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Effect of the eluent on enantiomer separation of controlled drugs by liquid chromatography–ultraviolet absorbance detection–electrospray ionisation tandem mass spectrometry using vancomycin and native β-cyclodextrin chiral stationary phases

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Abstract

Enantioseparation of nine amphetamine derivatives, methorphan and propoxyphene was studied by comparing two different chiral stationary phases, macrocyclic antibiotic vancomycin and native β-cyclodextrin (β-CD). Effects of 46 eluent compositions on enantioseparation in reversed-phase (RP) and polar organic phase modes were investigated. β-CD was found to be more suitable to phenethylamines in general and vancomycin for methorphan and propoxyphene. An eluent system capable of separating the enantiomers of all phenethylamines in one run was developed. Also, systems providing competitive analysis times for enantioseparation of methorphan and propoxyphene were reported. The suitability of the eluent systems to electrospray ionisation (ESI) was discussed and methods using a tandem mass spectrometric (MS/MS) detection were developed. The suitability of chiral LC–ESI–MS/MS was tested with 14 seized drug samples. The results were in agreement with conventional non-chiral methods. Repeatability of the methods was good and limits of detection were 25–100 ng/ml for most compounds using mass spectrometric detection.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Mobile phase composition; Vancomycin; β-Cyclodextrin; Amphetamines

1. Introduction

Enantiomers of different illicit or licit drugs can have very different pharmacological, pharmacokinetic and metabolic behaviour and therefore, chiral analysis of such drugs plays an important role in forensic research. Also, a few drug enantiomers are controlled under legal statutes only as racemic mixtures or as in one enantiomeric form only (e.g. methorphan, propoxyphene). Furthermore, chiral analysis of some illicit drugs (e.g. methamphetamine) may indicate possible synthetic routes and therefore, the origin of the drug [1]. Since amphetamine derivatives (phenethylamines) are the most important synthetic products of clandestine laboratories, their chiral analysis can provide additional means for investigation.

Recently, the main methods used for chiral analyses are liquid chromatography (LC) and capillary electrophoresis (CE), because of their high efficiency, speed, preparative capability and reproducibility [2]. The methods have been widely applied to enantiomeric separation of methamphetamine and related drugs [3–12]. The main chiral selector used in CE for phenethylamines has been native or modified β-cyclodextrin (β-CD) [8–11], but also other cyclodextrins [12–14] and, e.g. an ansamycin antibiotic, Rifamycin B [15], have been studied. The use of CE in chiral separations has been recently reviewed [16].

Chiral LC has been widely used in enantiomeric separations with different types of chiral stationary phases (CSPs). Their use in pharmaceutical applications has been

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reviewed recently [17–19]. Direct enantioseparation of phenethylamines with LC has been obtained with various CSPs: chiral crown ether [20,21], cyclodextrin or its derivatives [6,22–26], protein [25] and immunoaffinity [27] columns have been used. Indirect LC methods have also been of interest [3–5], but they suffer from inconveniences such as impure reagents and the fact that they are more time consuming.

In this study, native β-cyclodextrin and vancomycin, a glycopeptide belonging to macrocyclic antibiotic phases introduced by Armstrong et al. [28], were compared in the separation of enantiomers of nine amphetamine derivatives, methorphan and propoxyphene. Structures of macrocyclic antibiotics are very complex, e.g. the vancomycin molecule produced by *Streptomyces orientalis* contains 18 chiral centres with various functional groups surrounding its three pockets or cavities [29]. Possible interactions include π–π complexation, hydrogen-bonding, inclusion complexation, dipole interactions, steric interactions as well as anionic and cationic binding. Strengths of these interactions depend on the type of the mobile phase used [29]. Vancomycin has many of the separation characteristics of protein based stationary phases with exceptional stability and higher sample capacity [30]. To the best of our knowledge, vancomycin has not been applied to chiral analysis of the compounds studied in this work. β-CD was chosen as a reference column, since it is the most used chiral selector and it has already been applied to some amphetamine derivatives studied herein, i.e. to methamphetamine, amphetamine [23,26,31], MDEA, MDMA, and MDA [6,22,25].

In this work, enantioseparation of nine amphetamine derivatives, methorphan and propoxyphene is studied by comparing vancomycin and β-CD (Fig. 1) columns and by studying the effect of eluent composition on the enantioseparation in reversed-phase and polar organic phase modes. The final goal was to develop chiral LC–MS/MS method for the compounds studied. LC–MS has been seldom used in chiral analysis of drugs with a forensic interest.

2. Experimental

2.1. Chemicals and sample solutions

Acetonitrile (ACN), methanol (MeOH) and isopropyl alcohol (*i*-PrOH), HPLC grade, were purchased from Rathburn (Walkerburn, Scotland), triethylamine (TEA), analytical grade, from Fluka (Fluka Chemie AG, Buchs, Switzerland), ammonium acetate (NH_4OAc), reagent plus grade from Sigma–Aldrich (Milwaukee, WI, USA) and acetic acid (HOAc), analytical grade, from Riedel-de Haën (Sigma–Aldrich, Seelze, Germany). Water was purified with a Milli-Q purifying system (Millipore Corp., Bedford, USA). The eluent compositions studied are presented in Table 1.

Rasemethorphan, levomethorphan (*R*,*R*,*R*-methorphan) (University's Pharmacy, Helsinki, Finland), levopropoxyphene (*R*,*S*-propoxyphene) (USP Inc., Rickville, MO, USA), dextropropoxyphene (*S*,*R*-propoxyphene), racemic *para*-chloroamphetamine (PCA) (Sigma, St. Louis, MO, USA), amphetamine (AM), methamphetamine (MA), *para*-methoxyamphetamine (PMA), *para*-methoxymethamphetamine (PMMA) (Lipomed AG, Arlesheim, Switzerland), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethylamphetamine (MDEA) (United Nations, Wien, Austria), and norephedrine (NE) (Knoll AG, Ludwigshafen, Germany) were purchased as pure reference materials.

Structures of the compounds are presented in Table 2. Authentic sample material was from the drug seizures made by the Finnish Police. Standards were dissolved in methanol (1.0 mg/ml, stock solution) and dilutions were made with deionised water. The samples were dissolved in MeOH, sonicated for 10 min, diluted with deionised water, and filtrated (GHP Acrodisc, Pall Gelman Laboratory, Ann Arbor, USA) to autosampler vials. All solutions were stored at −20 °C.

2.2. Instrumentation

The LC–MS consisted of an Agilent 1100 Series HPLC system with an autosampler, a diode array detector

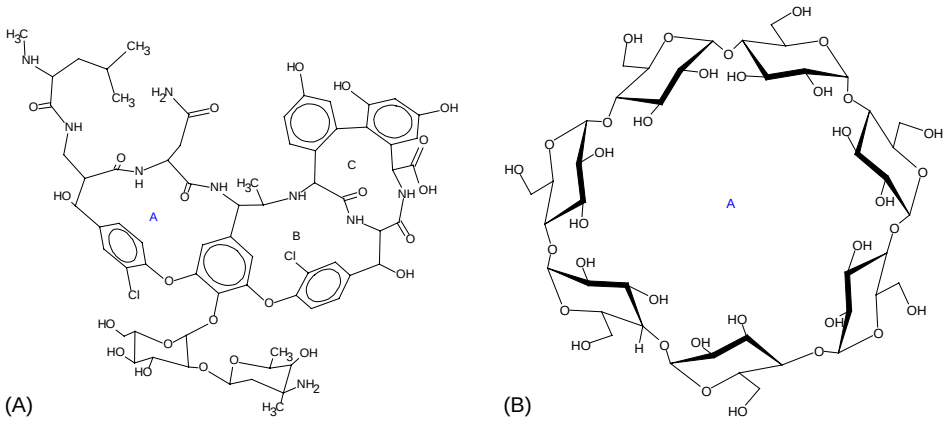


Fig. 1. The structures of (A) amphoteric glycopeptide vancomycin (MW = 1449) and (B) β-cyclodextrin (MW = 1135).

Table 1
The eluent systems investigated in the reversed-phase (RP) and in the polar organic phase (POP) modes with vancomycin and β-CD columns

Number	
Vancomycin (RP)	
1	ACN:0.1% TEAA 30:70, pH 4
2	ACN:0.1% TEAA 50:50, pH 4
3	ACN:0.1% TEAA 70:30, pH 4
4	ACN:0.1% TEAA 90:10, pH 4
5	MeOH:0.1% TEAA 30:70, pH 4
6	MeOH:0.1% TEAA 40:60, pH 4
7	MeOH:0.1% TEAA 50:50, pH 4
8	MeOH:0.1% TEAA 70:30, pH 4
9	MeOH:0.1% TEAA 80:20, pH 4 (5, 6, 7)
10	MeOH:0.1% TEAA 90:10, pH 4
11	*i*-PrOH:0.1% TEAA 30:70, pH 4
12	*i*-PrOH:0.1% TEAA 50:50, pH 4
13	*i*-PrOH:0.1% TEAA 70:30, pH 4
14	*i*-PrOH:0.1% TEAA 90:10, pH 4
Vancomycin (POP)	
15	MeOH:ACN:HOAc:TEA 70:30:0.03:0.02
16	MeOH:ACN:HOAc:TEA 70:30:0.03:0.04
17	MeOH:ACN:HOAc:TEA 70:30:0.06:0.02
18	MeOH:ACN:HOAc:TEA 70:30:0.5:0.05
19	MeOH:ACN:HOAc:TEA 70:30:0.6:0.2
20	MeOH:ACN:HOAc:TEA 70:30:0.9:0.1
21	MeOH:HOAc:TEA 100:0.03:0.02
22	MeOH:HOAc:TEA 100:0.06:0.02
β-CD (RP)	
23	ACN:1.0% TEAA 5:95, pH 4
24	ACN:1.0% TEAA 10:90, pH 4
25	ACN:1.0% TEAA 15:85, pH 4
26	MeOH:1.0% TEAA 5:95, pH 4
27	MeOH:1.0% TEAA 10:90, pH 4
28	MeOH:1.0% TEAA 15:85, pH 4
29	*i*-PrOH:1.0% TEAA 5:95, pH 4
30	*i*-PrOH:1.0% TEAA 10:90, pH 4
31	*i*-PrOH:1.0% TEAA 15:85, pH 4
32	ACN:100 mM NH_4OAc 5:95, pH 4
33	MeOH:100 mM NH_4OAc 5:95, pH 4
34	MeOH:100 mM NH_4OAc 15:85, pH 4
β-CD (POP)	
35	MeOH:ACN:HOAc:TEA 10:90:0.03:0.02
36	MeOH:ACN:HOAc:TEA 15:85:0.03:0.02
37	MeOH:ACN:HOAc:TEA 20:80:0.03:0.02
38	MeOH:ACN:HOAc:TEA 25:75:0.03:0.02
39	MeOH:ACN:HOAc:TEA 30:70:0.03:0.02
40	MeOH:ACN:HOAc:TEA 15:85:0.3:0.2
41	MeOH:ACN:HOAc:TEA 15:85:0.3:0.4
42	MeOH:ACN:HOAc:TEA 15:85:0.6:0.2
43	MeOH:ACN:HOAc:TEA 15:85:0.9:0.1

(DAD), and an Agilent 1100 Series LC/MSD Trap ion trap mass spectrometer (Bremen, Germany). In LC–DAD–ESI–MS/MS experiments, the flow was splitted after column by an Accurate splitter (LC Packings, San Fransisco, CA, USA) so that 1/10 of the eluent was directed to MS and 9/10 to DAD.

The columns were Chirobiotic V (vancomycin) and Cyclobond I 2000 (β-CD) (both 150 mm × 4.6 mm × 5 μm, Advanced Separation Technologies Inc., Whippany, USA). The flow rate was 0.8 ml/min and the column temperature was 15 °C. The injection volume was 1.0 μl when the eluents were investigated with a DAD detector and increased to 20 μl for the final MS methods. The eluents were degassed with vacuum. The monitored wavelengths were 233, 257 and 285 nm. $t_0 = 2.41$ min was measured from the first baseline disturbance in the UV signal.

The ionisation technique used was electrospray ionisation (ESI) operated in positive ion mode. The operation parameters of the ESI ion source were the following: drying gas (nitrogen) temperature 350 °C, drying gas (nitrogen) flow rate 8 l/min, nebuliser gas pressure 207 kPa (30 psi) in reversed-phase (RP) and 241 kPa (35 psi) in polar organic phase (POP), endplate voltage −3500 V, endplate offset −500 V, capillary exit 67.4 V and skim1 17.4 V. Nitrogen was generated by a Whatman (Haverhill, MA, USA) model 75–72 nitrogen generator. The mass spectrometer was used in precursor ion monitoring mode and the protonated molecule was selected as a precursor ion (Table 2). Isolation width was 4.0 *m*/*z* and for NE and MA 2.0 *m*/*z*. The scan range was *m*/*z* 50–220 for amphetamines and *m*/*z* 50–350 for propoxyphene and methorphan. The accumulation time was 50 ms, averages 2, rolling averaging off and ion charge control on. The autotune fragmentation amplitude was set to 1.0. Helium (4.6, 99.996%) was used in the trap as damping and collision gas.

3. Results and discussion

3.1. Effect of mobile phase

Vancomycin and β-CD CSPs can be used in normal–phase, reversed-phase and polar organic phase modes [28,32]. ESI is compatible only with polar eluent systems with high enough conductivity and therefore, non-polar eluent systems were not tested in this work. Methorphan, propoxyphene and MA, were used as model compounds in the comparison of the eluent compositions with vancomycin and β-CD columns and in the development of chiral LC–MS methods. The results of MA were used in choosing suitable conditions for other amphetamines.

3.1.1. Reversed-phase mode using vancomycin column

The effect of the eluents 1–14 (Table 1) on the resolution and retention of enantiomers of MA, propoxyphene and methorphan was studied in the RP mode using the vancomycin column (Fig. 2). Propoxyphene was not enantioseparated with any of the eluents 1–14. Methorphan was at least partially enantioresolved with all these eluents and the best resolution ($R_S = 2.6$) was achieved with 90% MeOH (eluent 10). The results with MA indicate that MeOH and *i*-PrOH provided enantioseparation while the enantiomers were not separated with ACN. These results were confirmed by running all the amphetamine derivatives with 70:30 ACN:0.1% TEAA. Matching the results with

Table 2
The precursors and recorded product ions and enantioresolution (R_s), retention factor (k) and separation factor (α) obtained with the best eluent compositions of each mode (vancomycin in RP and POP, β-CD in RP and POP)

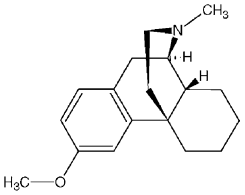
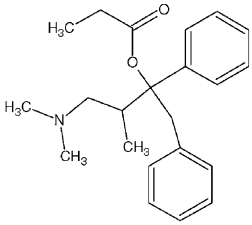
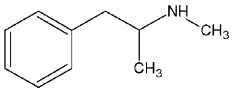
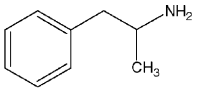
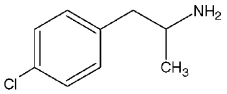
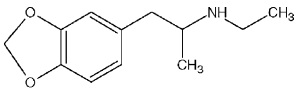
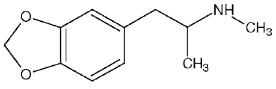
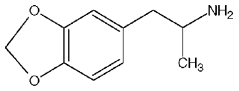
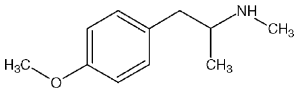
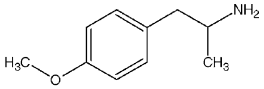
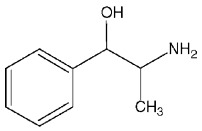
Compound	$[M–H]^+$ and product ions	R_s	k (1)[a]	α	Column	Mode	Eluent number
 **Methorphan**	272 → 215	2.58	4.7	1.18	Vancomycin	RP	10
		3.33	2.2	1.21	Vancomycin	POP	21[b]
		1.30	12.8	1.11	β-CD	RP	25
		1.82	14.8	1.12	β-CD	POP	35
 **Propoxyphene**	340 → 266	0	0.7–9.7	1.00	Vancomycin	RP	1–14
		0.89	3.6	1.05	Vancomycin	POP	15[b]
		0	1.2–7.2	1.00	β-CD	RP	23–31
		0.69	0.9	1.09	β-CD	POP	40
 **Methamphetamine (MA)**	150 → 119	1.02	2.1	1.07	Vancomycin	RP	9
		1.30	5.1	1.07	Vancomycin	POP	21
		1.45	3.2	1.11	β-CD	RP	26
		0	1.7–20.0	1.00	β-CD	POP	23–31
		1.26	4.4	1.10	β-CD	RP	33[b]
 **Amphetamine (AM)**	136 → 119	1.26	1.7	1.09	Vancomycin	RP	9
		1.44	3.3	1.08	Vancomycin	POP	21
		1.00	2.3	1.09	β-CD	RP	26
		–	–	–	β-CD	POP	Not run
		1.00	3.4	1.08	β-CD	RP	33[b]
 ***p*-chloroamphetamine (PCA)**	171 → 153	0	1.9	1.00	Vancomycin	RP	9
		0.50	3.9	1.03	Vancomycin	POP	21
		0.98	6.2	1.08	β-CD	RP	26
		–	–	–	β-CD	POP	Not run
		0.80	8.1	1.06	β-CD	RP	33[b]
 **3,4-methylenedioxy-ethylamphetamine (MDEA)**	208 → 163	0	1.9	1.00	Vancomycin	RP	9
		0	4.0	1.00	Vancomycin	POP	21
		1.50	11.8	1.14	β-CD	RP	26
		–	–	–	β-CD	POP	Not run
		1.48	17.8	1.13	β-CD	RP	33[b]
 **3,4-methylenedioxy-methamphetamine (MDMA)**	194 → 163	0.62	2.4	1.05	Vancomycin	RP	9
		0.94	5.9	1.04	Vancomycin	POP	21
		1.57	8.9	1.13	β-CD	RP	26
		–	–	–	β-CD	POP	Not run
		1.50	12.9	1.12	β-CD	RP	33[b]

Table 2 (*Continued*)

Compound	$[M–H]^+$ and product ions	R_s	k (1)[a]	α	Column	Mode	Eluent number
 **3,4-methylenedioxy-amphetamine (MDA)**	180 → 163	0.79	2.0	1.06	Vancomycin	RP	9
		0.65	3.7	1.04	Vancomycin	POP	21
		1.13	7.4	1.10	β-CD	RP	26
		–	–	–	β-CD	POP	Not run
		1.12	10.6	1.09	β-CD	RP	33[b]
 ***p*-methoxy- methamphetamine (PMMA)**	180 → 149	0.98	2.2	1.07	Vancomycin	RP	9
		1.49	5.5	1.08	Vancomycin	POP	21
		1.34	6.2	1.11	β-CD	RP	26
		–	–	–	β-CD	POP	Not run
		1.24	9.3	1.10	β-CD	RP	33[b]
 ***p*-methoxy-amphetamine (PMA)**	166 → 149	1.10	1.8	1.08	Vancomycin	RP	9
		1.43	3.5	1.08	Vancomycin	POP	21
		0.98	4.9	1.08	β-CD	RP	26
		–	–	–	β-CD	POP	Not run
		0.94	7.1	1.08	β-CD	RP	33[b]
 **Norephedrine (NE)**	152 → 134	No enantioresolution with any system tested.					

[a] The retention factor of the first eluting enantiomer.
[b] The eluent optimised for MS detection.

MA, none of the enantiomers of the amphetamine derivatives were separated. These results are in agreement with the earlier observation: MeOH provides superior enantioseparation for basic drugs with a vancomycin column [33], although ACN provides more efficient enantioseparation for other type of compounds than MeOH [32]. The effect of the amount of the organic modifier on retention gave U-shaped retention curves that are typical for glycopeptide columns (Fig. 2) [28].

The best enantioseparation for MA and methorphan was obtained with the eluents 9, 10 and 14. However, due to more non-polar character of *i*-PrOH compared to MeOH, the retention times with the eluent 14 were significantly longer than with the eluents 9 and 10, and also the backpressure was higher. The best enantioresolution for MA with a reasonable retention factor ($k = 2.1$) was achieved with the eluent 9 (80% MeOH), which was chosen for further studies. The eluent 9 showed an acceptable resolution also for all the other amphetamines, except for the enantiomers of PCA, MDEA and NE. The latter two were not separated with any solvent composition studied using the vancomycin column. The best separation for PCA was obtained with the eluent 6 ($R_s = 0.6$). The resolution and retention factors for the amphetamines studied with the eluent system 9 are presented in Table 2.

The effect of pH on enantioseparation and retention (Fig. 3A and B) was studied using the vancomycin column and eluent composition MeOH:0.1% TEAA 80:20 (eluent 9). The pH, adjusted with acetic acid, was studied between 4–7, which is the usable pH range of the vancomycin column. The resolution was increased only slightly, whereas the retention was increased significantly when the pH was increased. The increased retentions are obviously due to the changes in charge states of vancomycin. The ionisation state of the carboxylic acid group of vancomycin is increased when the pH is increased from 4 to 7. It follows that ionic interactions between fully ionised amphetamines (pK_a values are between 9.2 and 10.6 [34] or calculated with the Pallas 1.2 software (CompuDrug Chemistry, Budapest, Hungary, 1994)) and vancomycin become more important. To ensure a fast analysis and the sufficient resolution between enantiomers, pH 4.0 was found to be the most suitable.

3.1.2. Polar organic phase mode with vancomycin column

The polar organic phase was tested using the vancomycin column and eight different mobile phase compositions (eluents 15–22, Table 1). The selection of initial mobile phase (eluent 15) was based on an earlier study for clenbuterol [35] and this was modified by varying the acid/base ratio, and finally the MeOH/ACN ratio. In POP, the acid/base ratio and their concentrations are presented to be the most important parameters in controlling resolution and retention, respectively [29].

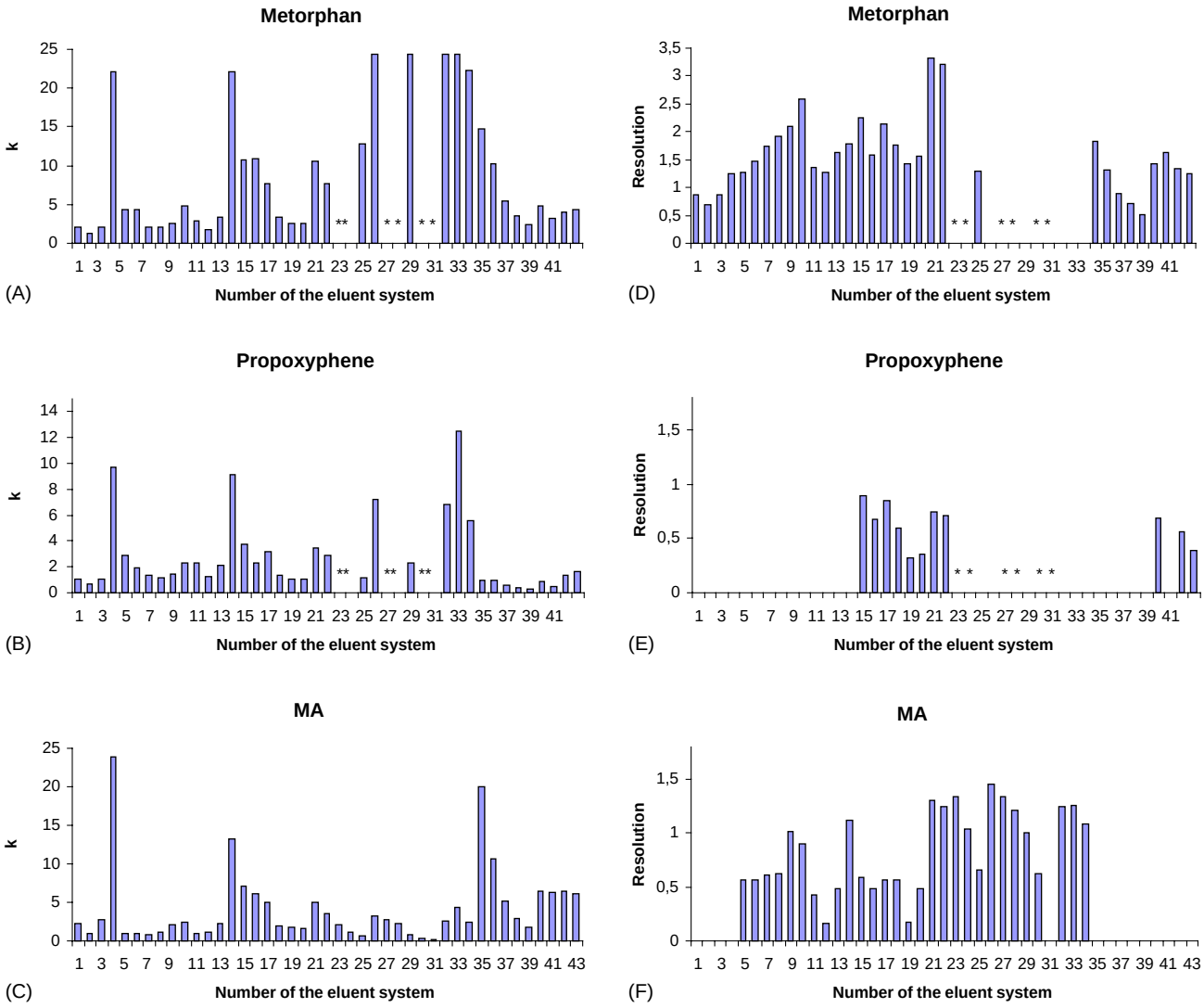


Fig. 2. The effect of the eluent system on retention (A–C) and enantioresolution (D–F) of methorphan, propoxyphene and MA using the vancomycin and β-CD columns. The eluent compositions are presented in Table 1 and (∗) = not run.

Vancomycin using POP is the only method providing separation of enantiomers of all the three test compounds. The best resolution for MA ($R_s = 1.3$) and methorphan ($R_s = 3.3$) with acceptable retention ($k = 5.1$ and 2.2, respectively) was obtained with eluent 21. The eluent 21 provided also a reasonable resolution for enantiomers of propoxyphene ($R_s = 0.7$), although a slightly better resolution was obtained with the eluents 15 and 17 ($R_s = 0.9$ for both, Fig. 2). The results show that the resolution of enantiomers of MA and methorphan was decreased with an addition of acetonitrile indicating the importance of a hydrogen bonding capability of the hydroxyl group of MeOH (eluents 15–20).

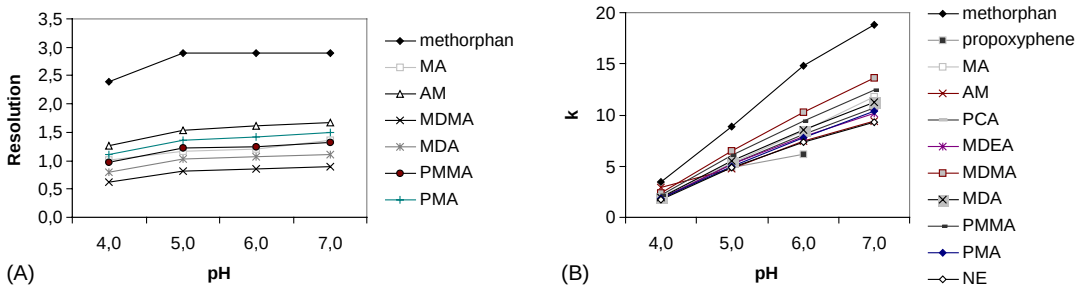


Fig. 3. The effect of pH on (A) enantioresolution and (B) retention with 80:20 MeOH:0.1% TEAA.

The results obtained with the test compounds were confirmed by running all the amphetamines with the eluents 15–22. As a result, the eluent 21 provided the best resolution, although MDEA and NE were not separated with any of these eluents (Table 2). The importance of using 100% MeOH instead of MeOH:ACN mixture was recognised particularly with MDMA, MDA and PMA which were not enantioresolved with any of the eluents containing ACN (data not shown), but were resolved with 100% MeOH (eluents 21 and 22). The exception to other amphetamines was PCA showing better enantioseparation with an increased amount of ACN (data not shown).

3.1.3. Reversed-phase mode with β-cyclodextrin column

The RP eluents 23–34 with the β-CD column provided an acceptable separation for MA, but not for methorphan and propoxyphene (Fig. 2). As in the earlier studies [22], it was evident, that the amount of the organic modifier must be low (<15%) and buffer concentration relatively high (1% TEAA) in order to achieve an acceptable resolution for phenethylamines. The retention increased together with the resolution when polarity of the solvent system was increased. The order of the elution strength of the organic modifier was *i*-PrOH > ACN > MeOH (Fig. 2) indicating that the β-CD column behaves as a RP column unlike vancomycin.

The best results for MA were obtained with MeOH as an organic modifier (eluents 26–28). The best resolution for MA with reasonable retention was achieved with the eluent 26, which provided acceptable resolution also for all the other amphetamines, except for NE (Table 2). Furthermore, this eluent enabled a chromatographic separation of the amphetamines and their enantiomers from each other allowing their analysis as a mixture by UV-detection. Sadeghipour and Veuthey [6] and Brunnenberg and Kovar [25] presented that with the RP mode and native β-CD, the *R*(−)-enantiomer always eluted before *S*(+). This is obviously also true in our experiments with β-CD.

3.1.4. Polar organic phase mode with β-cyclodextrin column

MA showed no enantioseparation using β-CD in POP (Fig. 2) and therefore, the other phenethylamines were not run in this mode. For propoxyphene, acceptable but weak enantioseparation ($R_s < 0.7$) with reasonable retention factors ($k < 3.1$) was obtained with the eluents 40, 42 and 43. Propoxyphene showed no enantioresolution when the concentration of TEA was higher than that of HOAc (eluent 41). Methorphan showed acceptable resolution with all the eluents (35–43) tested with the β-CD column in POP. The resolution and retention of methorphan decreased significantly when the amount of MeOH was increased (eluents 35–39), but the concentration ratio of HOAc and TEA (eluents 36 and 40–43) had no significant effect on the resolution. However, the retention time was significantly longer with low (eluent 36) than with high (eluents 40–43) buffer concentrations. As a conclusion, the most suitable eluent for the separation of enantiomers of methorphan with the β-CD column in POP was the eluent 41, which provided acceptable resolution and retention ($R_s = 1.6$, $k = 3.2$).

3.2. Effect of molecular structure on resolution and comparison of the methods

The effect of an alkyl chain length near the chiral centre of the phenethylamines on the enantioseparation was studied comparing MDA, MDMA, and MDEA with each other, PMA and PMMA with each other, and AM and MA with each other (Table 2). The comparison with the vancomycin column in the RP and POP shows that the enantioresolution decreased when the alkyl chain length increased and the ethyl substituent (MDEA) prevented the chiral separation totally. The exception to this was MDMA and MDA in POP. The reason may be that a longer alkyl chain causes an increased steric hindrance for interaction between amino groups of amphetamines and acidic groups of vancomycin. With β-CD, the effect of the alkyl chain length was not significant in enantioresolution. However, the size of the molecule had significant effect on the retention, as the amphetamines eluted from the β-CD column in an increasing order of size.

The effect of the electrophilic substituents in the aromatic ring of the phenethylamines can be studied by comparing AM, PMA, MDA and PCA with each other and MA, PMMA and MDMA with each other. With the vancomycin column in the RP and POP, the enantioresolution of the phenethylamines was clearly influenced by the electrophilic substituents at the aromatic ring. The compounds without substituents (AM, MA) showed best resolution, but also the methoxy compounds (PMA, PMMA) showed practically a similar resolution. The dimethoxy substitution in MDA and MDMA decreased the resolution significantly and chlorine prevented totally the separation of enantiomers of PCA with the eluent 9 in the vancomycin column. These results suggest that π–π interactions between the aromatic ring of the amphetamines and vancomycin have an effect on the resolution. With β-CD, the electrophilicity of the substituents in the phenyl ring affected the resolution only little. Interestingly, NE having a hydroxyl group at the carbon adjacent to the chiral centre did not show enantioseparation with any of the methods tested.

The best method for the enantioseparation of the amphetamine derivatives using one system was the β-CD column using the RP eluent system 26, which allowed enantioseparation of all the phenethylamines, except for NE. The system 26 was also able to separate the amphetamines from each other far more better than the vancomycin column. However, the vancomycin column provided better enantioresolution for AM, PMMA, and PMA as individual compounds (Table 2). Also, the vancomycin column using the polar organic phases 22 and 15 are the most suitable methods for methorphan and propoxyphene, respectively,

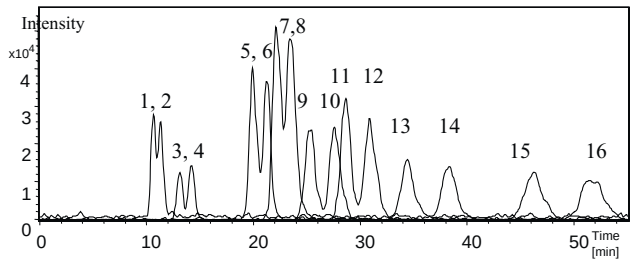


Fig. 4. Extracted product ion chromatograms of eight amphetamine derivatives. Method: 5:95 MeOH:100 mM NH_4OAc (eluent 33) and β-CD column. Compounds: (1) *R*-AM, (2) *S*-AM, (3) *R*-MA, (4) *S*-MA, (5) *R*-PMA, (6) *S*-PMA, (7) *R*-PCA, (8) *S*-PCA, (9) *R*-PMMA, (10) *S*-PMMA, (11) *R*-MDA, (12) *S*-MDA, (13) *R*-MDMA, (14) *S*-MDMA, (15) *R*-MDEA, and (16) *S*-MDEA. Sample concentrations: 35–130 μg/ml.

providing acceptable enantioseparations and reasonable retention factors.

3.3. ESI–MS

As the best method for the separation of enantiomers of phenethylamines, was the β-CD column with the RP eluent of 26, the suitability of the method with LC–ESI–MS/MS detection was tested. However, the method was not directly suitable, since a high TEAA concentration (1%) caused severe suppression of ionisation and lead to decreased sensitivity and rapid contamination of the ion source. Therefore, 1% TEAA (72 mM) was replaced by 100 mM ammonium acetate (eluent 33) which showed a nearly comparable resolution, but also somewhat increased retention (Table 2 and Fig. 2). Sensitivity was better with ammonium acetate than with TEAA due to decreased suppression. However, the low amount of TEA (0.02%) did not show significant ion suppression, and the POP eluent systems 22 and 15 were used in chiral LC–MS/MS analysis of methorphan and propoxyphene, respectively. Fig. 4 illustrates extracted product ion chromatograms for phenethylamine enantiomers and Fig. 5 for methorphan enantiomers.

The LC–ESI–MS/MS method was evaluated by determining the limit of detection, repeatability of the peak areas of the product ion chromatograms and retention times. The method is intended only for identification of the compounds studied and therefore, a complete quantitative evaluation was not performed. The relative standard deviations of the peak

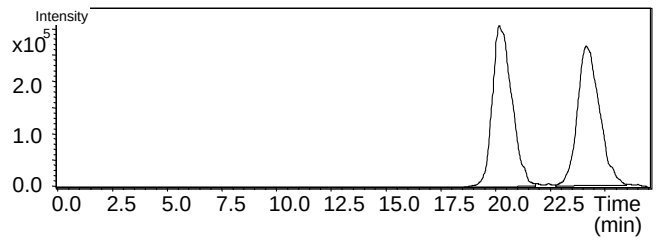


Fig. 5. Extracted product ion chromatogram of methorphan using the vancomycin column and the eluent 22 (elution order *S*, *S*, *S* before *R*, *R*, *R*), sample concentration was 20 μg/ml.

Table 3
The repeatability and limits of detection for the compounds studied using LC–ESI–MS/MS

Compound	System number	R.S.D. (t_r)	R.S.D. (area)	*N*	LOD (ng/ml)
Methorphan	22	0.6%	2.8%	5	25
Propoxyphene	15	0.2%	7.3%	5	25
Amphetamines	33	0.3–1.3%	2.4–11.4%	6	100, 1000 (AM)

The concentrations in the repeatability tests were 10 μg/ml.

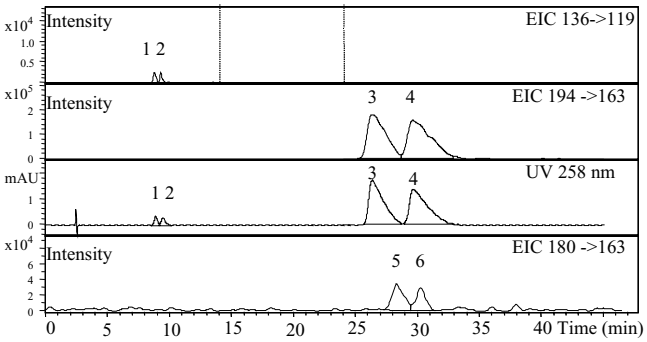


Fig. 6. An ecstacy tablet analysed using β-CD column and the eluent 33 (see Table 1). Extracted product ion chromatograms of 1 and 2 AM, 3 and 4 MDMA and their corresponding UV trace. The lowest trace is from a second injection of a more concentrated sample, 5 and 6 MDA.

areas were between 2.4 and 11.4% and for retention times 0.2–1.3% showing acceptable repeatability of the method. The limits of detection were between 25 and 1000 ng/ml being acceptable for a forensic analysis (Table 3). Splitting of the eluent 1/10 before MS decreased contamination of the ion source allowing more robust analysis than without splitting.

The LC–ESI–MS/MS method using the β-CD column and the RP eluent system 33 was tested with 14 authentic samples from police seizures. All the results were in agreement with the results obtained with standard non-chiral LC–UV and GC–MS methods. Fig. 6 illustrates as an example an analysis of amphetamine derivatives in an ecstacy tablet by LC–UV–ESI–MS/MS using the β-CD column and the RP eluent 33. The extracted product ion chromatograms show that the sample included a high concentration of MDMA, and MDA and amphetamine as impurities. The high amount of MDMA overloaded the column causing peak tailing and a shift in the retention times. Also, in order to detect MDA and get reasonable peak shape for AM and MDMA a more concentrated sample had to be injected separately (Fig. 6).

4. Conclusions

Comparison of different eluent compositions on enantioseparation showed that even small changes in the composition may affect the resolution significantly. The β-CD column, using a reversed-phase eluent system, was more suitable for chiral analysis of amphetamine derivatives than

the vancomycin column. However, the vancomycin column, using a polar organic phase was more suitable for enantioseparation of methorphan and propoxyphene than the β-CD column. The β-CD column allowed not only separation of enantiomers of the amphetamine derivatives, but also separation of the amphetamines from each others using UV detection. The high concentrations of non-volatile buffers caused severe signal suppression in ESI–MS. However, it was shown that non-volatile TEAA can be replaced with ammonium acetate without decreasing resolution. The repeatability and sensitivity of the LC–ESI–MS/MS method were shown to be acceptable and its applicability to authentic sample material was assessed.

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