solutions volumetrically by adding appropriate volumes of the analytes to an Eppendorf vial and making it up to a final volume of 1000 μL. Final concentrations of the analytes were 7.5 μg/μL (v/v) for midazolam, 15 μg/μL (v/v) for bupropion, hydroxy-bupropion, hydroxyflurbiprofen, omeprazole, hydroxy-omeprazole, dextromethorphan, dextrorphan, and hydroxy-midazolam, 30 μg/μL (v/v) for fexofenadine and 75 μg/μL (v/v) for caffeine, paraxanthine, and flurbiprofen.

2.5 | Sample preparation

Blank human plasma and solvent (190 μL) were spiked with 10 μL of the analyte mixture and extracted by a simple 3-step protein precipitation procedure. During the first step 200 μL of acetonitrile was added to the spiked plasma and solvent mixtures, vortex mixed for 5 min (Lasec Vortex Genie2), and sonicated for 5 min (Bran Sonic 52 ultrasonicator) followed by two more additions of 100 μL acetonitrile each and the vortex mixing and sonication steps repeated. After protein precipitation, the mixtures were centrifuged (Beckman Coulter Microfuge 16 centrifuge) at 14 000 *xg* for 10 min to remove the precipitated proteins. The supernatant (80 μL) was pipetted (Eppendorf pipette) into clean amber 2 mL LC vials, containing 200 μL glass tapered autosampler vial inserts, with 20 μL of IS mix, containing 0.75 μg/μL (v/v) probenecid and imipramine. The final solution was made up to 200 μL by adding 100 μL of pyrogen-free double deionized water to make up a 50:50 methanol: aqueous mixture at 4 different conditions, namely 0.1% formic acid, 5 mM ammonium bicarbonate, 10 mM ammonium acetate and 10 mM ammonium formate just prior to LC-MS/MS analysis.

2.6 | LC-MS/MS analysis

Isocratic chromatographic separation of all analytes and IS was achieved on two different columns with similar column dimensions, a Kinetex C18 and a Kinetex biphenyl column (100 × 2.1 mm, 2.6 μm particle size) at two different mobile phase conditions. The mobile phases consisted

TABLE 1 Mobile phase and sample vial composition with measured pH.

	Mobile phase composition	Measured pH
A	0.1% Formic acid	2.7
B	10 mM Ammonium formate (NH_4COOH) acidified with 1 M solution of formic acid	3.9
C	10 mM Ammonium acetate (NH_4COOCH_3)	6.5
D	5 mM Ammonium bicarbonate (NH_4HCO_3)	8.3

of methanol: water (60:40) or acetonitrile: water (40:60) with the flow rate set at 100 μL.min^{-1} and the sample injection volume 10 μL. Under these conditions the elution strength is approximately similar. The column temperature was controlled at 40°C ± 3°C. Each injection was done in triplicate and average retention times recorded for all analytes under different conditions. Retention factors were calculated and scatter plots were drawn comparing the logarithm of the retention factors (log *k'*) for all analytes on the biphenyl column against their respective log *k'* values on the C18 column for both mobile phase conditions. The slopes and correlation coefficients were determined from linear regression analysis to expose alternative column selectivity.

Next, the effect of different mobile phase conditions on the state of ionization and retention behavior was assessed, aiming to optimize the separation selectivity and stability of the acid-label omeprazole. The column temperature (Kinetex biphenyl 100 mm × 2.1 mm, 2.6 μm particle size) was kept constant at 40°C ± 3°C, with the mobile phase (methanol:water [60:40]) flow rate at 100 μL/min and the sample injection volume 10 μL. The four different mobile phase conditions and measured pH are given in Table 1. The average of the retention factors (k'), from triplicate injections, of all the analytes were plotted against the four different mobile phase conditions at different aqueous phase pH.

2.7 | Statistical analysis

The effect of the composition of the solvent in the LC vial and the effect of the mobile phase composition on the analyte peak area (signal intensity) and the interaction between these two independent variables on the signal intensity was evaluated with repeated measures of two-factor analysis of variance (ANOVA) with Geisser-Greenhouse correction to determine statistical significance. The distribution was determined with a Shapiro-Wilk normality test. Normal and lognormal distributions were compared and data transformed where a lognormal distribution was more likely before the ANOVA test was performed. Matched values were both spread and stacked across a row and simple effects were compared within rows. The effect was deemed significant if the *F* statistic was greater than the critical *F*-value ($\alpha < 0.05$). Post hoc Tukey tests were conducted to establish the source of variability by multiple comparisons using hypothesis testing. The statistical analysis was carried out with GraphPad Prism version 8.0.2 statistical software for Windows (GraphPad Software, www.graphpad.com).

2.8 | Ethical considerations

The study was approved by the University of Pretoria's Research and Ethics Committee (209/2016) and informed consent was obtained from all study participants who donated plasma.

3 | RESULTS AND DISCUSSION

3.1 | Comparison of analyte selectivity between a C18 and biphenyl column

The average retention times (t_R) of all the analytes, their respective retention factors (k'), and the calculated percentage variance for k' for each analyte, under the two different mobile phase conditions, are summarised in Table 2. Where 40% acetonitrile was used as the eluent, the results show a good correlation between the separation of the analytes on the biphenyl and the C18 columns as expected. Under these conditions, the interaction of analytes with the stationary phases was probably controlled by a common separation mechanism. It is well known that acetonitrile suppresses the π-π interaction between the analytes and the biphenyl groups present in the stationary phase as a result of its C-N triple bond [19, 20]. This is highlighted when comparing the extracted ion chromatograms (XIC) of the analytes on the different columns, as shown in Figure 1(I).

The separation of the analytes between the two columns with 60% methanol as the eluent, showed significant differences in the retention behavior of caffeine, 5′-hydroxy-omeprazole, dextromethorphan, midazolam, fexofenadine, and α-hydroxymidazolam (Table 2). The π-π interactions between the non-polar analytes and the stationary phenyl groups were favored and possibly enhanced under these conditions. Figure 1(II) highlights the alternative selectivity differences of the analytes on the two different columns. The scatter plot (Figure 2) drawn from this data infer that the biphenyl phase has more non-polar interactions when methanol is used compared to acetonitrile as a mobile phase. The slope of the respective linear regression analysis is indicative of the relative strengths of

TABLE 2 Average retention times (t_R) and retention factors (k') of analytes separated on a Kinetex C18 or Kinetex Biphenyl column (+ESI) under different isocratic mobile phase conditions.

Acetonitrile:Water (40:60)					
Analyte	**Kinetex C18**		**Kinetex Biphenyl**		**% Variance for k'**
	Average t_R (min)	**k'**	**Average t_R (min)**	**k'**	
Paraxanthine	3.44 (± 0.002)	0.274	3.28 (± 0.003)	0.215	0.18%
Dextrorphan	3.45 (± 0.002)	0.278	3.53 (± 0.010)	0.307	0.04%
5′-Hydroxyomeprazole	3.47 (± 0.005)	0.285	3.50 (± 0.007)	0.296	0.01%
Hydroxybupropion	3.54 (± 0.003)	0.311	3.64 (± 0.009)	0.348	0.07%
Caffeine	3.58 (± 0.002)	0.326	3.53 (± 0.003)	0.307	0.02%
Omeprazole	3.73 (± 0.047)	0.381	3.99 (± 0.019)	0.478	0.46%
Bupropion	3.93 (± 0.003)	0.456	4.14 (± 0.011)	0.533	0.30%
Dextromethorphan	4.34 (± 0.003)	0.607	4.94 (± 0.014)	0.830	2.47%
α-Hydroxymidazolam	4.37 (± 0.009)	0.619	4.69 (± 0.015)	0.737	0.70%
Midazolam	4.38 (± 0.005)	0.622	4.98 (± 0.014)	0.844	2.47%
Fexofenadine	5.86 (± 0.006)	1.170	7.52 (± 0.019)	1.785	18.90%
Methanol:Water (60:40)					
Analyte	**Kinetex C18 column**		**Kinetex Biphenyl column**		**% Variance for k'**
	Average t_R (min)	**k'**	**Average t_R (min)**	**k'**	
Dextrorphan	3.49 (± 0.015)	0.293	4.02 (± 0.013)	0.489	1.93%
Paraxanthine	3.59 (± 0.006)	0.330	4.20 (± 0.009)	0.556	2.55%
Hydroxybupropion	3.75 (± 0.011)	0.389	4.17 (± 0.012)	0.544	1.21%
Caffeine	3.87 (± 0.005)	0.433	5.69 (± 0.007)	1.107	22.72%
Bupropion	3.93 (± 0.011)	0.456	4.66 (± 0.011)	0.726	3.66%
5′-Hydroxyomeprazole	4.04 (± 0.027)	0.496	5.58 (± 0.020)	1.067	16.27%
Dextromethorphan	4.35 (± 0.014)	0.611	8.42 (± 0.015)	2.119	113.61%
Midazolam	4.41 (± 0.013)	0.633	9.04 (± 0.013)	2.348	147.03%
Omeprazole	4.71 (± 0.029)	0.744	5.25 (± 0.009)	0.944	2.00%
Fexofenadine	4.83 (± 0.009)	0.789	13.37 (± 0.027)	3.952	500.22%
α-Hydroxymidazolam	5.04 (± 0.029)	0.867	10.49 (± 0.026)	2.885	203.72%

these separation interactions. The correlation coefficient (r^2) when using acetonitrile was 0.97 indicating a high degree of similarity between the interactions involved in the separation of the two stationary phases. The graph on the right shows more differentiation in retention when methanol was used with more scattered data and a flatter slope with a correlation coefficient of 0.74. Despite the slopes appearing approximately similar note that the y-axis scale is different. The compounds below the trendline, including all the hydroxylated metabolites are influenced by the electron-donating effects of the biphenyl stationary phase and are thus better retained resulting in improved resolution. While these π-π interactions are not the only parameter controlling the retention of phenyl-based stationary phase columns in methanol, they do provide a slight enhancement to complicated separations of closely related compounds with wide applicability to quantitative methods.

3.2 | Effect of altered sample vial solvent and mobile phase composition and pH on analyte stability, selectivity, and sensitivity

These experiments aimed to determine the best combination of sample reconstitution solution and mobile phase to optimize the stability while maintaining ionization efficiencies of all analytes with different physicochemical properties and maintaining optimum retention and selectivity during the chromatographic separation. In chromatographic terms, the quality of the separation is optimal where the retention factor k, influenced by different mobile phase polarities, is between 1 and 5 as this is where it has the greatest effect on the overall resolution. The retention factor range is, however, extended to between 2 and 10 for complex mixtures [21]. The results are shown in Table 3. With a mobile phase pH of 2.7 (0.1% formic acid), k' was between 1.19 and 10.99 with isocratic

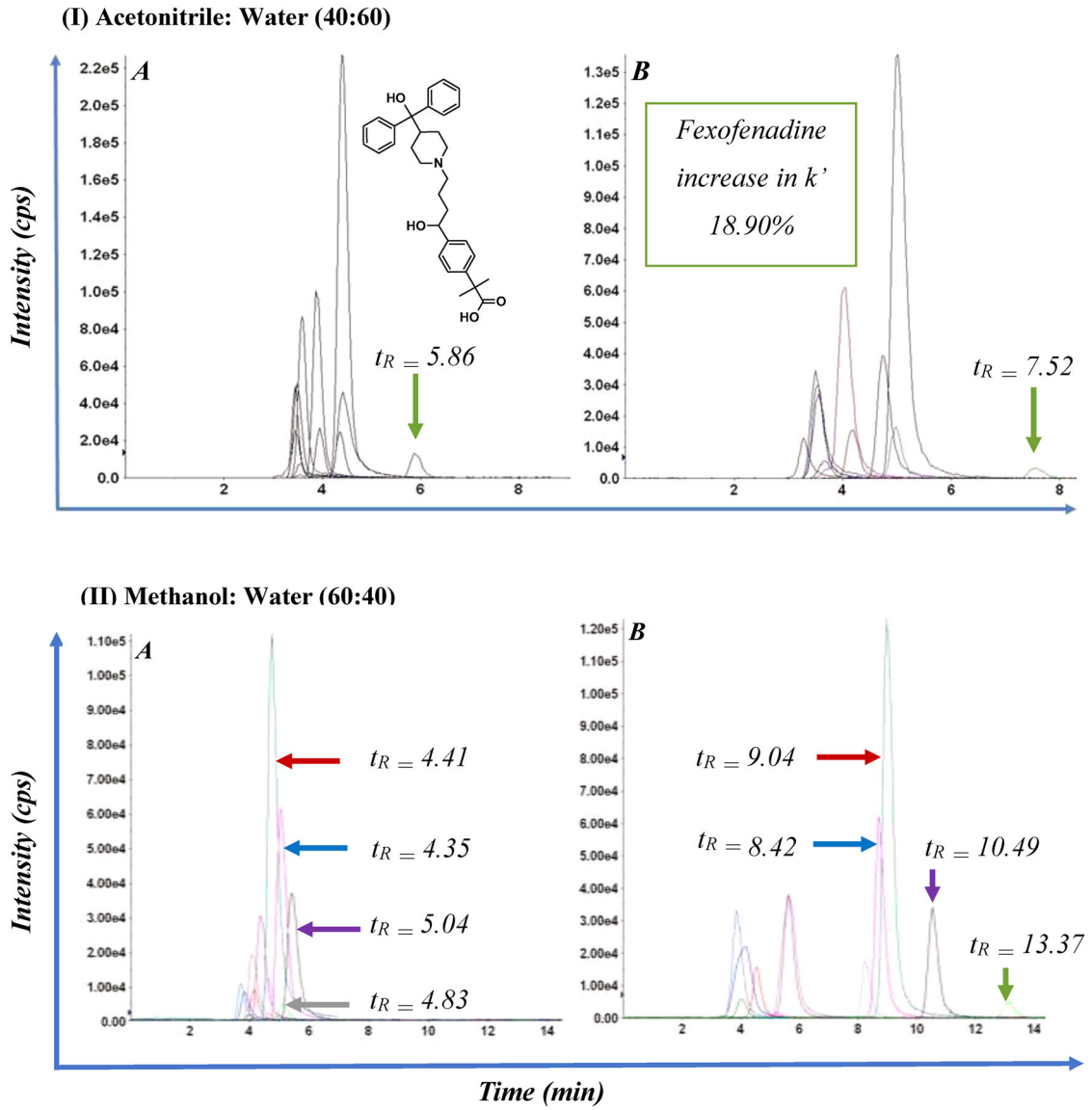

FIGURE 1 Extracted ion chromatograms (XIC) of multiple reaction monitoring (MRM)+ transitions on (A) Kinetex C18 and (B) Kinetex Biphenyl columns with isocratic acetonitrile:water (40:60) (I) and isocratic methanol: water (60:40) (II) as the mobile phase at a flow rate of 100 μL/min Increases in retention times are indicated by the colored arrows in (II); midazolam (red), dextromethorphan (blue), α-hydroxymidazolam (purple), and fexofenadine (green).

elution. When the mobile phase pH is increased to 3.9 (containing both 0.1% formic acid and ammonium formate) retention factors increase between 1.01 and 23.09. Under these conditions, the extended retention time will increase the overall analysis time with decreased peak height and band broadening. Figure 3 shows two XICs of the analytes at identical sample vial conditions with mobile phase pH 2.7 (A) and 3.9 (B) respectively with altered elution order due to different degrees of ionization at altered pH.

Plotting the mean retention factors (k') of the analytes (obtained from the four different sample vial conditions) against the pH of the mobile phase gives a graphical representation (Figure 4) of the relationship between the retention behavior at different mobile phase pH. Isocratic elution was necessary to avoid the pH shift observed during changes in organic mobile eluent under gradient elution conditions [11]. The pH was measured in the aqueous phase and it must be noted that the true pH for the different solvent mixes might differ due to the addition of the organic phase. The error bars represent the standard deviation of the mean retention factor observed between the four sample vial conditions. For neutral analytes, caffeine, and paraxanthine, changes in mobile phase pH had a minimal effect on their retention behavior as expected, however, for the ionizable basic analytes, midazolam, hydroxymidazolam, bupropion, omeprazole, and

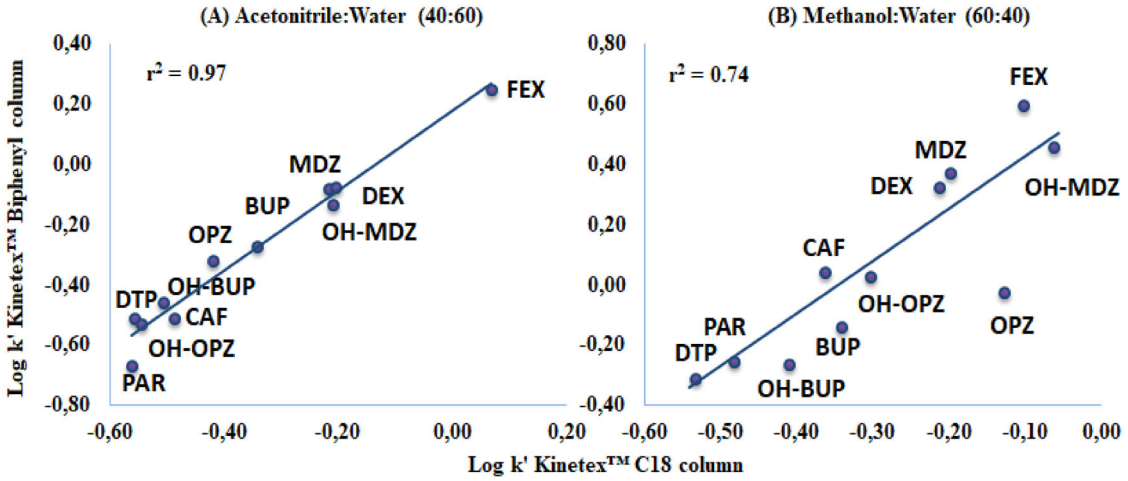

FIGURE 2 Scatter plots of the analyte retention factors on the biphenyl column against their retention factors on the C18 column for (A) acetonitrile:water (40:60) and (B) methanol:water (60:40) isocratic elution.

dextromethorphan, significant changes in retention were observed as a result of their degree of ionization at the different pH conditions.

Most of the analytes carry more than one functional group in their chemical structure, rendering them amphoteric with varied degrees of ionization depending on the pK_a of the basic or acidic functional groups. Fexofenadine for example has a carboxyl (strong acid), two alcohol functional groups (weak acid), and a nitrogen atom (weak base) with an overall predicted pK_a value of 4.04, a proton acceptor count of 5 and proton donor count of 3. This would explain why the retention times change significantly in pH ranges between 2.7 and 6.5. At a pH of 2.7, fexofenadine would have a positive charge on the nitrogen atom and would be ionized, rendering it more polar with a shorter retention time on the column. When the mobile phase pH increases to 3.9, the molecule would be 50% ionized since it is near the pKa value, thus increasing its retention time. This could also explain the peak broadening seen at this pH. At two pH units above the pK_a (at pH 6.5), the molecule would be 100% in its ionized form due to the loss of the hydrogen atom on the carboxyl group, again with a shorter retention time. Typical behavior was observed for the other basic analytes where the pH of the mobile phase increased their degree of ionization at lower pH, decreasing hydrophobicity with shorter retention factors. Large changes in the selectivity were observed for dextromethorphan, bupropion, and midazolam when the pH of the mobile phase changed from 6.5 to 8.3, with large changes in retention times. At basic pH, the acid functional groups would be deprotonated and the degree of ionization determined by how close the pH is to the pK_a of the analyte. Similarly, a change in the pH from 2.7 to 3.9 changed the retention times of midazolam, OH-midazolam omeprazole, OH-omeprazole, bupropion, OH-bupropion, dextromethorphan, and dextrorphan. This could be explained by the fact that the nitrogen atoms in their chemical structures are protonated at acidic pH. The formation of intermolecular hydrogen bonds should also be considered since their presence increases the hydrophobicity of the molecule and might influence the degree of net ionization. This is the case with omeprazole, for example, where an intermolecular hydrogen bond is formed between the oxygen atom on the sulfoxide group and the hydrogen atom on the nitrogen present in the 5-methoxybenzimidazole ring [22]. There are three possible acid/conjugate base pairs for omeprazole with the possibility of two protonations, however, the first acid/conjugate base pair from the di-cation to the cation was found to be very unstable and the pK_a for this species could not be determined with ultraviolet-visible spectrophotometry [22]. This study observed two distinct peaks at different retention times for omeprazole with the same MRM transitions (Figure S1). When the analyte solution was kept in acidic 0.1% formic acid 50:50 methanol: water solution within the sample vial, omeprazole degraded completely over time possibly due to the fact that omeprazole undergoes conversion to a cyclic sulphonamide under acidic conditions [23]. Omeprazole stability was found to be affected by the pH and composition of the sample vial and possibly different acid-base pairs forming at different pH.

When developing a quantitative analytical method for the simultaneous detection of analytes at low concentrations, from low-volume biological samples, it is important that the sensitivity and detection limits are optimized. Although the retention times for all analytes except omeprazole and its hydroxylated metabolite, were stable at the four different sample vial conditions, the ESI efficiency and hence the analyte sensitivity was influenced by both the composition of the solvent in the sample vial and the

TABLE 3 Mean retention factors (k') ± the standard deviation (SD) of analytes and their coefficient of variation (CV %) in extracted human plasma from triplicate injections over time at the same sample vial conditions and within different conditions at four different mobile phase conditions and pH levels.

pH	Composition of solution in LC-vial	CAF			PAR			BUP			OHBUP		
		Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)
2.70	0.1% Formic acid	2.38	0.02	0.64	1.11	0.01	1.04	1.71	0.01	0.58	1.23	0.01	0.47
	10 mM NH_4COOH	2.39	0.04	1.74	1.13	0.04	3.36	1.66	0.02	1.26	1.20	0.01	0.83
	10 mM NH_4COOCH_3	2.35	0.15	6.38	1.12	0.12	10.97	1.67	0.11	6.28	1.19	0.10	8.01
	5 mM NH_4HCO_3	2.31	0.02	0.90	1.08	0.06	5.16	1.61	0.03	1.80	1.14	0.02	1.52
	Average	2.36			1.11			1.66			1.19		
	CV (%)	1.44			1.86			2.58			3.05		
3.90	0.1% Formic acid	2.37	0.16	6.85	0.99	0.13	12.98	2.45	0.17	6.92	1.71	0.13	7.53
	10 mM NH_4COOH	2.35	0.05	1.95	1.01	0.05	4.90	2.47	0.07	2.64	1.72	0.05	2.99
	10 mM NH_4COOCH_3	2.24	0.17	7.78	0.94	0.14	15.41	2.34	0.21	8.89	1.64	0.16	9.90
	5 mM NH_4HCO_3	2.42	0.06	2.51	1.11	0.07	6.35	2.51	0.05	1.99	1.77	0.05	2.89
	Average	2.35			1.01			2.44			1.71		
	CV (%)	3.25			7.00			2.96			3.28		
6.50	0.1% Formic acid	2.47	0.05	2.01	1.14	0.08	6.85	2.78	0.04	1.27	1.83	0.08	4.26
	10 mM NH_4COOH	2.49	0.04	1.41	1.20	0.05	4.29	2.97	0.04	1.36	1.99	0.04	2.01
	10 mM NH_4COOCH_3	2.47	0.03	1.24	1.19	0.03	2.56	3.01	0.03	0.84	2.01	0.02	0.76
	5 mM NH_4HCO_3	2.54	0.12	4.69	1.20	0.10	7.94	3.03	0.07	2.20	2.03	0.06	3.01
	Average	2.49			1.18			2.95			1.96		
	CV (%)	1.41			2.57			3.97			4.72		
8.30	0.1% Formic acid	2.68	0.08	2.89	1.30	0.08	6.42	7.35	0.53	7.18	2.90	0.14	4.83
	10 mM NH_4COOH	2.68	0.12	4.33	1.30	0.08	6.43	9.40	0.26	2.81	3.72	0.10	2.81
	10 mM NH_4COOCH_3	2.68	0.05	1.94	1.27	0.04	2.84	9.30	0.17	1.86	3.71	0.13	3.37
	5 mM NH_4HCO_3	2.59	0.13	5.03	1.25	0.03	2.58	9.33	0.21	2.23	3.69	0.13	3.41
	Average	2.66			1.28			8.85			3.51		
	CV (%)	1.76			2.03			11.30			11.49		
pH	Composition of solution in LC-vial	OPZ			OHOPZ			DEX			DTP		
		Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)
2.70	0.1% Formic acid	–	–	–	–	–	–	5.61	0.04	0.78	1.01	0.00	0.00
	10 mM NH_4COOH	6.45	0.06	0.93	2.77	0.01	0.42	5.57	0.05	0.90	0.96	0.02	2.08
	10 mM NH_4COOCH_3	6.49	0.12	1.83	2.79	0.11	3.94	5.62	0.14	2.49	0.98	0.08	8.35
	5 mM NH_4HCO_3	6.37	0.05	0.81	2.73	0.04	1.60	5.53	0.01	0.21	0.91	0.03	3.16
	Average	6.44			2.77			5.58			0.97		
	CV (%)	0.90			1.17			0.75			4.20		
3.90	0.1% Formic acid	–	–	–	–	–	–	8.30	0.20	2.41	1.43	0.13	9.01
	10 mM NH_4COOH	11.90	0.40	3.36	3.84	0.12	3.13	8.17	0.25	3.08	1.43	0.04	3.05
	10 mM NH_4COOCH_3	11.70	0.36	3.08	3.69	0.23	6.36	8.03	0.29	3.59	1.36	0.16	11.46
	5 mM NH_4HCO_3	11.80	0.10	0.85	3.83	0.02	0.45	8.13	0.06	0.71	1.52	0.06	4.24
	Average	11.80			3.79			8.16			1.43		
	CV (%)	0.85			2.27			1.35			4.48		
6.50	0.1% Formic acid	–	–	–	–	–	–	8.60	0.14	1.64	1.50	0.07	4.71
	10 mM NH_4COOH	12.47	0.06	0.46	3.96	0.05	1.24	8.77	0.06	0.66	1.73	0.03	1.85
	10 mM NH_4COOCH_3	12.50	0.17	1.39	3.95	0.03	0.77	8.83	0.15	1.73	1.74	0.01	0.57
	5 mM NH_4HCO_3	12.60	0.20	1.59	3.99	0.09	2.18	8.90	0.20	2.25	1.80	0.06	3.47
	Average	12.52			3.97			8.78			1.69		
	CV (%)	0.55			0.48			1.47			7.81		

(Continues)

TABLE 3 (Continued)

pH	Composition of solution in LC-vial	OPZ			OHOPZ			DEX			DTP		
		Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)
8.30	0.1% Formic acid	–	–	–	–	–	–	13.83	0.40	2.92	2.41	0.11	4.55
	10 mM NH_4COOH	13.57	0.38	2.79	4.27	0.14	3.19	15.93	0.23	1.45	3.23	0.02	0.64
	10 mM NH_4COOCH_3	13.43	0.15	1.14	4.26	0.12	2.83	15.93	0.40	2.54	3.23	0.14	4.19
	5 mM NH_4HCO_3	13.37	0.23	1.73	4.16	0.16	3.87	15.90	0.30	1.89	3.22	0.11	3.42
	Average	13.46			4.23			15.40			3.02		
	CV (%)	0.76			1.45			6.78			13.45		
pH	**Composition of solution in LC-vial**	**MDZ**			**OHMDZ**			**IMIP**			**FEX**		
		Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)
2.70	0.1% Formic acid	6.37	0.04	0.55	8.63	0.06	0.67	8.27	0.06	0.70	11.03	0.15	1.38
	10 mM NH_4COOH	6.41	0.05	0.83	8.70	0.10	1.15	8.20	0.10	1.22	10.93	0.15	1.40
	10 mM NH_4COOCH_3	6.46	0.13	1.97	8.70	0.17	1.99	8.23	0.21	2.53	11.03	0.31	2.77
	5 mM NH_4HCO_3	6.37	0.02	0.24	8.60	0.00	0.00	8.17	0.06	0.71	10.97	0.15	1.39
	Average	6.40			8.66			8.22			10.99		
	CV (%)	0.62			0.58			0.52			0.45		
3.90	0.1% Formic acid	23.20	0.40	1.72	19.87	0.35	1.77	12.13	0.21	1.72	15.87	0.35	2.21
	10 mM NH_4COOH	23.20	0.56	2.40	19.83	0.65	3.28	12.00	0.40	3.33	15.73	0.75	4.77
	10 mM NH_4COOCH_3	23.00	0.56	2.42	19.53	0.60	3.09	11.83	0.40	3.42	15.43	0.60	3.91
	5 mM NH_4HCO_3	22.97	0.35	1.53	19.53	0.31	1.56	11.90	0.20	1.68	15.47	0.35	2.27
	Average	23.09			19.69			11.97			15.63		
	CV (%)	0.54			0.93			1.09			1.34		
6.50	0.1% Formic acid	39.50	0.00	0.00	21.45	0.07	0.33	12.65	0.07	0.56	12.95	0.07	0.55
	10 mM NH_4COOH	39.90	0.62	1.57	21.53	0.23	1.07	12.93	0.06	0.45	13.03	0.06	0.44
	10 mM NH_4COOCH_3	39.87	0.81	2.03	21.53	0.32	1.49	12.97	0.21	1.61	13.00	0.17	1.33
	5 mM NH_4HCO_3	40.10	0.44	1.09	21.80	0.30	1.38	13.17	0.23	1.75	13.17	0.23	1.75
	Average	39.84			21.58			12.93			13.04		
	CV (%)	0.63			0.71			1.65			0.71		
8.30	0.1% Formic acid	45.93	0.47	1.03	22.57	0.35	1.56	27.53	0.76	2.77	10.43	0.06	0.55
	10 mM NH_4COOH	48.37	1.16	2.40	23.83	0.49	2.07	28.23	0.51	1.82	10.83	0.32	2.97
	10 mM NH_4COOCH_3	47.87	0.65	1.36	23.53	0.25	1.07	28.00	0.35	1.24	10.70	0.17	1.62
	5 mM NH_4HCO_3	47.60	1.00	2.10	23.37	0.42	1.78	28.00	0.24	0.84	10.67	0.23	2.17
	Average	47.44			23.33			27.94			10.66		
	CV (%)	2.22			2.32			1.05			1.56		

NB: The – indicates that no peaks were detected for these analytes at this sample vial condition.

mobile phase used. The measured mean peak areas from triplicate injections ± the standard deviation, in counts per second, at each of the 16 possible combinations are given in Figure S2. The greatest overall sensitivity was achieved with a mobile phase at pH 3.9 (consisting of both formic acid and ammonium formate), despite the conditions of the sample vial, except for omeprazole and its hydroxylated metabolite that degraded in the sample vial consisting of 0.1% formic acid.

A repeated measure two-factor ANOVA, with Geisser-Greenhouse correction, tested both the effect of the two independent variables, that is, the composition of the solvent in the sample vial and the mobile phase conditions and pH, on the analyte sensitivity (peak area measured in cps) and the interaction effect between the two, on analyte sensitivity and ultimately ESI efficiency. Normal distribution was confirmed for most analytes at all four mobile phase conditions with the Shapiro-Wilk normality test ($\alpha = 0.05$). Normal and lognormal distributions were compared and data transformed where a lognormal distribution was more likely before the ANOVA test was performed. For the most, data conformed to a Gaussian

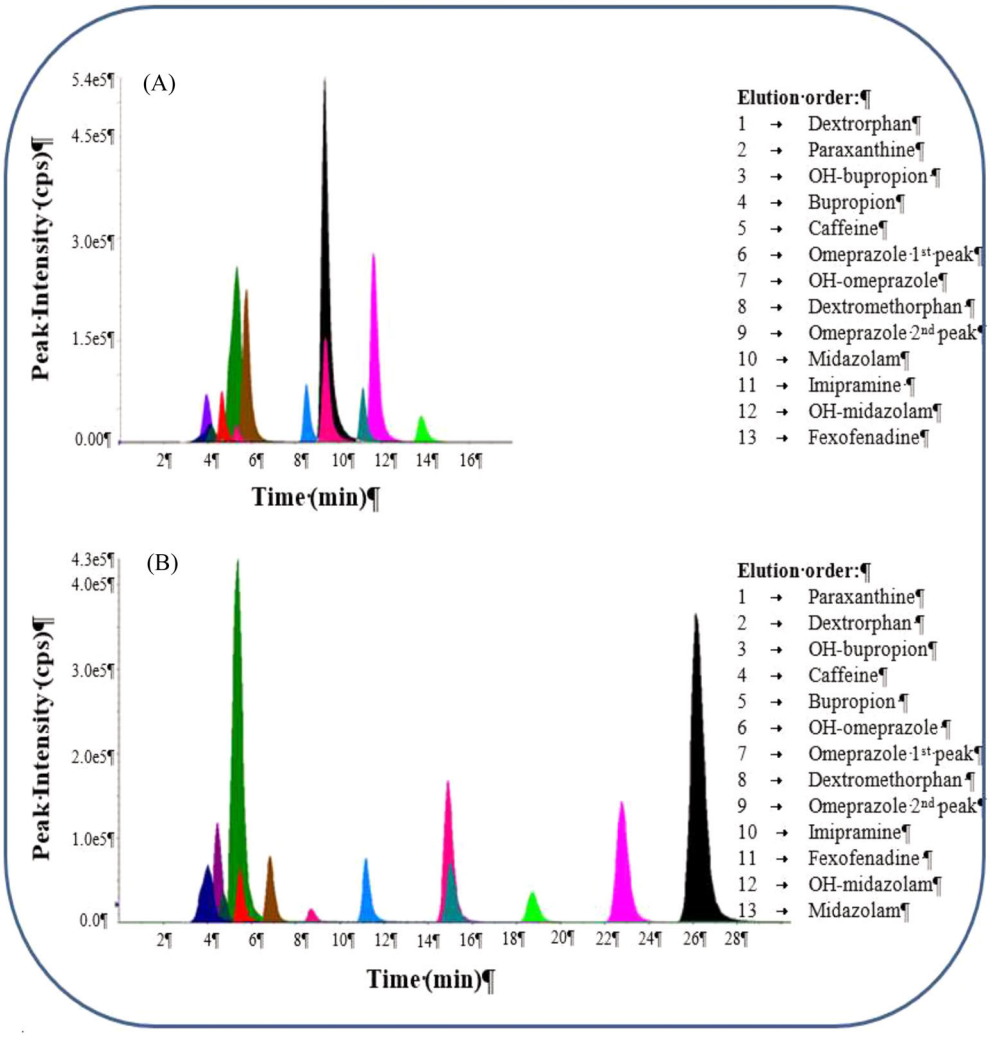

FIGURE 3 Extracted ion chromatograms (XIC) of analytes (100 ng/mL) from a sample in 50:50 methanol:water containing 10 mM ammonium formate separated by isocratic (100 μL/min) 60:40 methanol:water containing (A) 0.1% formic acid, pH 2.7 and (B) 0.1% formic acid with ammonium formate adjusted to pH 3.9 on a Kinetex Biphenyl column (100 × 2.1 mm, 2.6 μm).

distribution (40/48) and in which cases it did not, results for a repeated measures ANOVA, were confirmed using a repeated measures ANOVA for ranks.

The effect of the sample vial composition on the analyte peak areas of bupropion ($p = 0.0130$*), hydroxy-bupropion ($p = 0.0462$*), dextromethorphan ($p < 0.001$**), and internal standard imipramine ($p < 0.001$**) were found to be a source of variance, however, effect sizes were small (<1% of the total variance for bupropion and hydroxy-bupropion; 5.68% for internal standard imipramine; 11.11% for dextromethorphan). Acidic conditions (0.1% formic acid) in the sample vial influenced omeprazole and hydroxy-omeprazole, due to rapid acid hydrolysis and were subsequently excluded from further analysis to test the interaction of the other sample and mobile phase parameters.

The mobile phase composition was found to be the largest source of variance on analyte peak areas with the percentage contribution to overall variance 88.02% for fexofenadine ($p < 0.001$**), 95.08% for caffeine ($p = 0.003$**), 91.24% for paraxanthine ($p < 0.001$****), 97.20% for bupropion ($p < 0.001$***), 93.82% for hydroxy-bupropion ($p < .001$***), 96.52% for omeprazole ($p = 0.0014$**), 96.40% for hydroxy-omeprazole ($p = 0.007$**), 78.49% for dextromethorphan ($p = 0.002$**), 94.04% for dextrorphan ($p = 0.0013$**), 93.67% for midazolam ($p = 0.0023$), 88.64% for hydroxymidazolam ($p = 0.0122$*), and 82.33% for imipramine ($p = 0.0048$**).

The interaction effect between both the sample vial condition and the mobile phase pH was a source of variance for the following analytes: 6.99% for fexofenadine ($p = 0.0043$**), 2.73% for caffeine ($p = 0.0047$**), 4.24% for paraxanthine ($p = 0.0421$*), 1.60% for bupropion ($p = 0.0131$*), 3.91% for hydroxy-bupropion ($p = 0.0132$*), 7.22% for dextromethorphan ($p = 0.0278$*), 4.34% for dextrorphan ($p = 0.0065$**), 3.93% for midazolam (p 0.0169*),

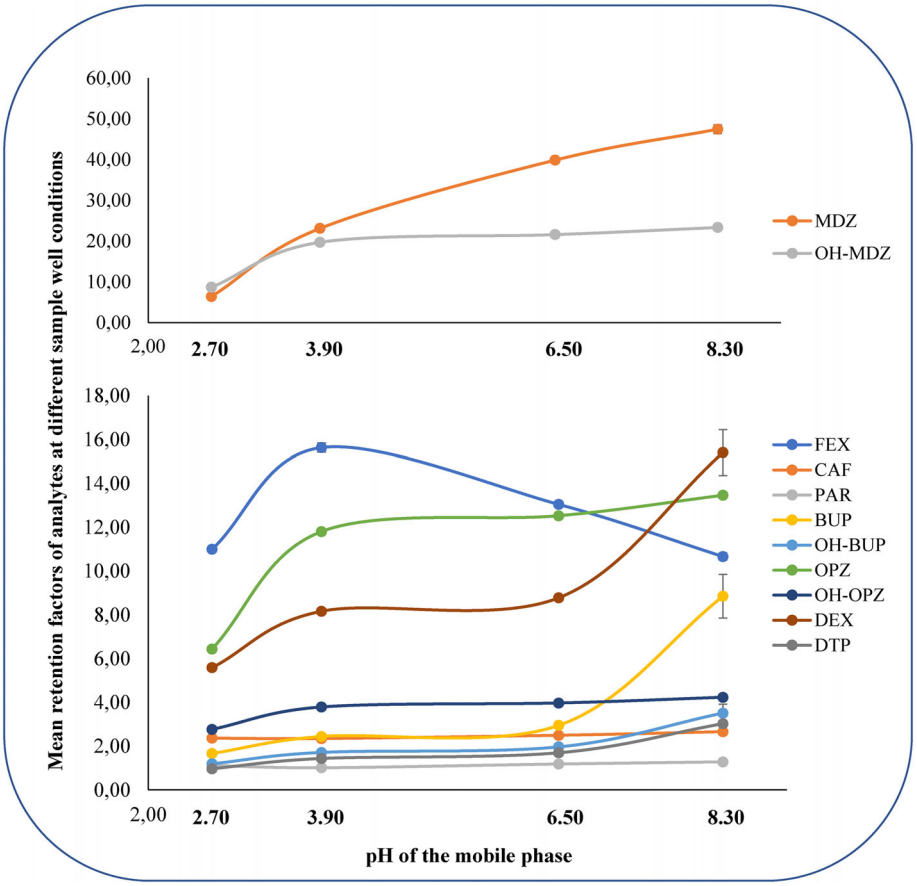

FIGURE 4 Relationship between retention behavior and the mobile phase pH under isocratic elution conditions, flow rate: 100 μL/min with methanol:water (60:40) on a Kinetex Biphenyl column (100 × 2.1 mm, 2.6 μm).

5.64% for hydroxymidazolam ($p = 0.0077$**), and 9.15% for imipramine ($p = 0.0046$**).

The repeated measures two-factor ANOVA revealed interaction effects between the conditions of the sample vial when paired with different mobile phase pH, affecting the analyte peak areas and thus the detection limits, sensitivity, and overall ionization efficiency of the analytes (Figure S2). Tukey's multiple comparisons tested multiple hypotheses with pair-wise comparisons between the mean difference of each of the different mobile phases for each independent sample vial condition. As expected, a mobile phase pH of 8.3 had a significant effect on the retention behavior and the sensitivity of the basic analytes. Other significant sources of variance were acidic conditions in the sample vial on the stability of omeprazole and hydroxy-omeprazole.

4 | CONCLUSION

This study provides a simple approach that can be used to optimize a bioanalytical method for the simultaneous quantification of the phenotyping probe drugs and their metabolites. Changes in analytical conditions caused statistically significant variation in the retention behavior, selectivity, column efficiency, stability, and sensitivity of the analytes of interest and therefore these parameters should be controlled for reliable, reproducible quantitative results during method validation. Considering all factors influencing the resolution of the analytes, a 10 mM ammonium formate in the sample vial with water and methanol as mobile phases A and B containing 0.1% formic acid (pH 2.7) were chosen for further method validation using a Kinetex biphenyl column as the stationary phase to overcome the analytical challenges previously experienced with this combination of analytes. With this combination, analyte sensitivity was sacrificed for a higher throughput method and better peak shape with optimized retention factors. The method was still sensitive enough with detection limits acceptable for all analytes of interest.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support thefindings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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