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Ion suppression effects in liquid chromatography–electrospray-ionisation transport-region collision induced dissociation mass spectrometry with different serum extraction methods for systematic toxicological analysis with mass spectra libraries

Claudia Müller, Patrick Schäfer, Mylène Störtzel, Susanne Vogt, Wolfgang Weinmann*

Institute of Legal Medicine, Klinikum der Albert-Ludwigs-Universität Freiburg, Albertstrasse 9, D-79104 Freiburg, Germany

Abstract

Ion suppression effects during electrospray-ionsation mass spectrometry (ESI-MS) caused by different sample preparation procedures for serum were investigated. This topic is of importance for systematic toxicological analysis for which LC–ESI-MS has been developed with transport-region collision-induced dissociation (ECI-CID) and mass spectra library searching. With continuous postcolumn infusion of two test compounds—codeine and glafenine—the ion suppression effects of extracted biological matrix obtained after a standard liquid–liquid extraction, a mixed-mode solid-phase extraction (SPE) method, a protein precipitation method and a combination of precipitation with polymer-based mixed-mode SPE have been investigated. Extracted ion chromatograms of codeine ($[M+H]^+$, m/z 300) and glafenine ($[M-H]^-$, m/z 371) were used for monitoring ion suppression. Severe ion suppression effects for codeine and glafenine were detected in positive and in negative ionisation modes, respectively, in the LC-front peak after serum clean-up with SPE (acid/neutral fraction) and protein precipitation as well as with protein precipitation combined with SPE. Less ion suppression of codeine in positive mode was found with liquid–liquid extraction of serum samples. No ion suppression was detected with the second fraction of the mixed-mode SPE (using RP-C_8 and cation-exchange phase) in both ionisation modes. All suppression effects were caused by polar and unretained matrix components, which were present after extraction and/or protein precipitation. However, no specific ion suppression was seen after elution of the polar LC-front throughout the whole gradient. It could be demonstrated, that ion suppression is not generally present at any retention time when using reversed-phase HPLC with rather long gradient programs, but may play an important role in case of high-throughput LC–MS analysis, when the analyte is not separated from the LC-front, or in flow injection analysis without chromatographic separation.

Keywords: Ion suppression effects; Systematic toxicological analysis; Mass spectra libraries

1. Introduction

Methods for a toxicological screening analysis using LC–MS with electrospray-ionisation transport-region collision induced dissociation (ESI-CID) have been developed in combination with mass spectra libraries of drugs, pesticides and explosives. To date,

*Corresponding author. Tel.: +49-761-203-6854; fax: +49-761-203-6858.

E-mail address: weinmann@sun11.ukl.uni-freiburg.de (W. Weinmann).

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the largest mass spectra library is the library of Marquet et al. with more than 1500 compounds [1,2], followed by our own library (430 compounds) [3,4] and the library of Schreiber et al. [5]. These ESI-CID-MS libraries of drugs, pesticides and explosives have been used for the identification of drugs in serum, urine, hair [1–4] and for the identification of pesticides and explosives in sewage-waters [5] using reversed-phase HPLC with gradient elution. Two different concepts for setting-up ESI-CID-MS libraries for general-unknown screening have been reported: one concept uses composite mass spectra which are obtained by averaging two spectra—one spectrum at low and one at high orifice voltage (the same procedure for positive and negative mode) [1,2]; the other concept uses three single mass spectra with different collision energies [3,5]. Glafenine [1] and haloperidol [3] have been used to check instrument performance and reference compounds have been suggested for a tuning procedure for ESI-CID to obtain a similar degree of fragmentation with different LC–MS instruments [6]. For quality control in pharmaceutical analysis, an LC–MS performance test kit consisting of aspartame, cortisone, reserpine and dioctylphthalate has recently been developed [7] for checking the chromatographic and mass spectrometric performance with regard to sensitivity for protonated molecules in positive mode and deprotoned molecules in negative mode.

Thus, the transport-region CID and the LC separation methods have been investigated and standardised, however, ion suppression effects in ESI-MS with these LC–ESI-CID screening procedures have not yet been investigated. In atmospheric pressure ionisation mass spectrometry (API-MS) ion suppression has recently been investigated for pharmacokinetic studies [8,9]. While atmospheric pressure chemical ionisation (APCI) did not show suppression effects, ion suppression was found with electrospray ionisation (ESI) due to interactions of matrix and analyte in solution when being sprayed by the ESI emitter. Ion suppression is a phenomenon which can be caused by matrix influences during the electrospray ionisation process, or by intermolecular charge transfer in the gas phase [9].

For this work, different sample preparation procedures for serum used in systematic toxicological analysis—liquid–liquid extraction (LLE) [10], solid-phase extraction (SPE) [11], protein precipitation and a combination of SPE and protein precipitation [1]—have been investigated for suppression effects when using ESI-CID-MS with gradient elution, which could be disadvantageous for the detection of the ''general-unknown'' in systematic toxicological analysis.

2. Experimental

The following instrumentation was used: API 365 triple–quadrupole mass spectrometer with turboionspray-source (Applied Biosystems/Sciex, Langen, Germany) Apple Macintosh G3 Power Computer, MASSCHROM 1.1.1 and MULTIVIEW 1.4 software. The mass spectrometer was operated in Q1-scan mode (50–600 amu), orifice voltage and polarity switching from scan to scan ($+20$ V/-20 V, $+80$ V/-80 V) as proposed by Marquet et al. [2]. The nitrogen drying gas (turbo-gas) flow was 4 l/min at a probe temperature of 350 °C. All other parameters were optimized by a standard tuning procedure using haloperidol [6].

A high-pressure gradient system (Shimadzu 10 AD, Duisburg, Germany) consisting of autosampler, two pumps and semimicro-mixing chamber was used with a flow-rate of 250 μl/min, a Synergy polar-RP phenyl-propyl column (Phenomenex, Aschaffenburg, Germany) (150×2 mm, 4 μm) with precolumn-cartridge and a binary gradient, which consisted of the following HPLC-grade solvents: A: 1 m*M* ammonium formate–0.1% formic acid (pH 3.1) and B: acetonitrile. The gradient was as follows: 0–1 min, 5% B; 1–5 min: 5–30% B linear; 5–19 min, 30–90% B linear; 19–21 min, 90% B.

Continuous infusion of codeine and glafenine (10 μg/ml) were performed by a syringe pump (Harvard, Quebec, Canada) and a 1-ml syringe (Hamilton, Bonaduz, Switzerland) with a flow-rate of 20 μl/min via a PEEK tee into the eluent from the LC column while an aliquot (20 μl, equal to one fifth of the concentrated extract of 1 ml serum) of blank human serum extract was analysed by gradient elution. The ion intensities of of codeine ($[M+H]^+$, m/z 300) and glafenine ($[M-H]^-$, m/z 371), which were continuously infused postcolumn, were used for monitoring ion suppression.

Postcolumn addition of acetonitrile (180 μl/min) was performed by a postcolumn HPLC pump (Pharmacia/LKB, Freiburg, Germany) to obtain stable spraying conditions and a stable baseline in scan mode even at low concentrations of organic modifier in the LC effluents (Fig. 1). Postcolumn addition [12] of acetonitrile had been used in our previous work to improve the sensitivity for ethylglucuronide with negative ESI-MS [13].

2.1. Extraction procedures

2.1.1. Liquid–liquid extraction [10]

A mixture of 1 ml serum sample and 1 ml saturated Na_2SO_4 was extracted by addition of 2 ml ethylacetate–diethylether (1:1, v/v), vortex-mixing, centrifugation and phase separation (organic phase: extract 1). The aqueous phase was then basified by addition of 100 μl 1 *M* NaOH, and extracted again using 2 ml ethylacetate–diethylether (extract 2). Both extracts (extract 1 and 2) were then mixed, evaporated to dryness (nitrogen gas, 45 °C) and redissolved in 100 μl HPLC solvents (A–B, 1:1, v/v). A 20-μl volume of the extract was injected into the LC–MS system. Suppression effects of this extraction procedure are shown in Fig. 2a.

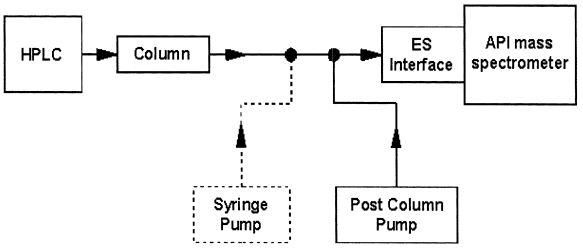

Fig. 1. Analysis set-up for the investigation of ion suppression: different blank serum extracts were chromatographed using gradient elution, while codeine and glafenine were infused continuously at the end of the column into the eluent. The degree of ion suppression due to matrix components in the eluent was monitored at any time during the gradient elution by continuously acquiring full-scan spectra of codeine and glafenine using single–quadrupole mass spectrometry. Decrease of signal intensities of the protonated molecules of codeine or glafenine means increase in ion-suppression (see Fig. 2). Postcolumn addition of acetonitrile was used to obtain stable spraying conditions even at low contents of organic solvent (acetonitrile) in the LC eluent.

2.1.2. SPE with two fractions: acidic/neutral and alkaline fraction [11]

An automated SPE device was used: RapidTrace (Zymark, Idstein, Germany) with a mixed-mode RP-C_8/cation-exchange SPE column (Chromabond Drug, Machery Nagel, Düren, Germany). Blank serum (1 ml) was mixed with 1 ml 0.1 *M* KH_2PO_4 (pH 6) and applied to a preconditioned SPE column (conditioning by 2 ml MeOH and 2 ml KH_2PO_4). The column was rinsed with 1 ml 0.1 *M* HAC and 0.1 ml MeOH. Then the first fraction (acidic/neutral fraction) was eluted by 1.5 ml acetone–dichloromethane (1:1, v/v).

Then the column was eluted with 1.5 ml of a mixture of dichloromethane–2-propanol–NH_4OH 25% (80:20:2, v/v) to obtain the alkaline fraction. The eluates were evaporated to dryness and were redissolved with 100 μl each (A–B, 1:1, v/v). 20 μl of each extract was injected into the LC–MS system. The suppression effects of these two extracts are shown in Fig. 2b and c.

2.1.3. Solid-phase extraction using Oasis combined with protein precipitation [1]

Oasis MCX (3 cc/30 mg, Waters, Eschborn/Germany), a polymer-based cation-exchange stationary phase, was used for the extraction of 1 ml serum after column conditioning with 1 ml methanol and 1 ml H_2O. The column was washed with 1 ml 0.1 *M* HCl, followed by drying with nitrogen gas for 1 min. The elution was performed with 1 ml methanol–NH_4OH 25% (98:2, v/v), the eluate was evaporated to dryness (Oasis SPE extract).

For protein precipitation 100 μl serum and 100 μl acetonitrile were vortexed and centrifuged. Either, a 20-μl aliquot of the supernatant was injected directly (suppression effects are shown in Fig. 2e), or an 100-μl aliquot of the supernatant was used to redissolve the Oasis SPE extract and 20 μl were injected (suppression effects are shown in Fig. 2d).

3. Results and discussion

The intensities of the protonated molecules of continuously infused codeine ($[M+H]^+$, m/z 300) and glafenine ($[M-H]^-$, m/z 371) were used for monitoring ion suppression. Severe ion suppression

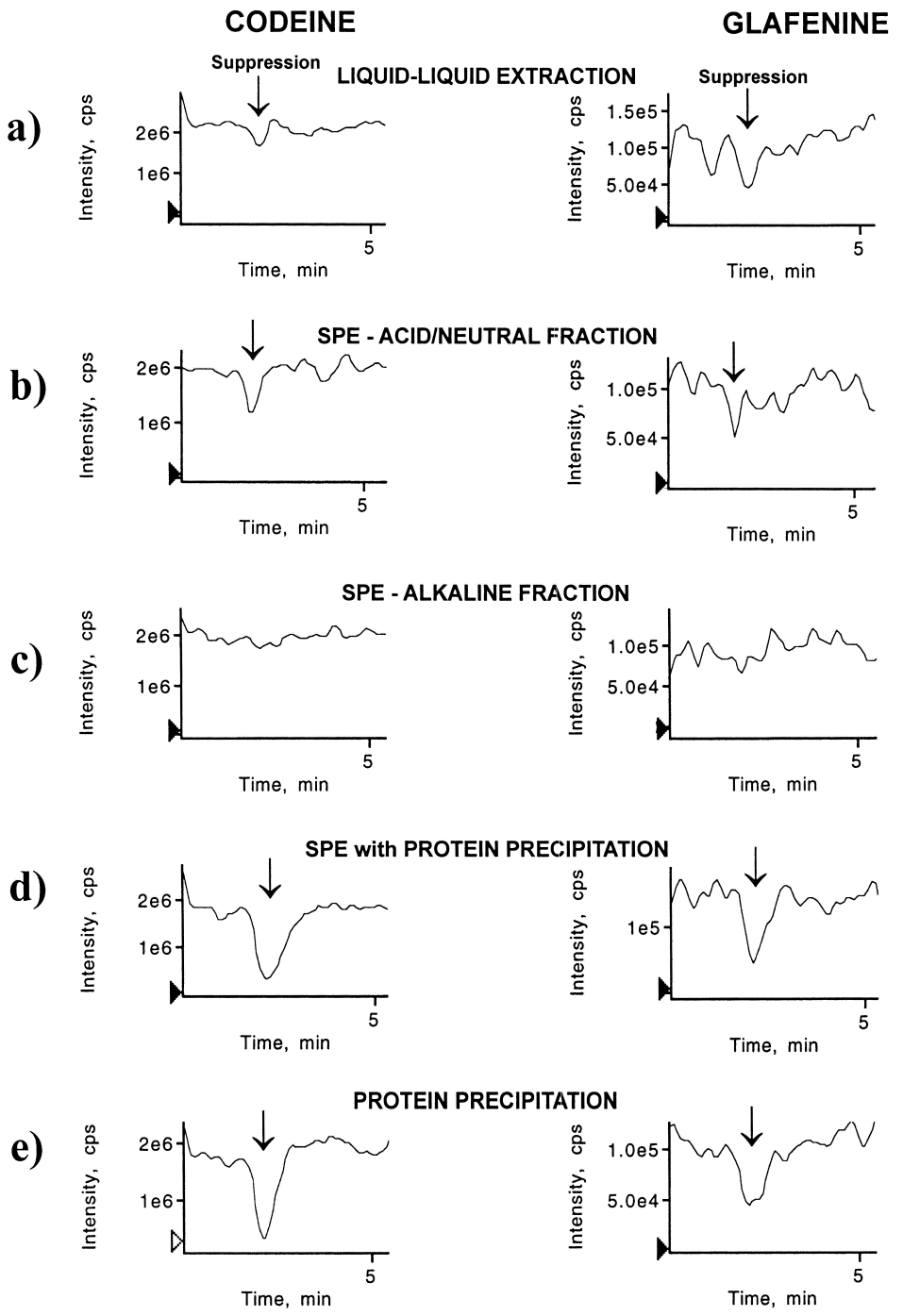

Fig. 2. Ion intensities of protonated codeine ($[M+H]^+$, m/z 300, positive ESI mode, figures on the left side) and deprotonated glafenine ($[M-H]^-$, m/z 371, negative ESI mode, figures on the right side) extracted from full-scan analysis during a postcolumn continuous infusion experiment. Different extracts of serum samples were injected into the LC system (a–e), resulting in ion suppression during elution of unretained, polar compounds in the LC front, which is monitored as a loss in signal intensities for codeine and glafenine. The regions with ion suppression are marked with arrows). No ion suppression was observable after elution of the LC front ($t_R > 2.9$ min).

effects for codeine and glafenine (see Fig. 2, marked by arrows) were detected in positive and in negative ionisation mode, respectively, in the LC-front peak (at $t_R = 2$ min) after serum clean-up with SPE (acid/neutral fraction, Fig. 2b) and protein precipitation (Fig. 2e) as well as with protein precipitation combined with SPE (Fig. 2d).

Less ion suppression of codeine in positive mode was found with LLE of serum samples using the mixture of diethylether–ethylacetate (1:1, v/v) (see Fig. 2a). Practically no suppression of the protonated molecules of codeine and glafenine was detected with the second fraction of the SPE extract (Fig. 2c) in positive and negative ionisation modes. Ion suppression was in all cases observed in the LC-front, when polar nonretainable substances eluted from the reversed-phase LC column. No signs for ion suppression were observed after the LC-front throughout the whole gradient (running from 10 to 90% acetonitrile), however, in positive ionisation the baseline was more stable than in negative ionisation—presumably due to the acidic pH value of the LC eluents, which is not optimal for negative ionisation.

The repetition of these experiments for control of reproducibility ($n = 3$) yielded the same results. No higher degree of transport-region CID of the analytes was detected in the scan-mode during the time of ion suppression, showing, that the substances do not just fragment into smaller, detectable fragment ions by transport region CID, but are completely suppressed by polar matrix components. Matrix components which can cause ion suppression are probably ionic substances or substances which show activity to the surface tension of the charged droplets and thus can either be involved in charge transfer processes in the gas phase or in the electrospray ionisation process. These experiments were not conducted, to differentiate between these two processes, but were performed to find out, which serum extraction methods used in clinical or forensic toxicology can result in severe ion suppression, making a detection of a compound impossible. It could be shown that the LLE method of Pfleger et al. at neutral and alkaline pH resulted in less (positive mode) to similar (negative mode) suppression as the lipophilic interactions-based solid-phase extract (acid–neutral fraction) with the mixed mode SPE. The second extract (alkaline fraction) of the mixed mode SPE did not contain any matrix compounds which were involved in ion suppression—probably because they had already been eluted with the first fraction. Protein precipitation and the combination of protein precipitation with Oasis SPE resulted in severe ion suppression in the LC-front peak but not after the LC-front had passed the LC column by gradient elution.

4. Conclusions

Ion suppression has been found to be critical in the LC-front peak with all tested serum sample preparation methods—except with the second fraction of the mixed mode SPE. However, with all tested extraction methods no specific suppression effects were detected during the rest of the gradient LC program. These results show that the fear of ion suppression should not be a general reason to reject ESI as a ionisation procedure suitable for use in systematic toxicological analysis when coupled to LC with gradient elution. However, for flow injection analysis without LC separation or for fast LC methods involving very short retention times—such as high-throughput analysis—the possibility of ion suppression of the target analytes has to be taken into consideration for method development. Due to ion suppression in the LC-front, LC–ESI-MS cannot be used with all presented extraction methods to detect polar, unretained compounds in the LC-front of serum extracts. Unretained compounds could be polar drugs or polar metabolites. These experiments have been performed with serum extracts, however, no studies have been performed with urine extracts or other biological matrices of forensic interest (hair, tissue extracts, etc.). Due to the higher polar matrix load of urine and the higher interindividual variability in matrix compounds of urine samples compared to serum samples, the applicability of LC–ESI-MS for urine analysis is more critical, and has to be investigated separately for different kinds of urine samples.

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