

## RETENTION BEHAVIOUR OF TWO BIGUANIDINES IN LIQUID CHROMATOGRAPHY BASED ON CYANO STATIONARY PHASE

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A new way of improving the retention of biguanidines by means of cyano based stationary phase, followed by mass-spectrometry as detection approach in order to be applied to the determination of low levels of biguanidines in complex samples, such as biological ones. Two biguanidines were studied in this paper: N,N-dimethylbiguanidine and N-methylbiguanidine. The retention study revealed an exponential functional dependence of the retention of target solutes on the mobile phase in accordance to a normal-phase mechanism: the retention time for both biguanidines increased with the increase of the organic modifier content (methanol, or acetonitrile).

### INTRODUCTION

Owing to their high hydrophilic character, biguanidine class compounds (with therapeutic effects) have almost no retention on classic chromatographic stationary phases, such as octyl- or octadecyl based silica materials.<sup>1</sup> In order to enhance their retention one possible solution is to apply an ion-pairing mechanism using anions with long hydrocarbonaceous chains (with more than six C atoms).<sup>2,3</sup> This mechanism was used to retain almost quantitatively several drugs belonging to this class on particles that had been treated with an ion-pair reagent of heptanesulfonate or dodecylsulfate, as a solid-phase extraction technique.<sup>4</sup> An assay based on cation exchange-based liquid chromatography - tandem mass spectrometry (LC/MS/MS) has been developed for the improvement of both retention and detection of metformin in biological samples.<sup>5</sup> On the other hand, these compounds absorb radiation in low UV domain and with a low sensitivity. Therefore, the enhancement of their detectability in LC is also another task in method development and applications to low levels of these compounds in different samples. For instance, derivatizations with p-nitrobenzoyl chloride,<sup>6,7</sup> or 9,10-anthraquinone-2-sulfonyl chloride<sup>8</sup> may improve

the sensitivities in absorption spectrometry with several orders of magnitude. Fluorescence precolumn labeling with desyl chloride or benzoin is a method of choice in detecting low ppb levels of these compounds.<sup>9</sup> In conclusion, their separations by LC followed by classic spectrometric detection are rather difficult. The improvement in sensitivity of detection can be achieved by mass-spectrometry.<sup>10-14</sup>

It is the aim of this paper to study a new way of improving the LC retention of biguanidines by means of cyano based stationary phase, keeping mass-spectrometry as detection approach in order to be used for determination of low levels of these compounds in complex samples, such as biological ones. The two studied compounds were N,N-dimethylbiguanidine (according to European Pharmacopoeia it is known as metformin) and N-methylbiguanidine (a related impurity substance in previous one, which can be used as internal standard for an LC-MS analytical method for metformin assay in biological fluids).

### EXPERIMENTAL

An Agilent 1100/1200 series LC system consisting of the following modules: degasser (G1322A), binary pump (G1312A), thermostated autosampler (G1329A/G1330B) and

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column thermostat (G1316A) coupled with an Agilent 6410 Triple Quad LC/MS mass spectrometer (Agilent Technologies - Waldbronn, Germany) were used for the present study.

A single end-capped Zorbax CN column from Agilent was used having 150 mm length, 4.6 mm internal diameter, 5  $\mu\text{m}$  particle size, 60  $\text{\AA}$  pore size. The column was thermostated at 25°C. The isocratic mobile phase pumped at a constant flow-rate of 0.8 mL/min consisted of aqueous acetate buffer (10 mM ammonium acetate at pH 3.5 with acetic acid) and acetonitrile or methanol as organic modifier. Mobile phase composition was variable in the range 10 – 90% organic modifier with a 10% step increase. The injected volume was 1  $\mu\text{L}$  of standard solution containing 500 ng/mL N,N-dimethylbiguanidine and 500 ng/mL N-methylbiguanidine in water/acetonitrile (20/80, as volumes).

Detection was performed with a triple quadrupole mass spectrometer in the ESI MRM mode. The ESI interface parameters were set to 325°C drying gas temperature, 10 L/min drying gas flow ( $\text{N}_2$ ), 60 psi nebulizer pressure ( $\text{N}_2$ ) and 1500 V capillary voltage. The full capabilities of the Agilent 6410 Triple Quad LC/MS system were exploited using MS/MS in the MRM mode, as follows: for N,N-dimethylbiguanidine the transition 130.2 (precursor ion) to 60.2 (product ion), while for N-methylbiguanidine the transition 116.2 (precursor ion) to 60.2 (product ion) were simultaneously monitored. The applied collision energy in the dissociation cell (Q2) was 15 eV. The fragmentor voltage used to focus and accelerate ions was set to 110 V. The dwell time used to monitor either of the two transitions was 50 ms.

All solvents were HPLC grade from Merck (Darmstadt, Germany). Reagents were analytical grade from the same producer. Water for chromatography (resistivity minimum 18.2M $\Omega$  and 170 TOC maximum 30 ppb) was obtained within the laboratory by means of a TKA Lab HP 6UV/UF instrument. N,N-dimethylbiguanidine and N-methylbiguanidine were purchased from European Pharmacopoeia, Council of Europe, Strasbourg, France.

## RESULTS AND DISCUSSION

During the optimization of an LC-MS method for N,N-dimethylbiguanidine (known as metformin) assay in plasma samples it has been observed that increasing organic modifier content (either acetonitrile or methanol) in the mobile phase leads to an exponential increase in the retention of this solute. A similar behavior has been observed for the internal standard used in these experiments (N-methylbiguanidine).<sup>14</sup> A complete study of the dependence of the retention time values for these compounds for two organic modifier (methanol and acetonitrile) has been then undertaken (the graphs are given in Fig. 1, while their exponential regression parameters are given in Table 1).

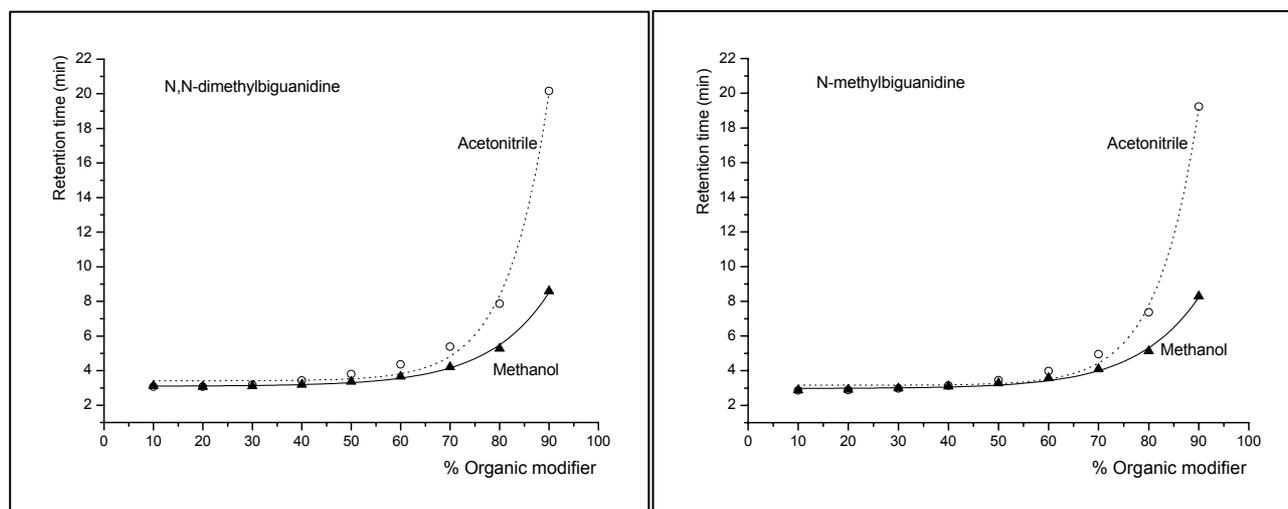


Fig. 1 – Retention time dependence on organic modifier content (acetonitrile or methanol) in mobile phase for N,N,-dimethylbiguanidine and N-methylbiguanidine.

Table 1

The values of exponential regression parameters:  $y = A_1 \cdot \exp(x/t_1) + y_0$

Compound	$y_0$	$A_1$	$t_1$	$r^2$
Organic modifier: acetonitrile				
N,N-dimethylbiguanidine	$3.401 \pm 0.187$	$0.00029 \pm 0.00023$	$8.213 \pm 0.596$	0.9954
N-methylbiguanidine	$3.151 \pm 0.158$	$0.00021 \pm 0.00016$	$8.020 \pm 0.521$	0.9964
Organic modifier: methanol				
N,N-dimethylbiguanidine	$3.131 \pm 0.050$	$0.00239 \pm 0.00089$	$11.648 \pm 0.562$	0.9976
N-methylbiguanidine	$2.967 \pm 0.063$	$0.00364 \pm 0.00160$	$12.366 \pm 0.747$	0.9963

The functional dependences of capacity factor on mobile phase composition are very similar for both solutes and for both tested organic modifiers (acetonitrile and methanol). An exponential function describes the retention behavior of N,N-dimethylbiguanidine and N-methylbiguanidine. The correlation coefficients are better than 0.995 for both solutes when using acetonitrile and methanol as well. Fig. 1 shows that the retention is always higher for acetonitrile than for methanol as organic modifier in the studied range of mobile phase compositions (10 – 90%). When using mobile phases richer in aqueous buffer (10% organic modifier) the retention time difference between acetonitrile and methanol is quite small; at the other end of the interval (90% organic modifier) the retention time difference between acetonitrile and methanol becomes higher.

The significant retention difference obtained for high content of organic modifier could be explained by difference between elutropic strengths of methanol (0.95) and acetonitrile (0.64).<sup>15</sup> Moreover, according to the same ref.<sup>15</sup>, the polarity parameter describing the solubilization power of these solvents is favorable to methanol (7.4) in comparison with acetonitrile (5.8): the polar-polar interactions are thus stronger for

methanol than acetonitrile leading to lower retention time values for the first mentioned organic modifier.

This behavior is opposed to reversed-phase LC retention mechanism, when solutes are retained less as the polarity of the mobile phase decreases (when increasing organic modifier content). This observation could lead to the idea that the two solutes are in fact retained by the cyano stationary phase in accordance to a normal-phase mechanism.

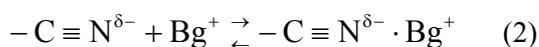
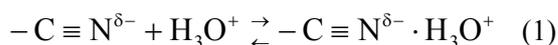
The normal-phase mechanism supposes a polar stationary phase, a non polar mobile phase and a polar solute. The cyano stationary phase of Zorbax CN column is a cyanopropyl modification of the silica support. Cyanopropyl radicals are in fact quite polar in character, but however behaving in both reversed-phase and normal-phase mechanisms. The mobile phase composed of aqueous acetate buffer and methanol or acetonitrile could be considered as having closed polarity to the cyano stationary phase; finally the considered studied solutes have similar polarity (or, very low log P values according to Table 2). All these considerations support the idea of a pseudo-normal phase separation mechanism for N,N-dimethylbiguanidine and N-methylbiguanidine, mainly for higher organic modifier content in the mobile phase.

Table 2

Some physico-chemical properties of N,N-dimethylbiguanidine and N-methylbiguanidine

Compound	log P (theoretical)	log P (experimental)	Solubility in water (mg/mL)	pK <sub>a</sub>
N,N-dimethylbiguanidine	-2.64	-0.5	2.25	12.4
N-methylbiguanidine	-2.85	-	3.85	-

Solute – stationary phase interactions are based primarily on  $\pi$ - $\pi$  interactions between  $-C=N-$  double bond from solute molecules and cyano group of the stationary phase. On the other hand, the major interactions are supposed to occur between dipole of cyano group in stationary phase ( $-C\equiv N^{\delta-}$ ) and different ions from mobile phase, such as  $H_3O^+$  and  $H_2N^+$  from solutes (denoted by Bg). The main equilibria occurring during the retention process can be summarized as following:



Although the eq. (2) can be more favorable owing to the stronger interactions and higher value of pK<sub>a</sub> of biguanidine than water, the high content

of water in mobile phase may influence these equilibria. The equilibrium (1) is thus influenced strongly by the pH of the aqueous component in mobile phase, a supposition sustained by the experimental influence of this parameter (given in Fig. 2).

Another explanation of the unusual behavior of N,N-dimethylbiguanidine and N-methylbiguanidine could rely on a reversed-phase mechanism which is denaturated by the specific hydrophobicity/solubility balance of the considered solutes. The hydrophobicity/solubility balance of a solute is the primary parameter which governs its retention in RP-LC. In the specific case of biguanidines this balance can be very much displaced in favour of the solubility parameter (water solubility) according to data from Table 2. The negative values for the hydrophobicity parameter log P and the high water solubility

constants show that these two compounds should have a poor interaction with the stationary phase

and an increased preference for an aqueous mobile phase.

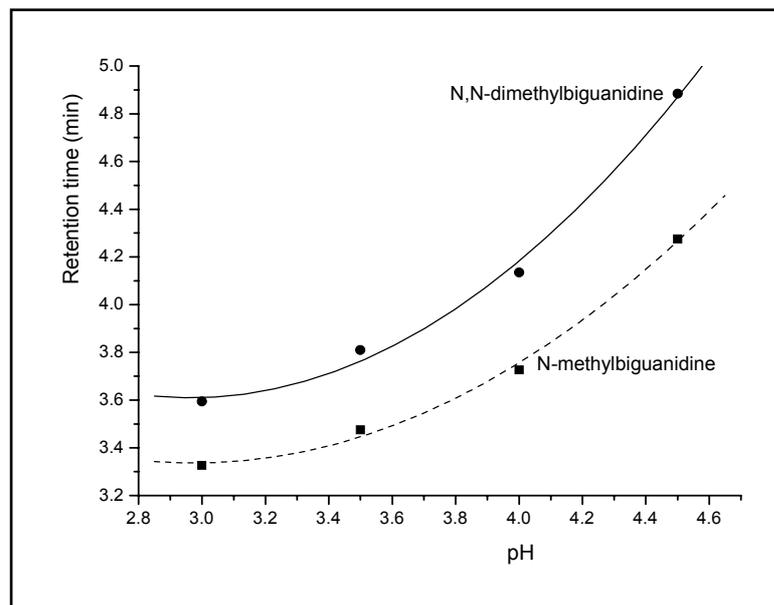


Fig. 2 – Dependence of the retention time values for studied biguanidines on pH of the mobile phase (using acetonitrile as organic modifier).

## CONCLUSIONS

The retention study of two biguanidines (N,N-dimethylbiguanidine and N-methylbiguanidine) carried out on a cyano based stationary phase revealed an exponential functional dependence of the retention of target solutes on the mobile phase in accordance to a normal-phase mechanism: the retention time for both biguanidines increased with the increase of the organic modifier content (methanol, or acetonitrile). Ion-dipole interactions between different species and cyano group in stationary phase are the only explanation for this unusual retention behavior of these compounds on this kind of stationary phase.

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