NANOELECTROSPRAY VERSUS ELECTROSPRAY IN CHIRAL ANALYSIS BY THE KINETIC METHOD

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Electrospray ionization generates trimeric diastereoisomeric clusters as the first important step in chiral analysis by mass spectrometry using the Cooks' kinetic method. Cu^{2+} and L-tryptophan were used as a central metal and as a chiral reference ligand, respectively. The comparison of electrospray and nanoelectrospray showed that although the electrospray system was generally more robust, the application of nanoelectrospray was essential for performing successful analysis in some cases, especially for real samples. Basically, no significant differences between the ion sources were observed for model samples of analytes (isoleucine, ephedrine, phenylalanine) without interfering matrix. On the other hand, model samples containing sodium chloride and a buffer containing a real sample (drug formulation Mucoseptonex E in which D-ephedrine is the active substance) could not be analyzed using ESI, whereas nano-ESI gave satisfactory results. An explanation is based on the differences of ionization processes occurring in the compared sources.

Keywords: Chiral analysis; Kinetic method; Mass spectrometry; Electrospray; Nanoelectrospray; Amino acids; Drugs; Diastereoisomeric clusters; Gas-phase.

Analysis of enantiomers is essential for understanding many processes in living organisms, e.g. action of drugs. One enantiomer can have a therapeutic effect while the other can be non-active or even toxic. The change in configuration at a chiral center can manifest itself in a different aroma or taste of optical isomers. Chromatographic and electrophoretic methods are often used for separation of enantiomers but also other techniques, including mass spectrometry, have been successfully used in their discrimination.

Different mass spectrometric approaches for the distinction of enantiomeric molecules have been described so far[1,2]. The formation, mass-selective isolation and subsequent fragmentation of a trimeric diastereoisomeric cluster $[M(ref)_2A\text{-}H]^+$ (where M stands for a central metal ion, ref is an opti-

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cally pure reference ligand, and A is the enantiomer of an analyte) are essential steps of the kinetic method[3–7]. Two product ions have to be formed ($[M(ref)A\text{-}H]^+$ and $[M(ref)_2\text{-}H]^+$). If a significant chiral effect is operative, the resulting difference in the rate constants of these two fragmentation channels depends on the chirality of the analyte enantiomer present in a diastereoisomeric cluster, and the intensity ratio of product ions (R) is related to the ratio of enantiomers in the sample.

$$R = [M(ref)A\text{-}H]^+/[M(ref)_2\text{-}H]^+ \qquad (1)$$

A linear relationship exists between ln R and the enantiomeric composition:

$$\ln R = K\,(\%\ \mathrm{D}) + Q \qquad (2)$$

where % D stands for the corresponding proportion of D-enantiomer, K and Q are variable coefficients determined by calibration.

The chiral selectivity is expressed by an R_{chiral} value.

$$R_{chiral} = R_L/R_D \qquad (3)$$

A value closer to one indicates lower chiral selectivity (for easier comparison, the values are presented as higher than one in this paper). R_L and R_D are obtained by measurement of a sample of pure L- or D-enantiomer, respectively.

In some cases, the deviations from linearity were observed. Zhang et al.[8] explained this observation by competition of enantiomers in the process of the trimeric cluster formation and proposed a correction that employs three points from the calibration curve (for pure enantiomers and a racemic mixture). A correction that includes all calibration points was recently described by us[9] and the calibration curve can accordingly be expressed by Eq. (*4*).

$$\ln R = A\,C\,(\%\ \mathrm{D})/[1 - (1 - C)\,(\%\ \mathrm{D})] + B \qquad (4)$$

where A, B, and C are coefficients of a non-linear dependence. Better fit to experimental data was achieved in this case but the mentioned competition

might be only one of the reasons for non-linearity and this phenomenon has to be further investigated. The results obtained by the kinetic method can also be influenced by the presence of interfering complexing metals (e.g. magnesium) in a sample. For example, a magnesium complex ion and its product ion isobaric to a target diastereoisomeric cluster and to $[M(ref)A\text{-}H]^+$, respectively, can deteriorate quantitative analysis[10].

The kinetic method has been successfully applied in chiral analysis of different classes of compounds. For example, amino acids[4,11], chiral drugs[6], enantiomers of peptides[12], nucleosides with antiviral activity[13], antibiotics[5], α-hydroxy acids[14] and sugars[15] were discriminated. Discrimination of peptides with different sequence of amino acids is also possible[16,17]. An interesting modification of the kinetic method is the utilization of fixed ligands[5] or its use in flow systems (FIA/ESI-MS/MS and HPLC/MS)[9].

The satisfactory signal intensity of a trimeric diastereoisomeric cluster is essential for the successful analysis by the kinetic method. Except for already described interferences[10], the negative influence of salts present in the sample can render analysis impossible. Since the mechanisms of electrospray and nanoelectrospray ionization are different (as a consequence, sensitivity and tolerance to salt contamination are better for nano-ESI)[18], the investigation and evaluation of the potential of these two ion sources can extend the applicability of the kinetic method in isomeric discrimination.

EXPERIMENTAL

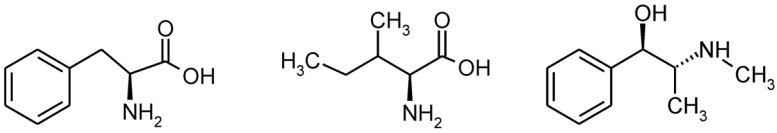
Instrumentation

An LCQ mass spectrometer equipped with a standard electrospray ion source (Thermo Finnigan, San Jose, CA, USA) was used for analyses performed by direct infusion. Experiments with nanoelectrospray were carried out with the same mass spectrometer but, instead of electrospray, a nano-ESI source was implemented (NSI probe, Thermo Finnigan, San Jose, CA, USA). Inner diameter of metal-coated nanospray tip was 2 ± 1 μm (PicoTip emitter GlassTip; New Objective, Woburn, MA, USA).

Chemicals

L-Tryptophan (L-Trp; nominal mass 204), L-phenylalanine, D-phenylalanine (L-Phe, D-Phe; 165), L-ephedrine, D-ephedrine (L-Eph, D-Eph; 165), L-isoleucine, D-isoleucine (L-Ile, D-Ile; 131) (Fig. 1), NaCl, $CuCl_2$ (all analytical grade) and methanol (HPLC gradient grade) were purchased from Sigma–Aldrich (Prague, Czech Republic). $Mg(NO_3)_2$ (analytical grade) was obtained from Lachema (Brno, Czech Republic). Water for HPLC was prepared using Elgastat Maxima (Elga, Marlow, UK).

The working solution mixtures containing an analyte (isoleucine, ephedrine, phenylalanine, 5×10^{-5} mol l^{-1}), a reference ligand (L-Trp, 1×10^{-4} mol l^{-1}) and $CuCl_2$ (1×10^{-4} mol l^{-1}) were prepared by dissolution of compounds in a water–methanol mixture (1:1, v/v). NaCl and $Mg(NO_3)_2$ were used at concentrations 2×10^{-3} and 1×10^{-3} mol l^{-1}, respectively.



D-phenylalanine D-isoleucine D-ephedrine

FIG. 1
Structures of chiral compounds under study

Method

Experimental parameters were optimized to provide suitable efficiency of formation and transportation of target clusters through ion optics. The selected ESI-LCQ parameters were as follows: spray voltage +5.6 kV, sheath gas (N_2) 60 arb. units, heated capillary temperature 175 °C. Working solutions were infused with a syringe pump into the ion source at a 3 μl min^{-1} flow-rate. The nano-ESI-LCQ system was operated with heated capillary temperature held at 175 °C and no sheath gas. Spray voltage was set up to +2 kV.

Ion optics was tuned automatically. The collision energy 8.5% provided appropriate fragmentation of trimeric cluster ions and was applied in all CID experiments (10% corresponds to the amplitude of resonance excitation radio frequency voltage 0.5 V).

Five point calibration curves were generated separately for the ESI and nano-ESI system. The levels of 0, 25, 50, 75 and 100% of D-isoleucine, D-phenylalanine and D-ephedrine were used. All the experimental points as well as analyses of samples were repeated five times. NaCl or $Mg(NO_3)_2$ was added to sample solutions to examine the influence of salts.

Statistical evaluation of experimental data was accomplished using statistical software QC Expert (Trilobyte statistical software, Pardubice, Czech Republic).

RESULTS AND DISCUSSION

A comparison of ESI and nano-ESI mass spectra of standards demonstrated the positive influence of a nano-ESI source (Fig. 2). For the studied compounds, the highest increase in the trimeric cluster intensity was found for ephedrine (almost by one order of magnitude) whereas the lowest change in the intensity was determined for isoleucine (with practically the same intensities in both ion sources). The increase in absolute abundance of a target cluster offers better ion statistics for its subsequent collision-induced dissociation. This can improve the determination of the enantiomeric ratios and also be helpful in the analysis of samples of low concentrations.

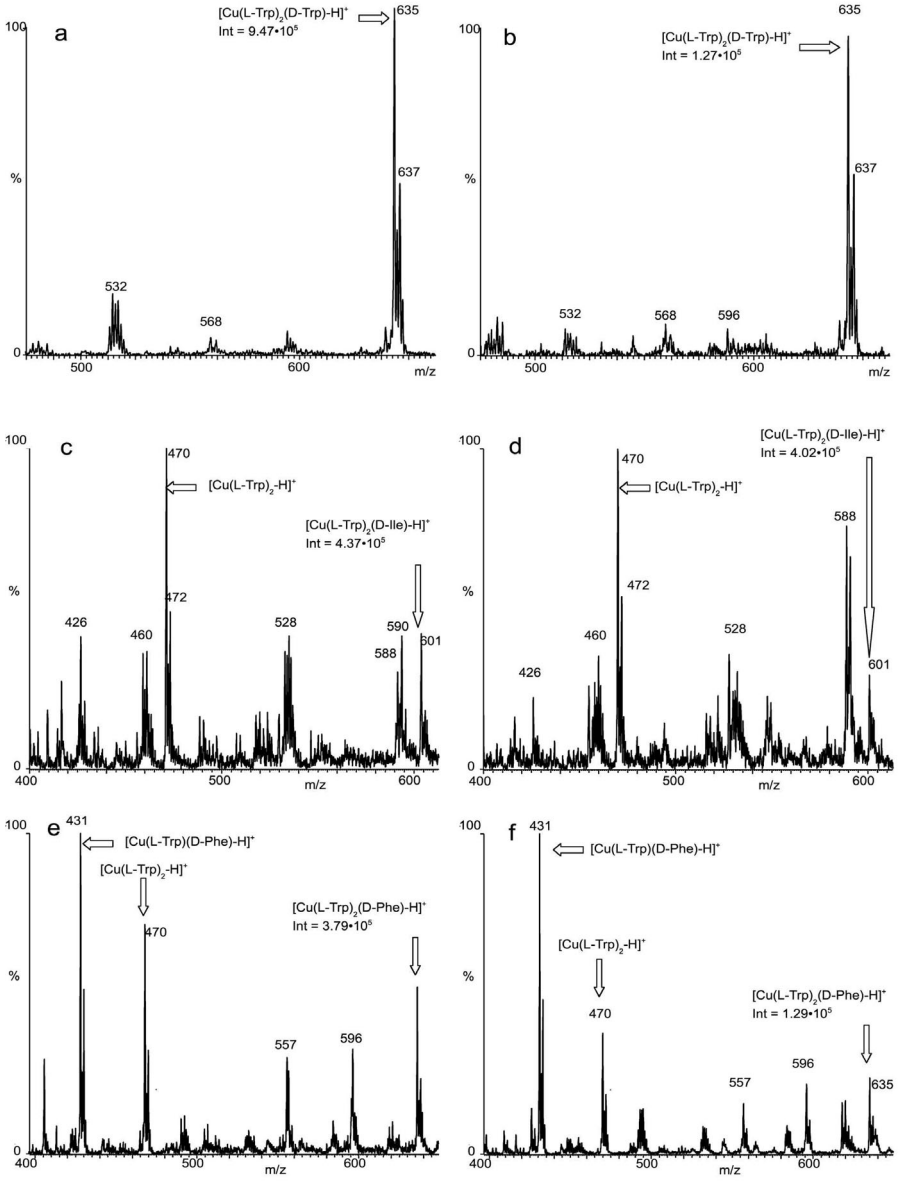


FIG. 2

The trimeric clusters of the studied chiral compounds generated by nano-ESI (a, c, e) and ESI (b, d, f)

Even if the absolute intensity was not significantly higher, an increase in relative intensity of target clusters and a resulting improvement of the signal-to-noise ratio was observed in all the spectra measured using nano-ESI. This ionization produced smaller droplets in comparison with ESI [18]. Such droplets disintegrated fewer times before transfer of ions to gas phase, therefore the increase in salt concentration in droplets and competition for the droplet surface and charges among the compounds present in a sample took place to a lesser extent. The species with a lower surface activity and lower affinity to charge were less discriminated in the process of nanoelectrospray ionization. This should be especially useful for analysis of more complex samples, such as salt solutions. The presence of NaCl hindered the formation of trimeric cluster in ESI but nano-ESI gave a sufficiently high signal intensity (Fig. 3).

The effect of the presence of $Mg(NO_3)_2$ in a sample was even more complex. The negative salt effect (as for sodium chloride) and competitive formation of magnesium complexes with the analyte and reference ligand made the analysis by ESI impossible due to an insufficient signal of a target

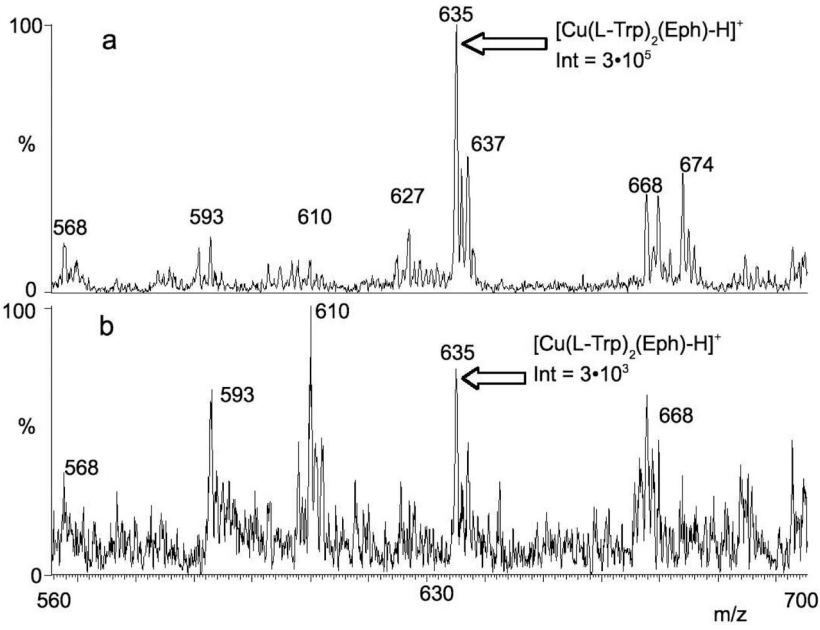


FIG. 3

The influence of salt addition (2×10^{-3} mol l^{-1} NaCl) on the formation of ephedrine trimeric cluster: nano-ESI (a), ESI (b)

TABLE I

Calibration equations (ln $R = A\,C\,(\%\ D)/[1 - (1 - C)\,(\%\ D)] + B$) generated by ESI and nano-ESI and analyses of model samples.

Analyte	Ion source	Calibration curve[a]			Determination coefficient	Sample (% D)[b]		R_{chiral}
		A	*B*	*C*		10	90	
Isoleucine	ESI	–0.021 (±0.006)	6.06 (±0.54)	1.0007 (±0.0002)	0.981	16.9 (6.9)	84.5 (5.5)	6.7
	nano-ESI	–0.054 (±0.016)	6.14 (±0.06)	1.0155 (±0.0341)	0.995	19.1 (9.1)	100.0 (10.0)	8.4
Ephedrine	ESI	–0.010 (±0.001)	–5.01 (±0.29)	1.0002 (±0.0003)	0.993	18.8 (8.8)	102.5 (12.5)	3.4
	nano-ESI	–0.023 (±0.004)	–1.57 (±0.25)	1.0087 (±0.0062)	0.994	10.5 (0.5)	104.1 (14.1)	3.5
Phenylalanine	ESI	–0.020 (±0.001)	3.76 (±0.61)	1.0008 (±0.0001)	0.960	20.4 (10.4)	107.5 (17.5)	6.6
	nano_ESI	–0.047 (±0.002)	4.05 (±0.17)	1.0079 (±0.0062)	0.975	16.2 (6.2)	91.0 (1.0)	7.5

[a] Confidence intervals. [b] Confidence differences from actual values are given in parentheses. Samples: aqueous methanolic solutions (1:1, v/v) of standards.

trimeric cluster. Nano-ESI gave a higher intensity of trimeric cluster signal but it did not provide satisfactory results either. Non-acceptable relative errors of tens of percent were obtained for nano-ESI. Besides the mentioned negative influences, magnesium formed a complex ($[Mg(L\text{-}Trp)_3\text{-}H]^+$, *m/z* 635) that overlapped with the target phenylalanine or ephedrine trimeric cluster ($[Cu(L\text{-}Trp)_2Phe\text{-}H]^+$, $[Cu(L\text{-}Trp)_2Eph\text{-}H]^+$, *m/z* 635 for both) and above all the isobaric product ions were produced by a loss of tryptophan molecule. This interference described previously by us[10] deteriorated quantitative determinations of the enantiomeric ratio for both ion sources.

Nonlinear calibrations[10] were performed to get an acceptable coefficient of determination for both ion sources (Table I). Chiral selectivity was sufficient for all analytes and both ion sources (see R_{chiral} in Table I). Quantitative analysis of standard solution mixtures showed statistically significant difference only for one sample (10% D-ephedrine). Nano-ESI gave better result. Nevertheless, it can be expected that both sources allow determination of the enantiomeric ratio with similar errors for samples without any interferences and with sufficiently high analyte concentrations. The advantage of the application of nano-ESI was proven for samples contaminated with

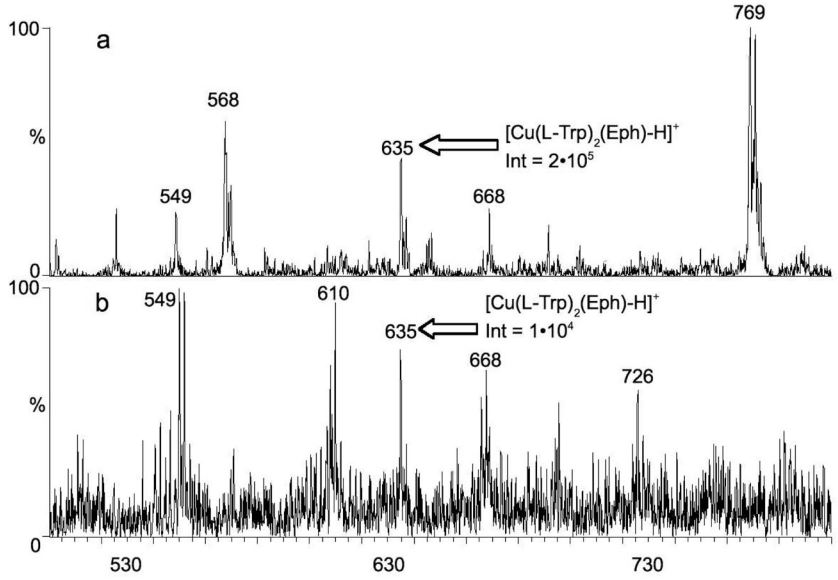


FIG. 4
Trimeric cluster formation in analysis of a real sample (pharmaceutical formulation Mucoseptonex E): nano-ESI (a), ESI (b)

salts. A low signal intensity of the ephedrine-containing trimeric cluster obtained using ESI in the presence of sodium chloride made the analysis impossible. The signal intensity obtained by nano-ESI was higher by two orders of magnitude (Fig. 3). The determined value was 8.5 and 93.6% for samples containing 10 and 90% D-ephedrine, respectively. A similar result was obtained for a real sample represented by a pharmaceutical drug formulation Mucoseptonex E containing D-ephedrine (10 mg l^{-1}) in phosphate buffer. A Mucoseptonex solution was diluted 1:1 (v/v) with methanol and directly analyzed. The matrix negatively influenced the formation of a trimeric cluster due to the formed sodium adducts and competition among the matrix components and the analyte in the electrospray process. A too low signal appeared in the case of ESI whereas nano-ESI allowed the enantiomeric ratio determination (Fig. 4). The determined value was 90% D-ephedrine which was sufficient for screening purposes (discrimination among pure enantiomers and racemic mixture).

CONCLUSIONS

The kinetic method as a tool for analysis of isomers including enantiomers was used with electrospray ionization that produces required trimeric clusters for subsequent fragmentation. Nano-ESI can be used as a viable alternative to commonly employed ESI. The former offered robustness and satisfactory results for sufficiently concentrated analytes in simple matrices that did not disturb the target diastereoisomeric cluster formation. In many cases, especially in real samples, matrix components (e.g. salts) negatively influence the formation of the target clusters. This effect was observed in nanoelectrospray ionization to a lesser extent. Analysis of a model sample enriched in sodium chloride and a real sample containing phosphate buffer (Mucoseptonex E) clearly demonstrates the potential and usefulness of nano-ESI application. The enantiomeric ratio was successfully determined in samples that could not be analyzed by ESI. Nanoelectrospray ionization produces charged droplets with a smaller initial diameter. The low number of droplet decomposition steps before the transfer of ions to gas phase results in a lower influence of matrix constituents competing for charge and space on the droplet surface with target analyte ions.

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