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RESEARCH ARTICLE

Analysis of highly potent amidine containing inhibitors of serine proteases and their *N*-hydroxylated prodrugs (amidoximes)

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Abstract

The development of serine protease inhibitors often results in the discovery of new lead compounds containing strong basic amidine functions that usually suffer from poor absorption from the intestine. In order to improve oral bioavailability of these drugs, prodrug principles such as the conversion of amidines into amidoximes may be applied. In this work, two HPLC-based separation methods of serine protease inhibitors (amidines) and their *N*-hydroxylated prodrugs have been developed and characterised. This was performed by evaluating 11 distinct amidine-amidoxime pairs with different physicochemical parameters (clogP: −3 to 5.1). The HPLC methods developed allowed excellent separation of the compound pairs examined. Also, the possible selection of different separation techniques (i.e. adsorption- and ion-pair-chromatography) permits universal application. Moreover, both techniques are compatible with mass spectrometry and are superior to the previously described methods. In summary, both HPLC methods are suitable for the separation of most amidoxime-prodrugs currently in clinical or preclinical development.

Keywords: Serine protease inhibitor, prodrug, amidine, amidoxime, HPLC

Introduction

Amidines are found in several drug-candidates with diverse medical indications, such as anticoagulants, antimicrobial, and antimetastatic drugs. Amidine containing drugs are often found in the development of trypsin-like serine protease inhibitors and have much potential for therapy in various diseases. These amidine residues mimic the arginine or lysine residues, which are the common substrates of trypsin-like serine proteases, and interact with the free carboxyl group of aspartate in the binding pocket [1].

Inhibitors of the matriptase and urokinase-type plasminogen activators (uPA) represent new classes of anti-invasive agents that have showed promising effects in animal studies by reducing the malignancy of different cancer cell types [2–5]. Antagonists of the glycoprotein IIb/IIIa receptor as well as inhibitors of thrombin and factor Xa (FXa) have become useful tools for the treatment of thromboembolic diseases as indicated by the recent approval of the direct thrombin inhibitor dabigatran etexilate (Pradaxa®) and also rivaroxaban (Xarelto®), a direct FXa inhibitor [6–8]. Other indications of amidine-drugs such as pentamidine and diminazene, include the treatment of *pneumocystis jiroveci* pneumonia, leishmaniasis, or trypanosomiasis [9].

Although many amidines show excellent effects in both *in vitro* assays and animal models, the clinical use of amidine-drugs in humans is still very restricted. The main reason for this is the generally poor oral bioavailability of these compounds that results from the strong basic amidine function, which is protonated under physiological conditions and consequently positively charged. In order to overcome these poor absorption properties, prodrug principles for amidines may be applied [10]. One of the

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most intensively investigated prodrug principles for these compounds is the conversion of amidines into amidoximes (*N*-hydroxy-amidines) [11]. After absorption from the intestine, amidoxime-prodrugs are rapidly converted into active amidines. This conversion is catalysed by an enzyme system consisting of cytochrome b_5, NADH cytochrome b_5 reductase and a third component identified in mitochondria as a previously unknown molybdoenzyme, which has been named mitochondrial amidoxime reducing component (mARC) and is the fourth human molybdenum containing enzyme [12,13]. A great advantage of this activation pathway is that the conversion of the amidoxime-prodrugs into their active forms avoids interaction with the cytochrome P450 isoenzymes. Thus, this prodrug principle has no risk of cytochrome mediated side-effects, such as the drug-interactions that can occur if diverse drugs are metabolised by the same cytochrome isoenzymes and have to compete with each other [14].

The enormous number of newly discovered amidine drugs and their amidoxime-prodrugs require selective and robust separation techniques. Therefore, we have developed appropriate analytics that fulfill these criteria.

Within this work, we have developed and characterised HPLC-based separation methods for a large number of amidine-drugs—predominantly potent inhibitors of trypsin-like serine proteases (FXa, matriptase, uPA, and thrombin) —and their *N*-hydroxylated prodrugs that are currently in clinical or preclinical trials. By analysis of 11 distinct pairs of amidines and amidoximes with clogP values between –3 and 5.1, two simple and universally applicable HPLC methods have been developed. The choice of ammonium acetate and trifluoroacetic acid (TFA), resulted in different separation methods (i.e. adsorption- or ion-pair-chromatography) and offers a broad field of possible applications. In addition, both techniques are compatible with mass spectrometry and are superior to the previously described methods using other mobile or stationary phases.

In summary, both methods would be suitable for the separation of most amidoxime-prodrugs currently in clinical or preclinical development.

Experimental

Materials

All reagents were purchased at the highest available purity. Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (Munich, Germany), ammonium acetate, hydrochloric acid, potassium phosphate, and sodium hydroxide from Merck KGaA (Darmstadt, Germany). High-performance liquid chromatography (HPLC) grade acetonitrile was purchased from Mallinckrodt Baker (Griesheim, Germany).

The applied test compounds were kindly supplied by The Medicines Company (Leipzig, Germany; CJ-463, CJ-929, CJ-930, CJ-1200, CJ-1331, CJ-1332, CJ-1707, CJ-1764, CJ-1802, CJ-1803, CJ-1817, CJ-2026), Wilex AG (Munich, Germany; WX-UK1, WX-671 (Mesupron®)), Hoffmann-La Roche (Basel, Switzerland; Ro 48-3656, Ro 44-3888) and AstraZeneca (Mölndal, Sweden; melagatran, ethyl-melagatran, *N*-hydroxy-melagatran, ximelagatran). Syntheses are described elsewhere [2,15,16,17,18]. Benzamidine was obtained from Sigma-Aldrich (Munich, Germany). *N*-hydroxy-benzamidine (benzamidoxime) was synthesised from benzonitrile and hydroxylamine as described previously [19]. All the structural formulas are summarised in Table 1.

Preparation for analysis

All the test compounds were dissolved in 30% acetonitrile to a final concentration of 100 µM. These dilutions were used as standards for the HPLC determinations. To obtain two independent HPLC methods with diverse advantages, two different mobile phases for either the ion-pair- or adsorption-chromatography were chosen. Both HPLC methods were evaluated by investigating the separation of each amidine-amidoxime pair in order to draw a general conclusion for the subsequent separation of the new amidine-amidoxime pairs. In the initial studies, the percentage of acetonitrile in the mobile phase was varied until both the compounds were well separated within less than 15 minutes. The chromatograms obtained were analysed and the chromatographic parameters (retention time, selectivity and resolution) were calculated for all the runs. The percentage of acetonitrile was plotted against the calculated lipophilicity (clogP) of the amidoximes and the resulting correlation curves were calculated for both methods.

In subsequent trials these correlations curves were used to calculate the required percentage of acetonitrile for the separation of every amidine-amidoxime-pair on the basis of their clogP. Thus, separation of all the compound pairs was performed using the mobile phases with these calculated acetonitrile concentrations. Afterwards, the chromatograms obtained were analysed and the chromatographic parameters (retention time, selectivity, and resolution) were calculated.

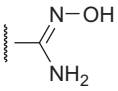
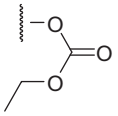
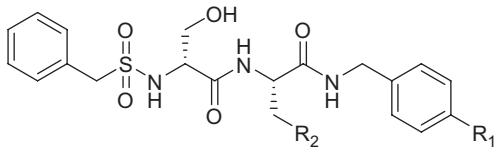
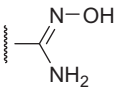
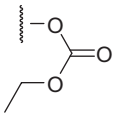
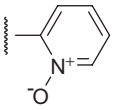
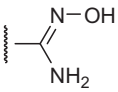
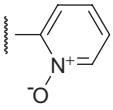
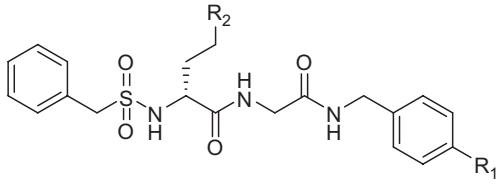
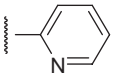
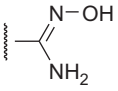
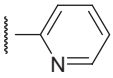
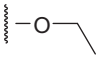
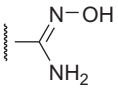
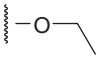
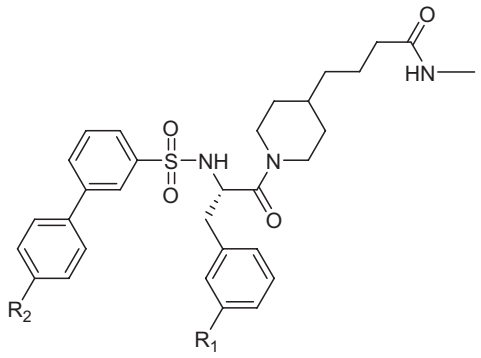
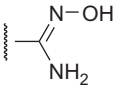
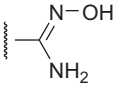
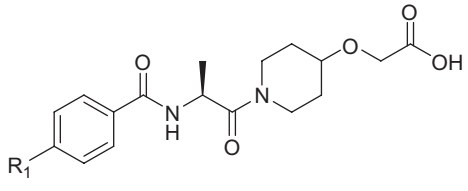
HPLC analysis

The separations were performed with a Waters Alliance™ HPLC system (Waters, Milford, MA, USA) consisting of a Waters e2695 XC separation module, a Waters 2998 Photodiode Array detector (Waters, Milford, MA, USA), and the Empower™ 2 software. The mobile phases were filtrated through a 0.45 µm Sartolon® membrane filter (Sartorius, Göttingen, Germany) and degassed. All analysis were carried out isocratically by a LiChrospher® 60 RP-select B column (125 × 4 mm; 5 µm, Merck KGaA) and a RP-select B guard column (4 × 4 mm; Merck KGaA). The flow rate was maintained at 1 mL/min, the injection volume was 10 µL, and the detection wavelength was 220 nm for all determinations. The column temperature was set to 25°C.

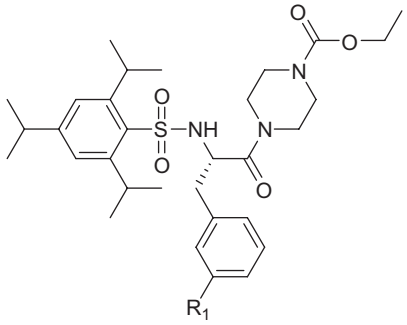
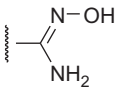
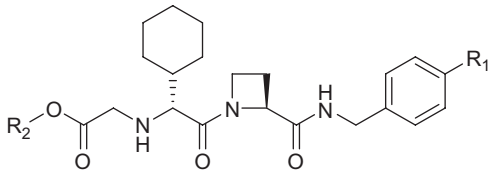
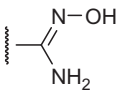
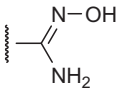
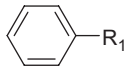
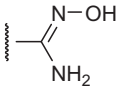
Method A (Adsorption chromatography): Separation with mobile phases containing ammonium acetate

The mobile phase of 20 mM ammonium acetate (pH 4) was applied with varying percentages of acetonitrile.

Table 1. A summary of the clogP values and chemical structures for all the amidine-amidoxime pairs.

No	Name	clogP*	R_1	R_2	Structure
1a	CJ-463	−2.23			
1b	CJ-1200	−2.30			
2a	CJ-930	−1.38			
2b	CJ-929	−1.46			
3a	CJ-1332	−2.93			
3b	CJ-1331	−3.00			
4a	CJ-1707	0.46			
4b	CJ-1764	0.39			
5a	CJ-1803	3.82			
5b	CJ-1802	3.75			
6a	CJ-1817	5.10			
6b	CJ-2026	5.02			
7a	Ro 44-3888	−1.91		–	
7b	Ro 48-3656	−1.99		–	

Eluents with organic percentages between 5% and 50% amidine bearing compounds are predominantly used

No	Name	clogP*	R_1	R_2	Structure
8a	WX-UK1	4.30		–	
8b	WX-671 (Mesupron®)	4.23		–	
9a	Melagatran	–0.93			
9b	*N*-Hydroxy-melagatran	–1.01			
10a	Ethyl-melagatran	0.97			
10b	Ximelagtran	0.90			
11a	Benzamidine	0.32		–	
11b	*N*-Hydroxy-benzamidine	0.25		–	

*clogP was calculated for the uncharged compounds using the "Molinspiration Property Calculator" software.

were used to ensure the separations were within 15 min (Table 2).

Method B (Ion-pair chromatography): Separation with mobile phases containing TFA

The mobile phase contained TFA in a concentration of 0.1% and the pH was adjusted to pH 7. Acetonitrile was added to final concentrations in the range of 5% to 75% to achieve separations within less than 15 min (Table 3). In supplementary trials the addition of 20 mM potassium phosphate to the mobile phase was investigated.

Calculation of lipophilicity (clogP)

The lipophilicity of all the compounds was calculated, in their uncharged state, using the "Molinspiration Property Calculator" software (Molinspiration Cheminformatics, Slovensky Grob, Slovak Republic).

Results and discussion

In this study, two universal HPLC methods for the separation of amidines and their *N*-hydroxylated prodrugs (amidoximes) have been developed. These in the development of serine proteases inhibitors. Consequently, we set the focus on the separation of these compounds. Potent inhibitors of uPA (pairs 1, 2, and 8, Table 1), FXa (pairs 3 and 4), matriptase (pairs 5 and 6), and thrombin (pairs 9 and 10) were selected to establish and validate two independent HPLC methods. The aim of the study was to develop universally applicable HPLC methods for the separation of amidine-drugs and amidoxime-prodrugs. These should be easily transferable to other amidine-amidoxime pairs providing a time-saving tool for the future development of inhibitors of serine proteases.

The separation of all the compounds examined was achieved within relatively short runtimes by both methods with good selectivity and resolution characteristics. In general, HPLC method A showed a lower selectivity (1.21 to 1.83) and resolution (1.74 to 5.96) compared to those obtained by using method B (1.3 to 7.19 for selectivity and 2.31 to 19.26 for resolution). The essential chromatographic parameters (retention time, selectivity and resolution) for all the runs are summarised in Tables 2 and 3. Two representative chromatograms for the separation of the amidine-amidoxime pair 1 are

displayed in Figures 1 (method A) and 2 (method B) showing the peak shape and the changed order of elution resulting from the two different separation techniques (i.e. adsorption- and ion-pair-chromatography) used.

The investigated compounds were chosen because of their differing physico-chemical properties with clogP values ranging from –3 (compound **3b**) to 5.1 (compound **6a**) (Table 1). This high physico-chemical heterogeneity shows the suitability of both methods for other amidine-amidoxime pairs.

Table 2. Chromatographic parameters of HPLC method A.

Method A (tested/calculated)					
Pair	Compound.	ACN [%]	Retention [min]	Selectivity	Resolution
1	**1a**	10/8	7/10.5	1.34/1.56	4.57/5.14
	1b		9.1/15.9		
2	**2a**	20/12	6.9/-	1.56/-	5.96/-
	2b		10.3/-		
3	**3a**	15/5	6.7/-	1.53/-	4.77/-
	3b		9.8/-		
4	**4a**	20/20	5.6/5.6	1.42/1.42	2.86/2.86
	4b		7.6/7.6		
5	**5a**	35/35	7.7/7.7	1.48/1.48	3.88/3.88
	5b		11/11		
6	**6a**	40/41	9.5/9.8	1.43/1.26	3.61/2.24
	6b		13.2/12.1		
7	**7a**	5/10	5.4/2	1.54/1.83	4.07/4.17
	7b		7.9/3		
8	**8a**	47/38	7.8/-	1.29/-	3.48/-
	8b		9.8/-		
9	**9a**	8/14	6.6/2.8	1.33/1.3	1.85/1.74
	9b		8.5/3.4		
10	**10a**	22/23	12.1/5.9	1.21/1.27	2/2.01
	10b		14.5/7.3		
11	**11a**	5/20	2.9/-	1.38/-	2.46/-
	11b		3.7/-		

The analytical parameters of the empirically established method compared to those calculated. Separations of pairs 2, 3, 8, and 11 were insufficient when using the calculated conditions.

Correlation curves were generated between the clogP value of the examined amidoximes and the acetonitrile percentage of the mobile phases used for the separations. The influence of clogP on the acetonitrile percentage of the mobile phase is depicted in Figure 3. The obtained correlations were used to calculate the required organic percentage in the mobile phase for separation of the test compounds. HPLC analysis performed with these conditions resulted in good separations for most of the pairs of compounds. The resolutions of the separations performed are summarised in Figure 4. For method A only four pairs showed insufficient separations when

Table 3. Chromatographic parameters of HPLC method B.

Method B (tested/calculated)					
Pair	No	ACN [%]	Retention [min]	Selectivity	Resolution
1	**1a**	20/16	7.1/7.3	2.42/1.3	12.33/2.31
	1b		3.4/5.8		
2	**2a**	40/40	4.1/4.1	2.75/2.75	7/7
	2b		2/2		
3	**3a**	20/10	11.5/-	2.55/-	13.68/-
	3b		5/-		
4	**4a**	35/36	7.8/5.4	3.5/2.88	13.79/10.17
	4b		2.8/2.4		
5	**5a**	50/60	11.5/6	5.22/5.2	11.53/14
	5b		2.9/1.8		
6	**6a**	75/70	6.2/7.8	5.6/7	11.5/10
	6b		1.8/1.8		
7	**7a**	5/18	4.3/-	1.59/-	4/-
	7b		3./-		
8	**8a**	75/64	7.6/15.9	4.86/7.19	13.5/19.26
	8b		2.2/2.9		
9	**9a**	15/25	5.2/-	4/-	6.73/-
	9b		1.9/-		
10	**10a**	40/39	6.4/7.3	3.29/3.61	7.88/9.49
	10b		2.5/2.6		
11	**11a**	20/35	4/3.5	1.52/2.7	3.14/5.48
	11b		2.9/1.8		

The analytical parameters of the empirically established method compared to those calculated. Separations of pairs 3, 7, and 9 were insufficient when using the calculated conditions.

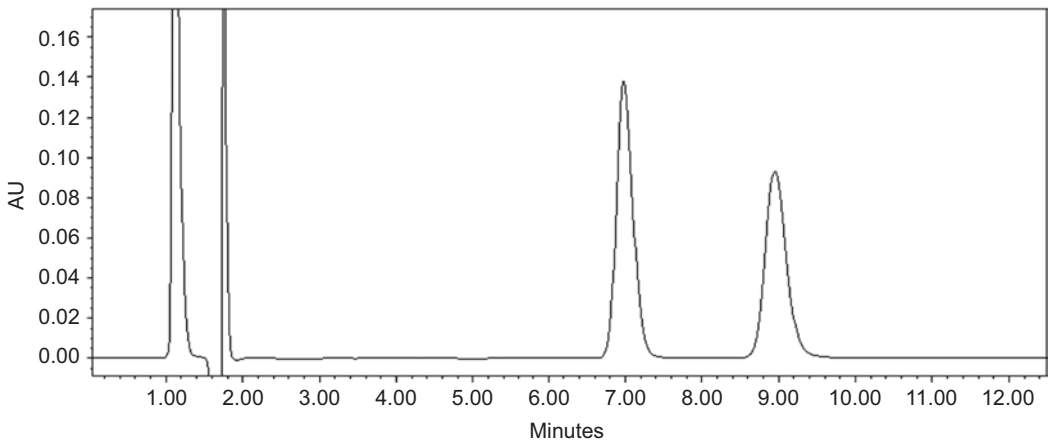


Figure 1. Representative chromatogram for the separation of CJ-463 (7 min) and CJ-1200 (9.1 min) using a mobile phase consisting of ammonium acetate buffer (20 mmol/L, pH 4) and acetonitrile (90/10, v/v) (method A). The chromatographic parameters are shown in Table 2.

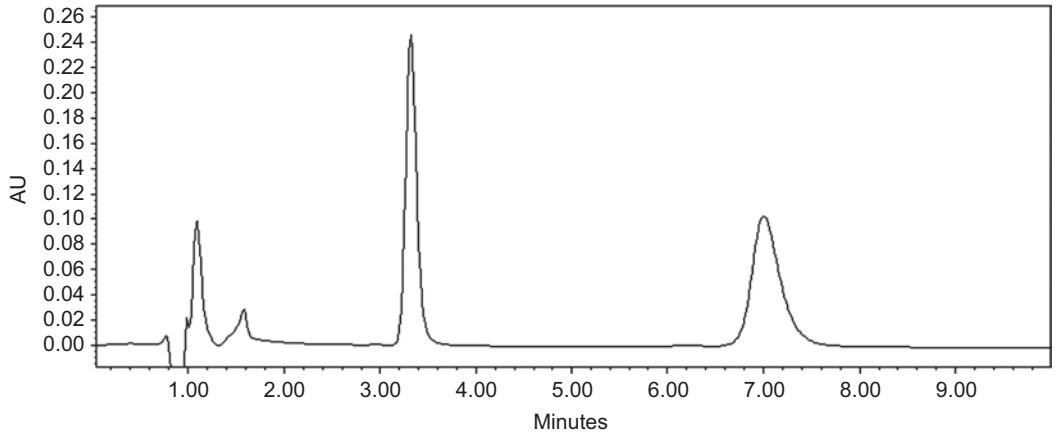


Figure 2. Representative chromatogram for the separation of CJ-1200 (3.4 min) and CJ-463 (7.1 min) using a mobile phase consisting of TFA (0.1%) in distilled water and acetonitrile (80/20, v/v; pH 7) (method B). For chromatographic parameters see Table 3.

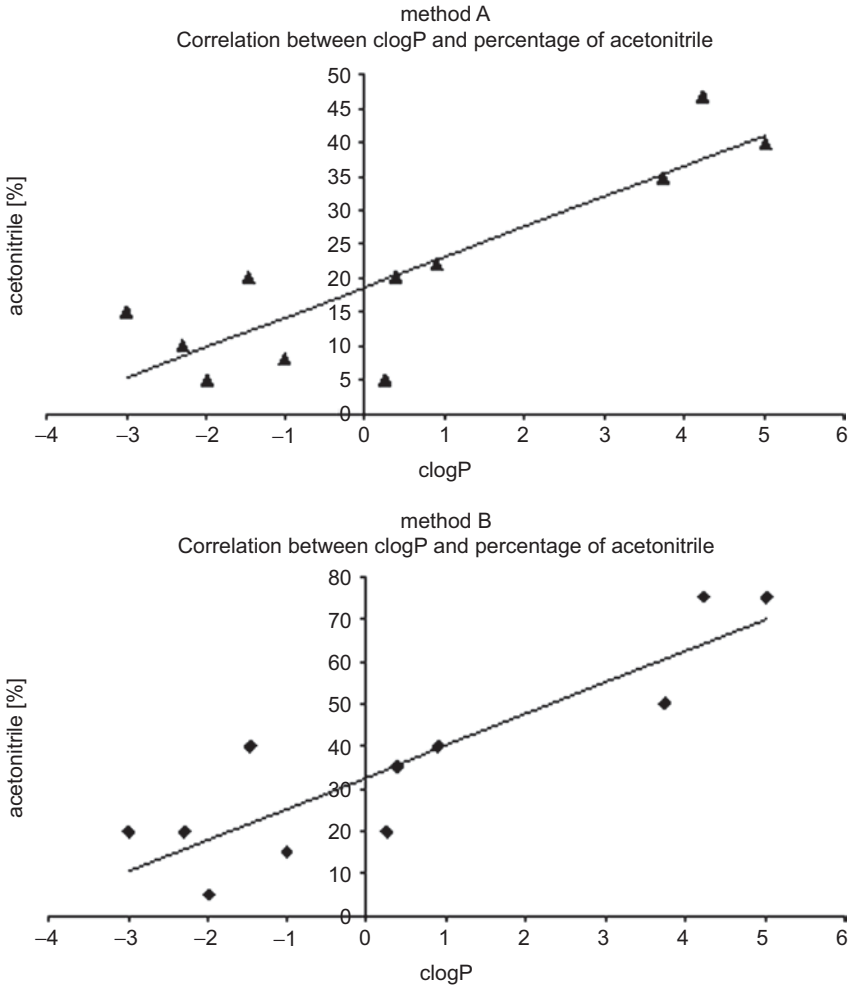


Figure 3. Correlations between clogP (amidoxime) and acetonitrile as a percentage of the mobile phase. The calculated linear equations are y = 4.46x + 18.70 (R^2 = 0.744) and y = 7.42x + 32.69 (R^2 = 0.783) for methods A and B, respectively.

using the calculated conditions, while for method B this was true for only three pairs. Accordingly, the calculated conditions for method B are not adequate for the separation of negatively charged compounds (pairs 3, 7, and 9) indicating that the usage of the calculated correlations is limited. Nevertheless, correlation curves established in this work are useful tools and facilitate the development of HPLC methods for newly discovered serine protease inhibitors as well as other amidine containing compounds.

Nowadays, the compatibility of HPLC to mass spectrometry is getting more and more important and has become a

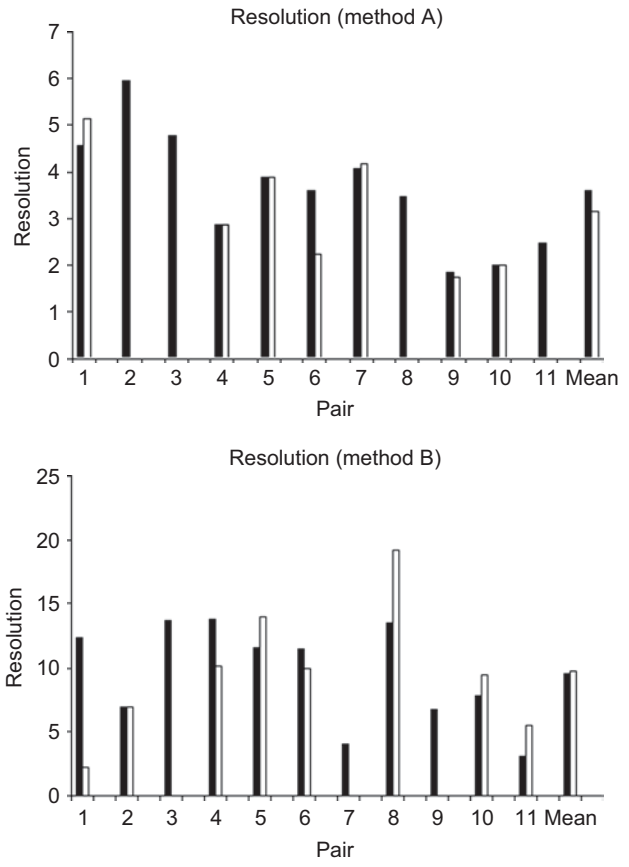


Figure 4. Resolution of HPLC methods A and B. Resolutions obtained with the experimentally established conditions (black bars) are compared to the calculated ones (white bars).

fundamental requirement for determinations in complex matrices such as blood or urine samples. One of the major limitations of the previously published HPLC methods for the separation of amidines and amidoxime-prodrugs is the lack of compatibility to mass selective detectors caused by the usage of unsuitable reagents like phosphate salts or ion-pair-reagents such as sodium octylsulphonate [20–23]. Compared to these techniques the methods described in this report show improved properties with respect to mass spectrometry and could facilitate analysis of samples with complex matrices.

Moreover, the choice of two different separation methods, adsorption- and ion-pair-chromatography, gives the advantage that the order of elution for the analytes can be reversed and therefore could be adapted to the particular analytical requirements. Separations with method B resulted in a fast elution of the amidoximes and a distinct retention of amidines, whereas method A showed short retention times for amidines and longer ones for amidoximes. The choice of whether to use method A or method B will depend on the particular need or sample usage required. Consequently, the choice of adsorption- or ion-pair-technique is important to achieve the best possible separations and could offer a widespread analytical usage.

When using TFA in the mobile phase as the ion-pair-reagent, the pH was adjusted to pH 7. At this pH, the compounds are charged or uncharged due to the different basicity of the amidines ($pK_B \approx 2–3$) and amidoximes ($pK_B \approx 9–10$) [24,25]. Amidines exhibit strong basic properties, whereas amidoximes are less basic and hence not protonated at pH 7. Consequently, only the amidines were positively charged under the described conditions and therefore able to form ion-pairs with TFA. This resulted in a significant increase of lipophilicity and elongated the retention time, which resulted in the excellent selectivity and resolution of method B (Table 3). However, the usage of TFA as an ion-pair-reagent resulted in slightly higher costs for waste disposal, because of an increased organic portion in the mobile phase in comparison to method A.

The addition of 20 mM potassium phosphate to the mobile phase led to a buffered solution at pH 7 and this improved the robustness of method B. However, with the addition of potassium phosphate this method loses its compatibility with mass spectrometry.

The limits of quantification were not optimised in this work because the main focus was on the separation of the amidines and amidoximes. For instance, the limit of quantification in porcine plasma was determined for compounds **3a** and **3b**. Only a slight optimisation of method A was necessary to achieve detection limits of 30 ng/mL using LC/MS with athmospheric pressure chemical ionisation (APCI). These data are published elsewhere [17].

All the amidine-amidoxime pairs examined were separated by the methods described within less than 15 minutes. Further optimisation of the runtimes could easily be done by adjusting the acetonitrile gradient or increasing the column temperature. First optimisation attempts showed significantly reduced runtimes without impairing the separations. For instance, when separating compound pair 5 with method B, by increasing the column temperature to 50°C the retention time of compound **5a** was decreased from 11.5 min to 9.8 min, while the retention time of compound **5b** was affected only marginally. Overall, these HPLC-based separation methods allowed fast separation and could be applied to standard high-throughput analysis.

Conclusion

In this work, two HPLC methods for the separation of amidines and amidoximes were successfully established using both adsorption- and ion-pair-chromatography. The use of reagents that are compatible with mass spectrometry is a great advantage compared to previously described analytical techniques. Both methods are versatile and may be easily optimised to achieve low limits of detection, especially in combination with mass spectrometry. A major advantage is that the established methods can be easily transferred to a large number of amidoxime-prodrugs. Thus would be a time-saving tool for the development of HPLC methods for amidoxime-prodrugs that are currently in preclinical or clinical trials.

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Declaration of interest

The authors report no conflicts of interests. The authors alone are responsible for the content and writing of the paper.

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