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Determination of (*R*,*R*)-glycopyrronium bromide and its related impurities by ion-pair HPLC

Dedicated to Professor Wilhelm Fleischhacker with the best wishes on occasion of his 75th birthday

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A simple, rapid and specific ion-pair HPLC method for the determination of (*R*,*R*)-glycopyrronium bromide and its related impurities is presented, and parameters affecting the chromatographic properties of these compounds are discussed. Optimal analyte separation was achieved on base deactivated Nucleosil at 40 °C, using phosphate buffer pH 2.30 with sodium-1-decanesulfonate (0.01 M)/methanol (35/65; v/v) as eluent for isocratic elution at a flow rate 1ml · min^{-1}. The analytical assay was validated according to international guidelines. The method is suitable for in-process control and as stability indicating assay.

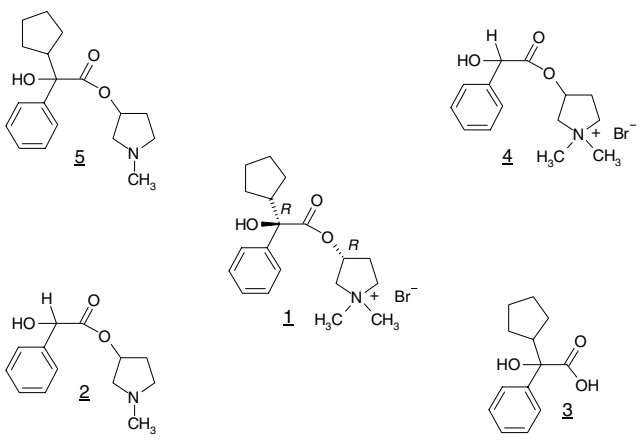
1. Introduction

3-[(Cyclopentylhydroxyphenylacetyl)oxy]-1,1-dimethyl-pyrrolidinium bromide (INN: glycopyrronium bromide; market as a racemic drug, often under the USAN-term glycopyrrolate) belongs to the synthetic anticholinergic agents, a class of drugs which competitively antagonise the effects of acetylcholine competing with this neurohormone for cholinergic receptor sites (Rama Sastry 1996). Consequently, the drug has been investigated for the management of chronic obstructive pulmonary disease and bronchial asthma (Cydulka and Emerman 1995; Gilman et al. 1990; Hansel 2005; Villetti et al. 2006), hyperhydrosis (Wohlrab 2003), urine storage failure (Levin and Wein 1982), treatment of allergic rhinitis (Weinstein and Weinstein 2000), management of peptic ulcer (Miettinen et al. 1985) and as postoperative medication (Biwas et al. 2002). In order to increase muscarinic receptor subtype selectivity and to abolish unwanted side effects, enantiomerically pure (*R*,*R*)-glycopyrronium bromide has been designed (Czeche et al. 1997; Noe et al. 1999). A new, improved route of (*R*,*R*)-glycopyrronium bromide synthesis has been developed in our group (Noe and Walter 2002), consequently appropriate methods to determine its potential impurities and to monitor the reaction process (Scheme) are needed. These analytical steps are important, especially since (*R*,*R*)-glycopyrronium bromide is currently clinically developed for the treatment of chronic obstructive pulmonary disease (COPD) by MEDA Pharma (Guera et al. 2006).

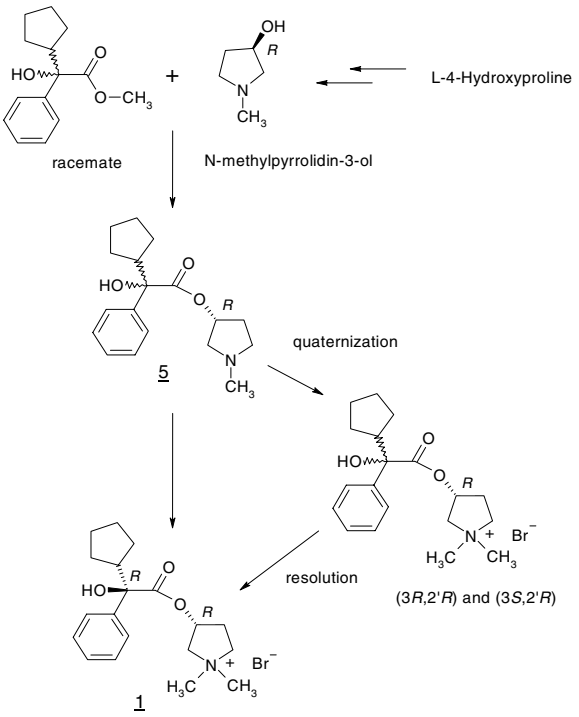
The final intermediate **5** as tertiary amine easily might pass the blood brain barrier and induce unwanted effects. Therefore, special emphasis should be placed on monitoring this potential impurity. Impurity **3** can result as decomposition product of (*R*,*R*)-glycopyrronium after hydrolytic cleavage of the ester functionality, and represents a biotransformation product of active compound as well.

Previously reported analytical techniques for determination of glycopyrronium bromide were based on HPLC/MS (Matassa et al. 1992), GC/MS (Murray et al. 1984), CE/MS (Tang et al. 2001); high-flow ion spray HPLC/MS (Hopfgartner et al. 1993); normal and reverse phase TLC (Ojanpera et al. 1991); and spectrophotometry (Ebeid et al. 1986). The USP 25 monograph "glycopyrrolate injection" employs an RP-HPLC assay using sodium-1-pentanesulfonate as an ion-pairing agent (USP 25th Ed. 1995). Chromatography with β-cyclodextrine as chiral stationary phase has been proposed for the determination of glycopyrronium bromide diastereomers (Demian and Gripshover 1990).

Investigations aimed at the determination of (*R*,*R*)-glycopyrronium bromide and its related impurities have not



Scheme



been published hitherto. The purpose of the present study was to develop a simple, rapid, and sensitive method for simultaneous determination of (*R*,*R*)-glycopyrronium bromide and its potential impurities which arise during the synthesis process, and as a stability indicating assay with respect to degradation product **3**.

2. Investigations, results and discussion

2.1. Optimization of the chromatographic conditions

Considering the structure of the polar and basic analytes severe peak tailing could be presumed when using unmodified RP phases, therefore a base deactivated end-capped stationary phase was included in the investigation. Initial studies were performed with two types of mobile phase modifiers: acetonitrile and methanol. Acetonitrile, due to its high elutive power, in all cases provided very fast elution and poor selectivity for all compounds studied. Therefore, all subsequent studies were performed employing methanol. As aqueous component of the eluent ammonium acetate buffer, 20 mM, pH 4.00; 4.50; 5.00 and 6.00, and phosphate buffer, pH 2.30, respectively, were tested for their suitability. The influence of the addition of ion pair reagents was studied as well as different column temperatures.

2.1.1. Eluent composition

Ammonium acetate buffer system pH 4.00; 4.50; 5.00; 6.00

Initial experiments indicated that the shape of peaks from (*R*,*R*)-glycopyrronium bromide and impurity **5** result as highly asymmetric, therefore, utilization of an ion pair additive was considered. Variations of the aqueous component of the eluent with respect to pH and to type of the ion pairing reagent proved that chromatographic retention

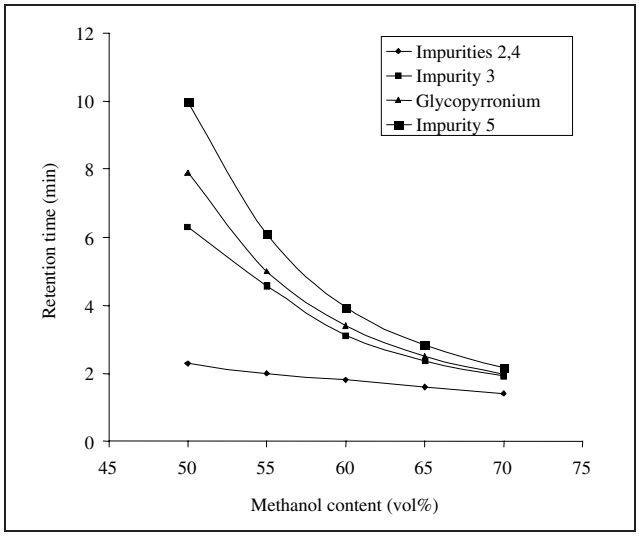


Fig. 1: Retension times of 1, 2, 3, 4 and 5 at different methanol content. Mobile phase: methanol (50–70%) : ammonium acetate buffer, 20 mM, pH 4.00; sodium pentanesulfonate (0.01 M)

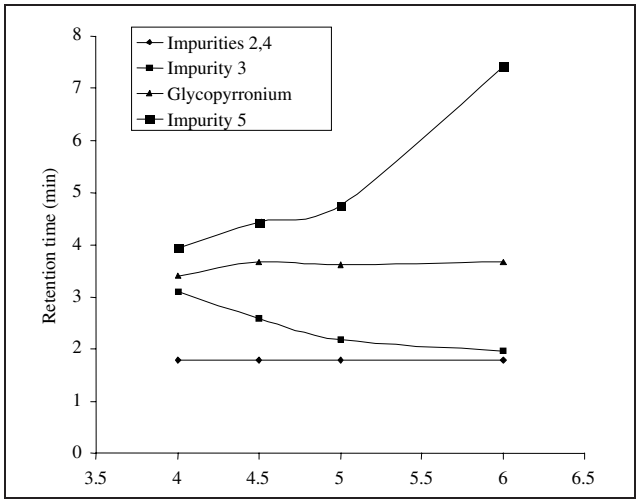


Fig. 2: Influence of the pH value on the retention of 1, 2, 3, 4 and 5. Mobile phase: methanol : ammonium acetate buffer, 20 mM, 60 : 40 (V/V); sodium-1-pentanesulfonate (0.01 M)

of (*R*,*R*)-glycopyrronium bromide was not affected by pH change but mainly by the lipophilicity of the ion pair created with the anionic reagent and/or the ion-exchange process involved with the sodium ions from sorbed reagent molecules at the stationary phase. Retention of compound **5** was strongly dependent from the ion-pair reagent type as well as the pH value. Naturally the influence of the ion pairing on the chromatographic behaviour of **5** was more distinct at lower pH, at pH values between 4.0 and 6.0 both factors were equally significant (Fig. 1). As evident, the retention of mandelic acid derivative **3** was affected by the pH value only (Fig. 2). The ion pairing with sodium-1-pentanesulfonate 0.01 M lead to separation of the critical peaks of **1** and **5**, nevertheless the peak symmetry was unsatisfactory. Though reasonable capacity factors for compounds 1 and 5 were found with eluents consisting of methanol: ammonium acetate; 60 : 40 (v/v), at pH 4.00 and 6.00, respectively, containing sodium-1-decanesulfonate, the assymetry factor did not satisfy. Mandelic acid derivative **3** eluted very fast ($k' < 2$) under these conditions.

Phosphate buffer pH 2.30

At all ratios of eluent components the cationic derivatives eluted very early without the use of ion pair reagents, peak tailing was observed especially with compounds **1** and **5**. Impurity **3** naturally had a high retention time. In order to allow isocratic elution, ion pair reagents were used to selectively increase the retention of the cationic compounds relative to impurity **3**. Sodium-1-pentanesulfonate increased the retention of all ionic and ionizable compounds, compound **3** naturally was not affected (Fig. 3). At higher methanol percentages critical peaks (**1** and impurity **5**) were not efficiently resolved, lower amounts of methanol led to unsuitably long retention times. Aiming for fast analysis with appropriate retention range, possibly $2 \leq k' \leq 5$, ion-pairing reagents of higher molecular size as sodium-1-dodecanesulfonate and sodium-1-decanesulfonate were considered, the latter proved to be better suitable (Fig. 4). At 65% methanol, this ion-pair reagent afforded baseline resolution for **1**, **3** and **5**, impurities **2** and **4** co-eluted.

Summarizing, it can be stated that lower pH of the eluent and higher modifier percentages together with a more hydrophobic ion pairing agent attained good resolution combined with satisfactory peak shapes. Accordingly, methanol/phosphate buffer pH 2.30 with sodium-1-decanesulfonate 0.01 M (65 : 35, v/v) was selected as the most suitable mobile phase solution which was used for further optimization studies.

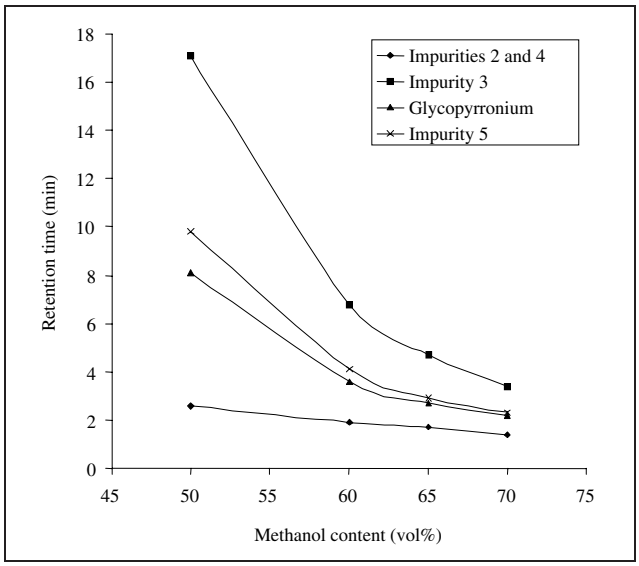


Fig. 3: Retension times of 1, 2, 3, 4 and 5 at different methanol content. Mobile phase: methanol (50–70%) : phosphate buffer, pH 2.30; sodium-1-pentanesulfonate (0.01 M)

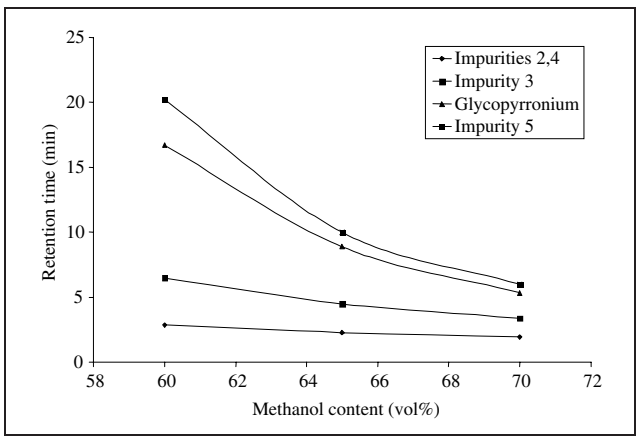


Fig. 4: Retension times of 1, 2, 3, 4 and 5 at different methanol content. Mobile phase: methanol (60–70%) : phosphate buffer, pH 2.30; sodium-1-decanesulfonate (0.01 M)

Table 1: Influence of stationary phase on peak asymmetry. Mobile phase: methanol : ammonium acetate buffer 20 mM, pH 4.00, 70 : 30 (v/v); sodium-1-pentanesulfonate (0.01 M)

Column type	Asymmetry factor	
	(*R,R*)-Glycopyrronium	Impurity **5**
Nucleosil 100-5C-18 HD	2.14	1.9
LiChrospher 100-RP 18	2.87	3.3

Table 2: Influence of stationary phase on peak asymmetry. Mobile phase: methanol : phosphate buffer, pH 2.30, 65 : 35 (v/v); sodium-1-decanesulfonate (0.01 M)

Column type	Asymmetry factor	
	(*R,R*)-Glycopyrronium	Impurity **5**
Nucleosil 100-5C-18 HD	1.22	1.31
LiChrospher 100-RP 18	1.99	1.91

2.1.2. Stationary phase

A common octadecyl silica, LiChrospher 100-RP 18 column was compared with a base deactivated Nucleosil 100-5C-18 HD column. As expected, the reduction of the influence of the free silanol groups considerably improved peak shapes. The selection of a lower eluent pH led to additional improvement of the peak symmetry (Tables 1 and 2).

2.1.3. Column temperature

The influence of temperature was studied in the range 20 to 60 °C. A temperature of 40 °C resulted as optimal affording a short analysis time (less than 7 min) and adequate resolution between critical peaks of glycopyrronium bromide **1** and impurity **5** ($Rs > 2$). All ensuing analyses for the quantification of impurities thereafter were carried out at 40 °C (Table 3 and Fig.5).

2.2. Method validation

This method has been substantiated with the determination of specificity, linearity, precision, accuracy, limits of detection and limits of quantitation, according ICH guidelines (ICH Harmonised Tripartite Guidelines 1994, 1996). The

Table 3: Influence of column temperature in chromatographic retention. (Mobile phase : methanol : phosphate buffer, pH 2.30, 65 : 35 (v/v); sodium-1-decanesulfonate (0.01 M)

Temp. °C	Retention time, min		
	3	**1**	**5**
20	4.69	9.15	11.70
25	4.18	7.30	8.66
30	3.91	6.17	7.29
40	3.56	5.00	6.00
60	2.80	3.30	3.40

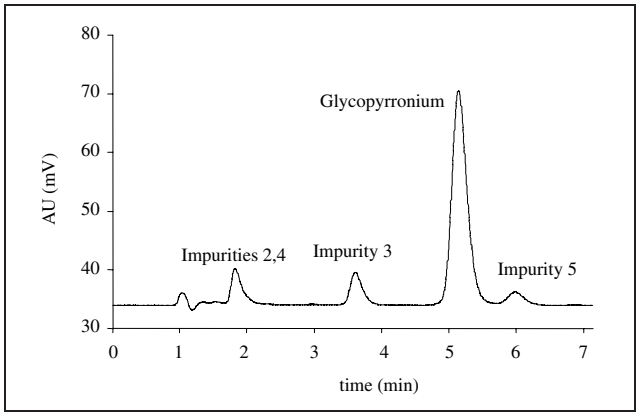


Fig. 5: HPLC of (*R*,*R*)-glycopyrronium bromide 100 μg ml^{-1}, spiked with 5% impurities (mobile phase: methanol : phosphate buffer, pH 2.30, 65 : 35 (v/v); sodium-1-decanesulfonate 0.01 M); Column temperature, 40 °C)

selectivity of the assay with respect to (*R*,*R*)-glycopyrronium bromide was assessed with injecting blank solution and (*R*,*R*)-glycopyrronium bromide, and for impurities in spiking experiments. No other signals appeared at the retention times of (*R*,*R*)-glycopyrronium bromide and its impurities.

Linear relationship was observed between detector signals as a function of concentration of glycopyrronium **1**, compounds **3** and **5**. Regression line equations with their corresponding errors and squared correlation coefficients are presented in Table 4. For impurity testing, spiking experiments with the known impurities have been used for the evaluation of accuracy, which is reported as percentage of recovery by the assay of known added amounts of compound **3** and compound **5**. At each study, triplicates of samples are evaluated. The mean of triplicates was expressed as recovery %. For glycopyrronium and compounds **3** and **5** repeatability was evaluated by expressing the relative standard deviation of triplicate injections of samples. Intermediate precision was evaluated for impurities 3 and 5. Accuracy (recovery values) at two different analysis days has been used to indicate the intermediate precision of method (Table 5). LOD and LOQ were assessed on the basis of the standard deviation of the response and the slope of calibration curve (Table 6 and Fig. 6).

Table 6: Limits of detection and limits of quantitation

	LOD, μg · ml^{-1}	LOQ, μg · ml^{-1}
(*R*,*R*)-Glycopyrronium bromide	3.5×10^{-3}	1.0×10^{-2}
Impurity **3**	1.5×10^{-3}	4.5×10^{-3}
Impurity **5**	2.7×10^{-3}	8.2×10^{-3}

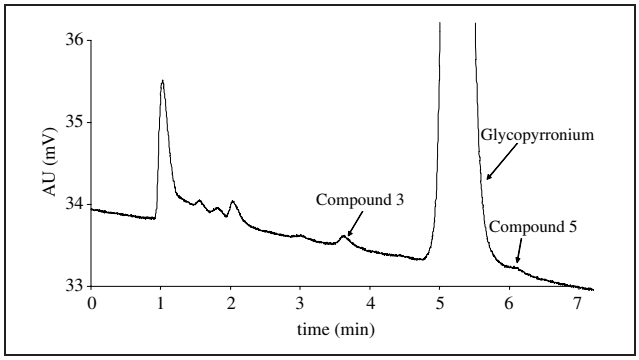


Fig. 6: (*R*,*R*)-Glycopyrronium bromide (100 μg ml^{-1}) spiked with 0.1% impurities

3. Experimental

3.1. Materials and reagents

(*R*,*R*)-Glycopyrronium bromide; compounds **2**, **3**, **4** and **5** were synthesized in our laboratory. Methanol and acetonitrile, HPLC grade, were purchased from Acros Organics, Belgium. Sodium-1-pentanesulfonate and sodium-1-decanesulfonate, analytical grade, Fluka Chemica GmbH, Switzerland; sodium-1-dodecanesulfonate, Merck, Germany. Phosphoric acid, Mallinkrodt Baker B.V. Holland, ammonium acetate, Riedel-De Haen AG, Hannover Germany, potassium dihydrogenphosphate, E. Merck, Darmstadt, Germany. HPLC grade water was purchased from Fisher Chemicals, UK, ammonium hydroxide and acetic acid from E. Merck Darmstadt, Germany.

3.2. Standard and sample preparation

A stock solution of (*R*,*R*)-glycopyrronium bromide (1 mg · ml^{-1}) was prepared by dissolving 100 mg (*R*,*R*)-glycopyrronium bromide standard in mobile phase (volumetric flask 100 ml). This solution was further diluted with mobile phase to result in the appropriate concentrations to establish the calibration function. The solution of highest concentration, 100 μg · ml^{-1}, was used as reference standard in spiking experiments with solutions of impurities (compounds **2**, **3**, **4** and **5**). Stock solutions of impurities (0.2 mg · ml^{-1}, compound **3** and 0.53 mg · ml^{-1}, compound **5**) were prepared dissolving accurately weighted substances in mobile phase and sub-

Table 4: Linearity parameters for compounds 1, 3, and 5

	Regression line Eq.	Sr	Sa	Sb	R^2
(*R*,*R*)-Glycopyrronium	A = 6095.9XC − 754.96	1750.41	21.10	967.7	1.00
Impurity **3**	A = 14208.13XC + 1487.80	2334.47	362.43	1487.0	0.9981
Impurity **5**	A = 7863.7XC − 225.93	634.56	35.54	344.3	0.9999

Table 5: Accuracy and repeatability of impurities 3 and 5

Impurity level, %	Impurity **3**						Impurity **5**					
	t_R, min		Concentration				t_R, min		Concentration			
	min	RSD %	Observed μg ± CI	RSD, % n = 3	% R	% R Second day	min	RSD %	Observed μg ± CI	RSD, % n = 3	% R	% R Second day
5.0	3.61	0	4.92 ± 0.13	1.1	98.4	96.0	5.99	0.08	5.24 ± 0.13	1.0	104.6	86.4
2.5	3.60	0.14	2.52 ± 0.12	2.05	100.2	97.6	5.95	0.60	2.68 ± 0.36	5.3	106.9	96.0
0.5	3.59	0.14	0.4 ± 0.02	2.5	80.0	88.0	5.88	0.14	0.6 ± 0.035	2.3	120.0	109.0
0.25	3.60	0.22	0.28* ± 0.05	7.5	111.0	108.0	5.84	0.41	0.29 ± 0.03	4.5	116.8	112.0
0.1	3.71	1.74	0.108* ± 0.03	11.5	108.0	116.0	6.24	3.5	0.124 ± 0.08	26	124.0	120.0
0.05	3.55	0.73	0.056* ± 0.006	0.41	112.0	–	–	–	–	–	–	–

* Recovery values and respective areas were calculated directly on the basis of (*R*,*R*)-glycopyrronium bromide peaks

sequently diluting with the same solvent to 5; 2.5; 0.5; 0.25; 0.1 and 0.05%, relative to the amount of parent compound (*R*,*R*)-glycopyrronium bromide, 100 $\mu g \cdot ml^{-1}$.
For construction of the calibration functions of impurities **3** and **5** their stock solutions were diluted with mobile phase to obtain appropriate concentrations.

3.3. Instrumentation

The HPLC consisted of dual pump LC-10AD, Shimadzu; Rheodyne Injector Cotati, California USA, SPD-10A Shimadzu UV-VIS detector, and column thermostat SpH 99, Holland. Nucleosil 100-5 C-18 HD, octadecyl base deactivated (5 μm, 125 × 4 mm I.D) and LiChrospher 100-RP 18, octadecyl, (5 μm, 125 × 4 mm I.D) columns were used.

3.4. Mobile phase and LC conditions

Phosphate buffer pH 2.30 was prepared dissolving 4.80 g phosphoric acid 85% and 6.66 g potassium dihydrogenphosphate in deionized water, adjusting to pH 2.30 with the phosphoric acid 85%, after that 1000 ml volume was accurately obtained adding deionised water. Ammonium acetate buffer, 20 mM was prepared dissolving 1.542 g of ammonium acetate in deionised water, adjusting the pH to the respective value with concentrated acetic acid or ammonia, deionized water was added to give 1000 ml.
Accurately weighed ion-pairing reagents were dissolved in aqueous portions of mobile phase which was further mixed with organic phase and this solution was finally filtered through a Millipore 0.5 μm filter and degassed in Ultrasonic Bath Starsonic 60 for 10 min before each run.
The chromatographic runs were performed in isocratic mode, mobile phase flow rate 1.0 $ml \cdot min^{-1}$, and volume of injection 20 μl.

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