HPLC ENANTIOSEPARATION OF PHENYLCARBAMIC ACID DERIVATIVES BY USING MACROCYCLIC CHIRAL STATIONARY PHASES

KATARÍNA HROBOŇOVÁ[1], JOZEF LEHOTAY[2], JOZEF ČIŽMÁRIK[3]

[1]*Institute of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, SK-812 37 Bratislava, Slovak Republic (katarina.hrobonova@stuba.sk)*
[2]*Faculty of Natural Sciences, University of ss.Cyril and Methodius, J. Herdu 2, SK-917 01 Trnava, Slovak Republic*
[3]*Comenius University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Odbojárov 10, SK-832 32 Bratislava, Slovak Republic*

Abstract: The HPLC by using chiral stationary phases based on macrocyclic antibiotics, dimethylphenyl carbamate cyklofructan 7 and β-cyclodextrin in terms of polar-organic separation mode (mobile phase methanol/acetonitrile/acetic acid/triethylamine) were used for enantioseparation of alkoxy derivatives of phenylcarbamic acid. The effect of the analyte structures on the efficiency of enantioseparation was investigated. The most suitable stationary phase was teicoplanin aglycone, where the separations of the enantiomers were obtained (the resolution value from 0.65 to 2.90, depending on the structure of the analyte). Significant effect on the resolution of the enantiomers has position of alkoxy substituent in the hydrophobic part of the molecule. The enantiorecognition was achieved for 3-alkoxysubstituted derivatives.

Key words: HPLC, chiral stationary phase, structure of the analyte, esters of phenylcarbamic acid

1. Introduction

Chiral separation is based on the ability of the chiral selector to form diastereoisomeric complexes preferably with one of the enantiomer of a chiral analyte. The higher affinity differences of selectors to enantiomers lead to greater efficiency of the chiral recognition. The formation of stable complexes between chiral selector and enantiomer, and their structural complementarity are the main requirement for selection of suitable chiral selector (ZHANG *et al*., 1979).

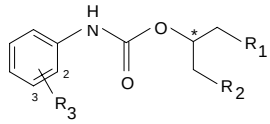
Chromatographic techniques, particularly high performance liquid chromatography (HPLC) have become the most used in the separation of enantiomers due to the availability of a large number of stationary phases containing different chiral selectors, high reproducibility, and often universality. The most challenging aspect in the development of chiral HPLC method is to select the chiral stationary phase (CSP). The many of available CSPs provides a more "degrees of freedom" for the selection of a suitable stationary phase. On the other hand, a testing of all available CSP for the analysis of all chiral molecules is time-consuming. The most commonly used are CSPs based on saccharides and macrocyclic antibiotic chiral selectors due to their multi-functionality and the ability to use a several separation modes (reversed-phase,

DOI 10.1515/nbec-2016-0002

normal-phase, and polar-organic phase mode). The main requirement for newly developed CSP is particularly high enantioseparation efficiency for a wide range of structurally different compounds, or an increase in separation efficiency compared with existing CSP (BEESLEY, 2011).

The racemic compounds used in this study are derivatives of alkoxysubstituented esters of phenylcarbamic acid which are potential local anesthetics (Fig. 1). Chemically, they are weak bases which molecules contain lipophilic and hydrophilic part interlinked with connecting chain. The various CSPs can be used for HPLC enantioseparation of local anesthetics of phenylcarbamic acid type. Normal-phase separation mode (NP) was suitable for enantioseparations on polysaccharide (ZHANG *et al*., 2005) and cyclofructan CSPs (SUN *et al*., 2009). Reversed-phase mode (RP) was especially suitable for cyclodextrin-based (HROBOŇOVÁ *et al*., 2003) and polysaccharide-based CSPs (PENG *et al*., 2010). Polar-organic (PO) mode was mostly used for separations on chiral stationary phases based on macrocyclic antibiotics (ROJKOVIČOVÁ *et al*., 2003; 2005). The selection of separation mode related with the solubility of analytes and type of matrix.

The goal of the work was to separate the enantiomers of alkoxyphenylcarbamic acid derivatives by HPLC using various types of chiral stationary phases in terms of polar-organic separation mode. Chiral stationary phases tested were based on I) the macrocyclic antibiotics (teicoplanin and teicoplanin aglycone); II) β-cyclodextrin and III) cyclofructan (3,5-dimethylphenyl carbamate of cyclofructan 7). All types of tested columns are multimodal in relation to the chromatographic conditions (NP, RP, PO separation mode). The influence of the structure of analytes on the efficiency of enantioseparation on different types of CSPs was studied as a contribution to the systematic approach to selection of suitable stationary phase.



Nr.	R_1	R_2	R_3	Nr.	R_1	R_2	R_3
1		OCH_3	2-OC_5H_{11}	15		OCH_3	2-OC_4H_9
2			3-OC_5H_{11}	16			3-OC_4H_9
3		OC_2H_5	2-OC_4H_9	17		OC_2H_5	2-OC_4H_9
4			2-OC_5H_{11}	18			2-OC_5H_{11}
5			2-OC_6H_{13}	19			2-OC_6H_{13}
6			3-OC_4H_9	20			3-OC_4H_9
7			3-OC_5H_{11}	21			3-OC_5H_{11}
8			3-OC_6H_{13}	22			3-OC_6H_{13}
9		OC_3H_7	2-OC_4H_9	23		OC_3H_7	2-OC_4H_9
10			2-OC_5H_{11}	24			2-OC_6H_{13}
11			2-OC_6H_{13}	25			3-OC_4H_9
12			3-OC_4H_9	26			3-OC_6H_{13}
13			3-OC_5H_{11}				
14			3-OC_6H_{13}				

Fig. 1. Chemical structures of alkoxysubstituted derivatives of phenylcarbamic acid under study.

2. Material and methods

2.1 Materials

The racemates of alkoxyphenylcarbamic acid derivatives were synthesized according the literature (BÚČIOVÁ *et al*., 1987; BÚČIOVÁ *et al*., 1991; BÚČIOVÁ *et al*., 1992). The stock solutions were prepared by dissolving the substance in the mobile phase (concentration of 1.0 $mg \cdot mL^{-1}$, storage at 4 °C).

Methanol and acetonitrile (HPLC grade, JT Baker), triethylamine (for synthesis, Merck) and glacial acetic acid (100 %, Merck) were used for the preparation of the mobile phase.

2.2 HPLC instrumentation and conditions

The liquid chromatograph Agilent 1100 series, consisting of a binary high pressure pump, injection valve Rheodyne, column thermostat, and diode array detector was used. Separation of the enantiomers were performed on Chirobiotic T (Astec), Chirobiotic TAG (Astec), 3,5-dimethylphenylcarbamate cyclofructan 7 (Astec), β-cyclodextrin - ChiraDex (Merck) (4×250 mm I.D., 5 μm) chiral chromatographic columns. The mixture of acetonitrile/methanol/acetic acid/triethylamine (20/80/0.3/0.2 v/v/v/v) was used as mobile phase. The flow rate was 0.8 $min \cdot mL^{-1}$, the injection volume 20 μL. The column temperature was maintained at 22 °C. The chromatograms were recorded at wavelength 240 nm.

All measurements were done in three replicates. The retention factor (k) values of first and second eluted enantiomer were calculated as the ratio of differences between retention time of enantiomer (t_R) and dead time (t_M) to dead time. The selectivity factor were calculated as the ratio of retention factors of second and first eluted enantiomer ($\alpha = k_2/k_1$). The resolution values (R_S) of enantiomeric forms were calculated by ratio of difference between retention times of enantiomers (t_{R1}, t_{R2}) to sum of peak widths at half peak height ($w_{05,1}$, $w_{05,2}$) as follow: $R_S = 1.18 * (t_{R2} - t_{R1}) / (w_{05,1} + w_{05,2})$.

3. Results and discussion

3.1 Chromatographic conditions

In the previous work (HROBOŇOVÁ *et al*., 2003), the β-cyclodextrin CSP in reversed-phase separation mode was used for separation of selected compounds. The mobile phase was a mixture of acetonitrile and 0.1 $mol \cdot L^{-1}$ sodium acetate, pH=5.2 (88/12, v/v). The enatioseparation only 2-alkoxysubstituted derivatives was achieved (the referred chromatographic conditions were not suitable for enantioseparation of 3-alkoxysubstituted derivatives, $R_S \sim 0$). The resolution values (R_S) ranged from 0.6 to 1.2, depending on the length of the alkoxysubstituent (C4 – C7). The highest resolution values were obtained for compounds with hexyloxy substituent, which, in terms of biological activity, have the highest local anesthetic activity. Dominant types of interactions responsible for the enantiorecognition were steric effects influenced by

the length and position of the alkoxy chain. (HROBOŇOVÁ *et al*., 2003) The mechanism of enantiomeric recognition on β-cyclodextrin CSP in RP mode was based on the formation of inclusion complexes driven by the hydrophobic interactions between the analytes and the cyclodextrin. The hydrophobic part of the analyte penetrated into the cyclodextrin cavity and displaced the molecules of solvent. Inside the cavity the complex is stabilized by Van der Waals interactions optionally by other hydrophilic interactions (hydrogen bonding, dipole-dipole interactions) with the hydroxyl groups of the cyclodextrin skeleton. The π-π interactions can be dominant for aromatic compounds. The inclusion into the cavity is related to the size of the molecule. (LÄMMERHOFER, 2010). Kopecky and coworkers in the micellization study of the local anesthetic drug carbisocaine present the inclusion of the carbisocaine C7 alkyl chain into the cyclodextrin cavity and the role of the hydrophobic interaction in complexation with β-cyclodextrin in aqueous solution (KOPECKY *et al*., 2002).

The present work was orientated on separation of the enantiomers of the whole group of substances (2-positioned and 3-positioned alkoxyderivatives), and therefore other types of stationary and mobile phases were tested. The polar-organic separation mode is most commonly used for CSP based on macrocycles. The mobile phase consists of a mixture of polar organic solvents (methanol, acetonitrile) with the addition of ionic modifiers (acids and bases). Methanol molecules are able to participate in the formation of hydrogen bonds, acetonitrile contributes to dipol interactions, which influenced the enantioseparation (value R_S). The acid/base ratio in the mobile phase influenced also the ionization of groups (amino, hydroxyl, carboxyl) in stationary phases and thus to affect the ion interaction with ionizable groups of alkoxyphenylcarbamic acid derivatives under study (quaternary nitrogen in piperidine or pyrrolidine heterocycle of phenylcarbamic acid derivative molecule) (MERIČKO *et al*., 2008).

The separation of the enantiomers of selected analytes was realized by using a methanol/acetonitrile/acetic acid/triethylamine 20/80/0.3/0.2 (v/v/v/v) as mobile phase at 22 °C**.** The ratio of the concentrations of ionic modifiers in the mobile phase, which is most effective for achieving the separation was 3:2 (glacial acetic acid:triethylamine). The acidic nature of the mobile phase supports the ionization of amino groups in the molecules of separated compounds and the stationary phase. It can be assumed that the charge interactions had a positive influence on enantioseparation.

3.2 Comparison of enantioseparation on different CSPs

The stationary phases with different types of chiral selectors were used for enantioseparations of alkoxyphenylcarbamic acids derivatives. The aim was to compare the selectivity and efficiency.

In the case PO mode is the inside of the β-cyclodextrin cavity blocked with the molecules of solvent of mobile phase, and therefore it is not available for the formation of inclusion complexes with molecules of the analyte. The hydrophilic interactions between the molecules of analyte and the polar surface of the β-cyclodextrin skeleton are supported. The enantioselectivity can be influenced by the

strength of polar interactions (hydrogen bonding, dipole-dipole interactions). (LÄMMERHOFER, 2010).

The variety of stereoselective interactions (hydrogen bonding, π-π, electrostatic interactions, hydrophobic, steric or repulsive interactions, formation of inclusion complexes) of macrocyclic antibiotic (teicoplanin, teicoplanin aglycone) CSPs related to the presence of many stereogenic centers, the heterogenity of functional groups and structural specificity of different types of macrocyclic antibiotic molecules. This group of CSP is predominantly used for the separation of enantiomers containing ionic groups at/or near the stereogenic center (WANG, 2000).

Cyclofructan 7 (CF7) is macrocyclic oligosaccharide consisting of seven β-linked fructofuranose units. The fructofuranose units are distributed internally or externally around the "crown" ether skeleton. The CF connected hydrophilic and hydrophobic groups. In particular, derivatized (aliphatic or aromatic) cyclofructan CSPs are effective for enantiomer separation of compounds with amine functional groups. Aromatically derivatized CF CSPs provide effective separation based on the use of hydrogen, π-π and dipole-dipole interactions supplemented by steric effects (SUN *et al.*, 2009).

Table 1 summarizes the retention factor, resolution, and selectivity factor for the separation of selected phenylcarbamic acid derivatives by using the four types of CSPs. Teicoplanin and teicoplanin aglycone CSPs in PO separation mode were appropriate for separation of enantiomers of 3-alkoxysubstituted derivatives while the 2-alkoxysubstituted derivatives were not separated. Highest selectivity factors were achieved generally by using the CSP without saccharide units in the molecule (Teicoplanin aglycone). The retention factors of the enantiomers ranged from1.29 – 6.30 and the resolution value from 1.09 to 2.90. The values of retention factors achieved on teicoplanin CSP were lower (1.03 – 4.31) in comparison with teicoplanin aglycone CSP. Resolution values ranged from 1.72 to 2.75. The most effective enantioseparation (*R*S = 2.90 for teicoplanin aglycone CSP, *R*S = 2.75 for teicoplanin CSP) was achieved for compound (2) (R1 = pyrrolidine, R2 = -OC3H7, R3 = -OC6H13) as is documented in Fig. 2. The most effective enantioseparation (R_S = 2.90 for teicoplanin aglycone CSP, R_S = 2.75 for teicoplanin CSP) was achieved for compound (2) (R_1 = pyrrolidine, R_2 = -OC_3H_7, R_3 = -OC_6H_{13}). It can be assumed that saccharide units of the native teicoplanin molecule may have negative influence on chiral recognition process of enantiomeric forms. It is probably the effect of steric hindrance (BERTHOD *et al.*, 2000).

By using β-cyclodextrin CSP only the partial separation (R_S = 0.36 and 0.44) of the compounds (2) and (16) (R_2 = -OCH_3) was achieved. Compounds with longer R_2 (ethoxy-, propoxy-) substituents near the stereogenic center were not resolved. This is probably due to steric hindrance and shading of stereogenic center. The values of retention factor obtained on β-cyclodextrin CSP were the lowest (k_1 = 0.08 – 0.38) in comparison with other CSP tested. The reason of poor enantioseparation was probably the unavailability inclusion of analyte molecules into the β-cyclodextrin cavity in the PO separation mode.

The cyclofructan CSP (DMP-CF7) in PO separation mode was not suitable for resolution of phenylcarbamic acid derivatives under study. The retention of analytes

was higher in comparison to cyclodextrin CSP (about two times), but any separation of enantiomers was observed.

Table 1. Chromatographic results (retention factor (k_1), resolution (R_S) and selectivity factor (α)) for alkoxysubstituted derivatives of phenylcarbamic acid obtained on four chiral stationary phases in the polar-organic separation mode.

Nr.	CSP									
	Teicoplanin			**Teicoplanin aglycone**			**β-cyclodextrin**			**DMP -CF7**
	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1
1	2.42			3.37			0.17			0.80
2	3.96	1.14	2.75	5.74	1.25	2.90	0.32	1.01	0.36	0.85
3	2.33			3.69			0.13			0.81
4	2.22			3.20			0.15			0.80
5	2.08			3.14			0.15			0.75
6	3.83	1.14	2.34	6.30	1.20	2.46	0.28			0.80
7	3.77	1.14	2.66	5.52	1.21	2.66	0.29			0.82
8	3.57	1.13	2.69	5.47	1.21	2.87	0.28			0.78
9	2.65			3.43			0.13			0.75
10	2.46			2.90			0.13			0.74
11	2.36			2.76			0.14			0.72
12	4.31	1.13	1.95	5.72	1.22	2.55	0.38			0.74
13	4.17	1.13	1.99	5.36	1.21	2.44	0.38			0.73
14	4.06	1.13	2.17	5.21	1.22	2.52	0.37			0.72
15	1.03			1.57			0.11			0.47
16	2.02	1.12	2.13	3.70	1.16	1.65	0.25	1.03	0.44	0.49
17	1.84			1.75			0.08			0.45
18	0.95			1.61			0.09			0.44
19	0.93			1.53			0.09			0.43
20	1.93	1.10	1.81	3.58	1.14	1.25	0.20			0.47
21	1.67	1.11	1.84	3.47	1.14	1.21	0.21			0.46
22	1.52	1.11	1.97	3.35	1.13	1.20	0.21			0.46
23	1.19			1.42			0.11			0.44
24	1.07			1.29			0.11			0.44
25	1.81	1.11	1.72				0.24			0.42

RSD ≤ 3 %, n = 3; Chromatographic conditions: mobile phase: acetonitrile/methanol/acetic acid/triethylamine (80/20/0.3/0.2 v/v/v/v); column temperature: 22 °C; flow rate: 0.8 min·mL^{-1}; detection: UV 240 nm; t_M determined as the retention time of acetonitrile; t_M(teicoplanin) = 3,81 min; t_M(teicoplanin aglycone) = 3.85 min; t_M(DMP-CF7) = 3.93 min; t_M(β-cyclodextrin) = 3.13 min.

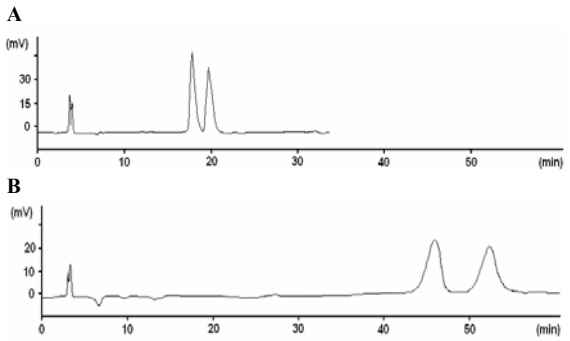


Fig. 2. HPLC chromatograms of compound (2) separated on teicoplanin (A) and teicoplanin aglycone (B) CSPs. Chromatographic conditions: as in Table 1.

3.3 The effect of structure of the analyte on the separation of enantiomers

The chemical structure of analyte has a dominant influence on the enantiomeric recognition. This may affect the availability to the chiral centers and the functional groups of the chiral selector, and the type of interactions responsible for chiral recognition. The work was focused on the investigation of the effect of structure alkoxyphenylcarbamic acid derivatives on efficiency of enantioseparation using macrocyclic antibiotics (teicoplanin, teicoplanin aglycone) CSPs. From the chiral separation point of view the influence of i) the position, ii) length of alkoxy substituent (R_3) in the lipophilic part of the molecule, and iii) the effect of alkoxy substituent (R_2) in the connecting chain of the molecule were evaluated.

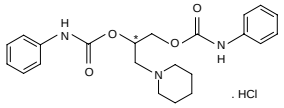
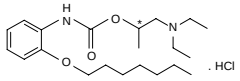
β-cyclodextrin CSP in the RP mode was suitable for the enantioseparation of 2-alkoxysubstituted derivatives, while the derivatives with 3-alkoxysubstitution provided no enantioseparation. The position of alkoxy substituent near stereogenic center was preferred from the reason of molecule compatibility for inclusion into the cyclodextrin cavity and separations results. (HROBOŇOVÁ *et al*., 2003) 4-alkoxysubstituted derivatives exhibit very low local-anesthetic activity and they were not synthesized. Table 1 has documented that the compounds which have a substituent R_3 in the 3-position on the aromatic ring are separated (R_S = 0.96 to 2.90) on glycoprotein (Teicoplanin, Teicoplanin aglycone) CSPs. The substituent near the stereogenic center (2-positioned derivatives) of the separated compound may intervene in the chiral recognition process by steric hindrance, where the substituent shades stereogenic center and blocking possible interaction sites. The values of the retention factors of 3-alkoxy derivatives were about two times higher in comparison with 2-alkoxy derivatives. The separation of enantiomers is slightly affected by non-polar interactions. Increase of lipophilicity of separated compounds (R_3: C4-C6) caused decrease of retention factors and resolution values of the enantiomers on both stationary phases. The hydrophilic part of the molecule contains the nitrogen heterocycle (R_1: piperidino, pyrrolidino). Higher values of resolution and retention factors were achieved for pyrrolidino derivatives on both tested columns. The

piperidino substituent is probably steric hindrance in chiral recognition process. The values of the retention factor and resolution decreased with increase of carbon atoms (C1 – C3) in the R_2 substituent.

3.4 Separation of the local anaesthetics

Diperodon (3-(1-piperidinyl)-1,2-propanediol bis(phenylcarbamate)) and carbisocaine ((2-(heptyloxy)phenyl)-2-(diethylamino)-1-methylethyl ester of carbamic acid) are a local anesthetic drugs used as the hydrochloride salt; applied to the skin for abrasions, irritations, and pruritus and intrarectally for relief of pain from hemorrhoids.(COHEN *et al*., 1977) Table 2 summarised the chromatographic parameters for Carbizocaine and Diperodon enantioseparation on a teicoplanin, teicoplanin aglycone, β-cyclodextrin, and 3,5-dimethylphenyl carbamate cyclofructan 7 CSPs by using the same mobile phase. Even though the retention of Carbisocaine was significantly higher on macrocyclic antibiotic CSPs in PO separation mode, the any enantiorecognition was observed. Carbizocaine is the the derivative of phenylcarbamic acid with heptyloxysubstitution in the 2-position on the aromatic ring (Table 2). CSPs and mobile phase used are not suitable for separate the enantiomers of compounds with substitution near the stereogenic center, in comparison to 3-positioned derivatives. Diperodon enantiomers were separated on CSPs based on macrocyclic antibiotics, where the highest value of resolution (R_S = 1.79) was achieved using teicoplanin aglycone column. Obtained results were compatible with thus obtained previously (part 3.3). Figure 3 shows the chromatogram of the enantioselective separation of Diperodon.

Table 2. Chromatographic results for Carbisocaine and Diperodon obtained on four chiral stationary phases in the polar-organic separation mode.

	CSP	k_1	α	R_S
Diperodon  . HCl	Teicoplanin	0.99	1.14	1.01
	Teicoplanin aglycone	2.86	1.29	1.79
	β-cyclodextrin	0.85	1.0	-
	DMP-CF7	0.11	1.0	-
Carbizocaine  . HCl	Teicoplanin	2.11	1.0	-
	Teicoplanin aglycone	2.32	1.0	-
	β-cyclodextrin	0.16	1.0	-
	DMP-CF7	0.76	1.0	-

RSD ≤ 3%; n = 3, Chromatographic conditions as in Table 1.

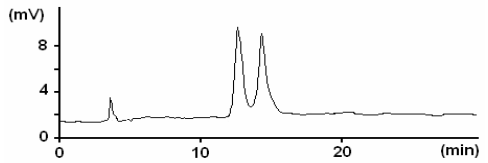


Fig. 3. HPLC separation of Diperodon enantiomers on teicoplanin aglycone CSP. Chromatographic conditions: as in Table 1.

4. Conclusions

HPLC enantioseparation of potential local anesthetics of alkoxysubstituted phenylcarbamic acid type was achieved in terms of a polar-organic separation mode using macrocyclic antibiotic CSP (teicoplanin, teicoplanin aglycone), in contrast to the β-cyclodextrin and 3,5-dimethylphenylcarbamate cyclofructan 7 based chiral stationary phases. Comparison of the results shows that while the position of the alkoxy substituent near stereogenic center significantly affected retention and recognition of enantiomers, the length of the substituent has only slightly effect. Enantiomeric separation was achieved for the 3-alkoxy substituted derivatives. Alkoxy substituent in the connecting chain of molecule had not significant impact on the separation of the enantiomers. Increasing of nitrogen heterocycle (piperidine, pyrrolidine) caused decrease of resolution value. Macrocyclic antibiotic columns in polar-organic separation mode show enantioselectivity for local anaesthetic drug Diperodone. These obtained results are complementary to study on β-cyclodextrin CSP in reversed-phase separation mode, where was reached the enanantioseparation only for 2-alkoxysubstituted derivatives (HROBOŇOVÁ *et al*., 2003; HROBOŇOVÁ *et al*., 2002b).

Acknowledgments: Support of this work by VEGA grant no. 1/0499/14 of the Scientific Grant Agency of the Ministry of Education, Science, Research, and Sport of the Slovak Republic and the Slovak Academy of Sciences. The authors are grateful to D.W. Armstrong for providing of macrocyclic and cyclofructan HPLC columns.

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Received 21 January 2016
Accepted 19 April 2016