Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 225–230
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Quantitative determination of pharmaceuticals using nano-electrospray ionization mass spectrometry after reversed phase mini-solid phase extraction

Lining Qi, Neil D. Danielson*

Department of Chemistry and Biochemistry, Miami University, Oxford, OH 45056, USA

Received 9 July 2004; accepted 13 September 2004
Available online 5 November 2004

Abstract

The pre-concentration effect of solid phase microextraction (SPE) with nano-electrospray ionization mass spectrometry (nano-ESI MS) for selected pharmaceuticals is presented. An analytical method is developed for the quantitative determination of dicyclomine in serum with cyclopentolate as the internal standard by off-line nano-ESI ion-trap MS with reversed phase mini-SPE. Homemade C18 and C4 mini-SPE cartridges of 0.5 and 1 cm in length and 1.55 mm i.d. have been tested for pre-concentration of samples originally 30 μL in volume. After SPE, the volume of the sample in methanol is about 1–2 μL and 0.5 μL can be injected into the nano-ESI MS instrument. Use of a 1 cm C18 cartridge lowered the detection limit of dicyclomine 100 times to 16.1 fmole. Dicyclomine spiked in serum can be determined by nano-ESI MS after protein precipitation and further clean-up on the 1 cm C18 cartridge. However, the slope of a calibration curve of dicyclomine standards spiked in serum is more than a factor of 10 less than that for a calibration curve of dicyclomine standards prepared in water indicating pre-concentration of pharmaceuticals could be compromised by a complex biological matrix.

Keywords: Solid phase extraction; Nano-electrospray ionization MS; Dicyclomine

1. Introduction

Nano-electrospray ionization (nano-ESI) is a miniaturized electrospray source with a nm droplet size and flow decreased to the sub μL/min range. Very small amounts (1–2 μL) of the sample can be loaded into the capillary with no need of solvent pumps or inlet valves for mass spectrometry (MS). With the flow rate lowered to the range of 20 nL/min to 1 μL/min, the transfer efficiency (number of ions detected/number of analyte molecules sprayed) for peptides can be substantially higher for nano-ESI than standard ESI. Off-line nano-ESI MS also has advantages of solvent choice over a wide range of composition and pH, an extraordinary tolerance to salt contamination, and a more uniform response for chemically very different analytes [1,2]. Nano-ESI tandem MS has been successfully applied for the determination of steroid sulphates in brain tissue [3], cholesterol [4], lipids in membranes [5], and underivatized oligosaccharides (particularly difficult for standard ESI MS) [6], as well as protein sequencing [7].

Microscale solid phase extraction (SPE) sample preparation has been developed primarily for matrix assisted laser desorption ionization-MS (MALDI-MS) and more recently for nano-ESI MS. C18-fused silica capillaries have been used for concentration and desalting of peptide samples before MALDI-MS [8]. Micro pipet tips packed with a 2 μL packed bed of Poros 50 R2 resin previously sized to 40–60 μm have been used for the clean-up and concentration of peptide samples before MALDI-MS [9]. Affinity chromatography using similar gel-loader pipet tips with a 0.2 μL bed of reactive red-120 resin and elution using a low speed centrifuge was applied to protein samples before MALDI-MS [10]. A com-

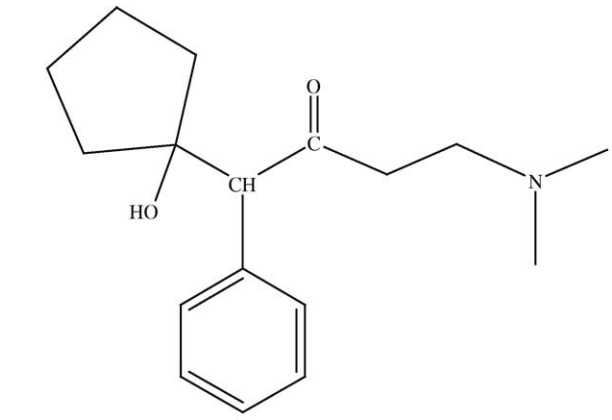
* Corresponding author. Tel.: +1 513 529 2872; fax: +1 513 529 5715.
E-mail address: danielnd@muohio.edu (N.D. Danielson).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.09.023

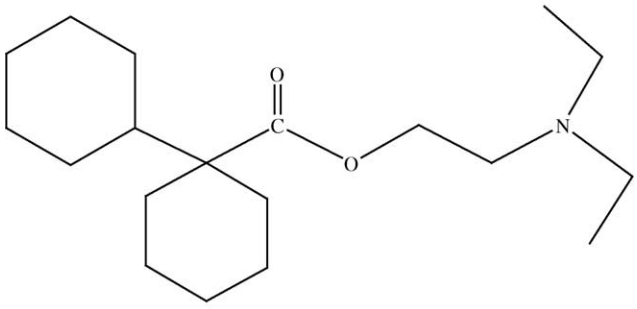
mercial version of this packed micro-tip for MALDI-MS is now available from the Millipore Corp. [11]. This device is called a ZipTip, a 10 μL pipette tip, which has only 0.6 μL of chromatographic media (C4, C18 or strong cation exchange) packed near the tip. The C18 ZipTip is most often used for desalting and concentrating peptides before MS analysis, such as MALDI-TOF and potentially nano-ESI MS. Applications showing use of these tips for nano-ESI MS are lacking. One application of off-line SPE using ion exchange columns of dimensions 5–8 cm × 0.4 cm facilitated the determination of neurosteroids in rat brain by nanoscale liquid chromatography (LC) ESI MS [12]. Several recent on-line micro SPE approaches include SPE of heterocyclic aromatic amines on a C18 particle packed capillary segment before capillary electrophoresis separation with nano-ESI MS detection [13], sample pre-concentration of *N*-acylhomoserine lactones on a 75 μm i.d. capillary column before nano-LC ESI MS [14], and protein pre-concentration on a 50 μm i.d. × 4 cm C18 SPE column acting as the sample injection loop for nano-LC ESI MS [15].

Dicyclomine, one of the amine anticholinergic pharmaceuticals, is a very important parasympatholytic agent and it has been used in the treatment of gastrointestinal disorders. Overdose of dicyclomine in adults could result in drowsiness and blurred vision and death in infants from an overdose of dicyclomine is possible [16]. Both GC and LC methods for dicycloamine are available. After extraction from human plasma with ether, GC with nitrogen-selective detection has been applied for the determination of dicyclomine [17]. A similar GC study for dicyclomine using chlorocyclizine as the internal standard has also been published [18]. Sensitive and selective UV–vis detection of dicycloamine after HPLC separation is not straightforward since only an isolated C=O moiety is present in the structure (Fig. 1). Microbore HPLC of tertiary amines using tris(bipyridyl)ruthenium(III) chemiluminescence detection has been reported for dicycloamine, cyclopentolate, and other anticholinergic pharmaceuticals [19]. Cyclopentolate (molecular weight 291.4) is similar in structure to dicycloamine (molecular weight 309.5) but is not a common prescription drug (Fig. 1).

To the best of our knowledge, pre-concentration of organic compounds, like pharmaceuticals using ZipTips or similar cartridges in conjunction with nano-ESI MS, is not evident in the literature. In the present study, we have determined dicyclomine with cyclopentolate as the internal standard by nano-ESI MS after reversed phase mini-SPE. Mini-SPE refers to cartridges dimensionally smaller (1–2 mm i.d. × 0.5–1 cm) than standard SPE ones but not fibers as used for micro-SPE. Two different lengths (0.5 and 1 cm) of C18 and C4 reserved phase SPE cartridges were packed and characterized for the pre-concentration effect. These packed beds of about 9 and 20 μL are considerably larger than those in the micro tips described previously for peptides and proteins. Experimentally, the technique is facile since the pipet tip to load the sample fits into the mini-SPE tip, which fits into the metalized needle used to introduce the sample into the nano-ESI inlet (Fig. 2).



Cyclopentolate



Dicyclomine

Fig. 1. Structures of dicyclomine and cyclopentolate.

2. Experimental

2.1. Chemicals and reagents

Dicyclomine hydrochloride (>99%), cyclopentolate hydrochloride (>99%) and porcine serum were obtained from Sigma Chemical Co. (St. Louis, MO). Geloader tips (1–10 μl) were purchased from Eppendorf (Hamburg, Germany). C18 (carbon 23%) and C4 (carbon 8%) reversed phase silica (40–63 μm) particles were obtained from Silicycle Chemical Division (Quebec, QC, Canada). Methanol (MeOH) was purchased from Fisher Scientific (Cincinnati, OH). Acetonitrile (ACN) was purchased from Fisher Scientific (Fair Lawn, NJ) and ethanol was purchased from Quantum Chemical Co. (Newark, NJ). Distilled, deionized, filtered water was generated using an E-Pure water treatment system (Barnstead, Dubuque, IA).

2.2. Sample preparation

Different volumes of a dicyclomine standard and a constant volume of the internal standard (2.5 μL of 100 mg/L cyclopentolate) were added to 25 μL of serum, which were then diluted with water 10 times to 250 μL. All solutions were

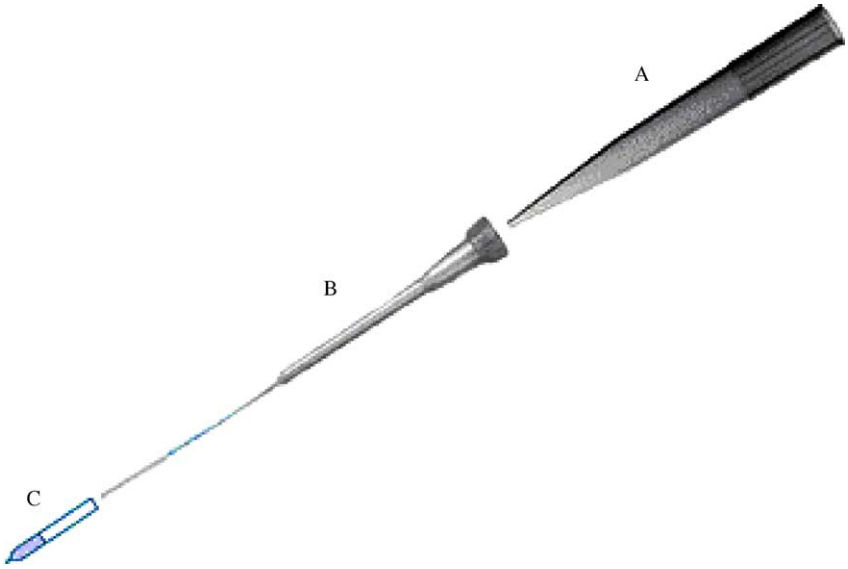


Fig. 2. Exploded diagram of large pipet tip (A) to load sample and elute fractions, the mini-SPE column made from a gel-loader tip (B), and the metalized needle (C) for interfacing to the nano-ESI MS port.

vortex-mixed. A volume of 100 μL ACN–ethanol (1:1, v/v) was added to a 25 μL portion of the spiked serum for protein precipitation and the solutions were vortex-mixed. After centrifugation (14,000 × *g* for 12 min), the supernatant was transferred to another microcentrifuge tube and evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was reconstituted with 100 μL water; any pre-concentration due to sample volume differences before mini-SPE was small. All final samples were filtered with a 0.2 μm syringe filter disk.

2.3. Off-line solid phase extraction

Samples were extracted using mini SPE cartridges, which were packed with different amounts of C18 and C4 sorbents in gel-loader tips (Fisher, Pittsburgh, PA). For the 1 cm columns, 9 mg was used to make a 20 μL packed bed and for the 0.5 cm long cartridges, 3.6 mg was sufficient to make 8.8 μL packed bed. Because of the taper of the gel-loader tip, the weight of packing used is not proportional to the column length (Fig. 2). The two ends of the cartridge were blocked with compressed glass fiber to prevent particle leakage.

SPE cartridges were pre-conditioned prior to sample application using 30 μL of deionized distilled water. The sample solution (30 μL) was applied to the cartridge and forced out slowly by air pressure with a 1 mL pipet until the cartridge appeared dry and no more water droplets came out. The analytes were eluted in a stepwise fashion with first 5 μL and then six successive 3 μL portions of MeOH using the 1 mL pipet at a flowrate of 30 μL/min for the 1 cm long cartridge. For the 0.5 cm cartridge, the analytes were eluted stepwise with seven successive 3 μL portions of MeOH using the same pipet and flowrate. Although a 3 μL volume of MeOH was added, some eluent fractions actually collected in the micro centrifuge tubes were less than 1 μL due to possible evaporation and/or trapped solvent. For characterization of the signal as a function of eluent volume, each fraction was loaded into the metalized needle and analyzed individually with nano-ESI MS.

2.4. Mass spectrometry

Off-line nano-ESI mass spectrometry detection was performed on an Esquire∼LC system (Bruker, Billerica, MA) operated in the positive ion mode. After optimization, the MS parameters were held constant for positive nano-ESI MS as: capillary voltage: 4500 V, capillary exit: 99.8 V, accumulation time: 4525 ms, scan range: 160–460 *m*/*z*, averages: 16 spectra, skim: 128.9 V, trap drive: 42.4. A 0.5 μL volume was loaded to the metalized needle each time. The ion intensities of the MH^+ ions for dicyclomine and cyclopentolate were monitored and ratioed as a function of dicyclomine concentration.

3. Results and discussion

3.1. Comparison of standard ESI and nano-ESI MS

Both standard ESI MS and nano-ESI MS without any pre-concentration step were first compared to check the quantitation of an aqueous standard solution of dicyclomine with the internal standard cyclopentolate. With standard ESI MS using a sample volume of 100 μL, a detection limit of 1 mg/L (Fig. 3) and a short linearity range for dicyclomine from 2 to 5 mg/L with the equation of the line $y = 0.63x - 0.75$, $n = 4$, $R^2 = 0.9878$, where y = peak intensity ratio dicyclomine/cyclopentolate and x = mg/L. R.S.D.

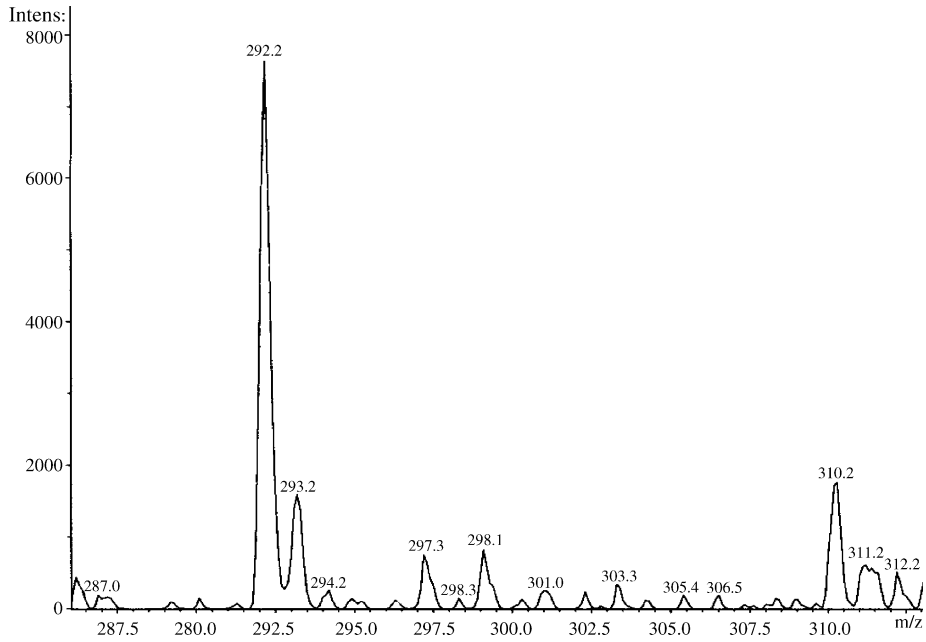


Fig. 3. Detection limit standard ESI MS spectrum of a 1 mg/L dicyclomine ($MH^+ = 310$) and 1 mg/L cyclopentolate ($MH^+ = 292$) mixture in H_2O (no SPE). Ion polarity: positive, averaged 8 spectra.

for these data points ranged from 4.5 to 9.6% with an average 7.5%. These calibration curve data were not considered satisfactory for clinical analysis. With nano-ESI MS, sample consumption was only 0.5 μL but the detection limit and linearity for aqueous samples were similar to those for standard ESI MS (data not shown). An unexpected potential advantage found for SPE was the nano-ESI MS response, which is about 2–4 times stronger in MeOH than H_2O (Fig. 4A and B). Acetonitrile was found to be not a good choice for mini-SPE cartridge elution in conjunction with nano-ESI MS probably because it cannot easily assist in protonation of organic amines. The relative peak intensity comparison using nano-ESI MS between no SPE and 1 cm C18 SPE is shown in Fig. 5. A definite 8–10 enhancement factor for the detectability of organic compounds after mini-SPE by nano-ESI MS is evident and should be due to both a pre-concentration effect and the change in solvent from water to methanol.

3.2. Nano-ESI MS with mini-SPE

The pre-concentration effect of different length C18 and C4 SPE cartridges was compared. With 1 cm C18 or C4 mini-SPE, dicyclomine and cyclopentolate could be detected from the fourth to sixth eluent fractions with nano-ESI MS (Fig. 6) with the fifth eluent fraction showing the best relativity intensity ratio for dicyclomine/cyclopentolate. The mass spectrum for a 10 μg/L solution for the fifth fraction, taken using the 1 cm C18 cartridge, is shown in Fig. 7. For Fig. 7, the relative intensity of dicyclomine and cyclopentolate is similar due to preconcentration as compared to Fig. 4B. As shown in Fig. 7, a detection limit of 10 μg/L has been reached, an improvement of about 100 over nano-ESI MS without mini-SPE. Using the 1 cm C18 cartridge, linearity was established from 0.05 to 5 mg/L with the equation of the line $y = 3.03x + 0.12$, $n = 6$, $R^2 = 0.9916$, where y = peak intensity ratio dicyclomine/cyclopentolate

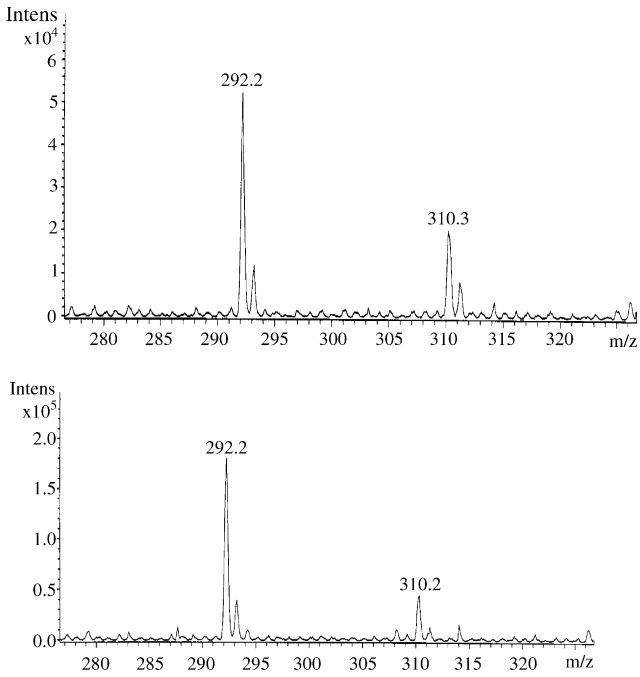


Fig. 4. (A) Nano-ESI MS spectrum of a 1 mg/L dicyclomine (310) and 1 mg/L cyclopentolate (292) mixture in water (no SPE). Averaged spectra: 8. (B) Nano-ESI MS spectrum of a 1 mg/L dicyclomine (310) and 1 mg/L cyclopentolate (292) mixture in MeOH (no SPE). Averaged spectra: 8.

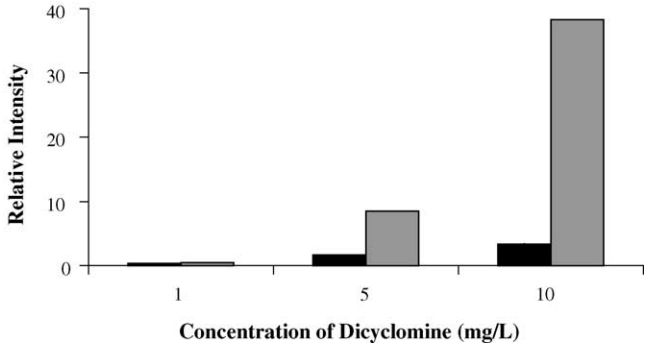


Fig. 5. Comparison of the relative intensity (dicyclomine/cyclopentolate) ratio with (gray) and without (black) mini-SPE using the 1 cm C18 cartridge.

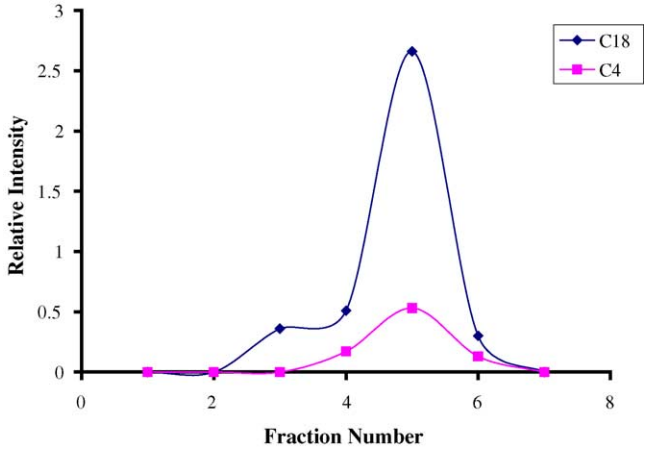


Fig. 6. Plot of relative intensity (dicyclomine/cyclopentolate ratio) vs. fraction collected for 1 mg/L dicyclomine and 1 mg/L cyclopentolate from 1 cm mini-SPE column. First fraction: 5 μL, successive fractions 3 μL. Points are connected for clarity.

and x = mg/L. Using the 1 cm C4 cartridge, linearity was established from 0.05 to 4 mg/L with the equation of the line $y = 0.48x + 0.11$, $n = 6$, $R^2 = 0.9887$, where y = peak intensity ratio dicyclomine/cyclopentolate and x = mg/L. The R.S.D. ($n = 3$) for all these data ranged from 1.0 to 8.2% with an overall average R.S.D. of 3.7%.

Using the 0.5 cm C18 or C4 cartridge, the best detection limit of 100 μg/L was found from the fifth eluent fraction similar to that shown for Figs. 6 and 7. This optimum fraction number is not less than that for the 1 cm cartridges due to the fact that 5 μL was the volume of the first fraction using the 1 cm cartridge but only 3 μL for the 0.5 cm cartridge. Linearity ($y = 0.65x - 0.19$, $n = 6$, $R^2 = 0.9931$, where y = peak intensity ratio dicyclomine/cyclopentolate and x = mg/L) was established from 0.5 to 5 mg/L with the C18 cartridge with a detection limit of 100 μg/L. Linearity ($y = 0.68x - 0.29$, $n = 5$, $R^2 = 0.9932$, where y = peak intensity ratio dicyclomine/cyclopentolate and x = mg/L) was established from 0.5 to 4 mg/L with the C4 cartridge with a detection limit of 100 μg/L. The R.S.D. ($n = 3$) for these data ranged from 0.9 to 17.7% with an overall average R.S.D. of 3.7%.

The possibility of a matrix effect causing ion suppression or enhancement for pharmaceuticals in plasma samples determined by LC–MS has been shown to be higher when the ESI MS interface is used as opposed to only the heated nebulizer required for atmospheric pressure chemical ionization [20]. Therefore, the pretreatment of biological samples is important for ESI MS methods. Because the 1 cm C18 mini-SPE cartridge was found to have the best pre-concentration effect (100 times) with an improved sensitivity (slope) of a factor of 6 over the C4 cartridge, it was chosen to determine spiked serum samples. Spiked synthetic serum samples were determined by nano-ES MS after protein precipitation and further clean-up using the 1 cm C18 mini-SPE cartridge. The relative dicyclomine/cyclopentolate intensity ratio for a given dicyclomine concentration in synthetic serum was significantly less than that for the same standard solution in water and an additional peak at 308 m/z was observed. Therefore, a set of standard dicyclomine solutions was prepared in serum matrix before 1 cm C18 SPE and nano-ESI MS. Although good linearity ($y = 0.15x + 0.74$, $n = 6$, $R^2 = 0.9986$, where y = peak

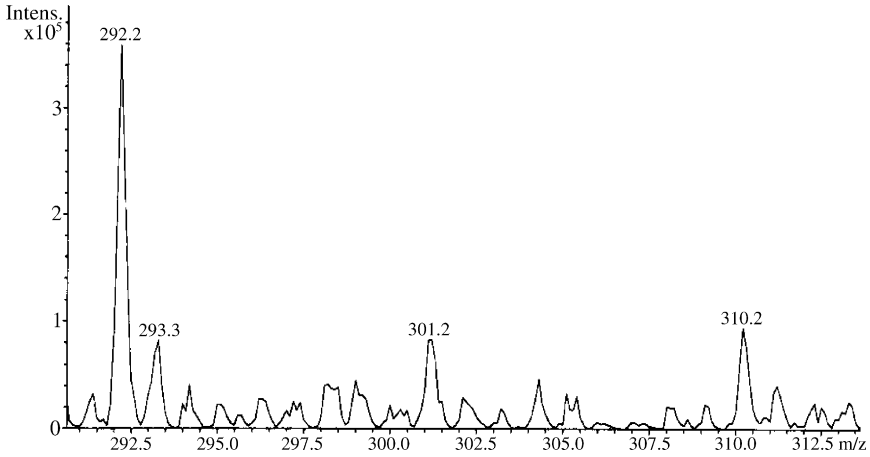


Fig. 7. Detection limit nano-ESI mass spectrum of a 10 μg/L dicyclomine (m/z 310) and 1 mg/L cyclopentolate (m/z 292) mixture originally in H_2O after 1 cm C18 mini-SPE. For (+) mode nano-ESI MS, 16 spectra averaged.

intensity ratio dicyclomine/cyclopentolate and x = mg/L) was observed for the calibration curve from 0.5 to 30 mg/L with a detection limit in only the low mg/L range for spiked dicyclomine/cyclopentolate in serum using the fifth collected fraction, the low slope value implies little pre-concentration probably occurred. The R.S.D. ($n = 3$) for these data ranged from 1.9 to 6.1% with an overall average R.S.D. of 4.1%. To demonstrate longer term reproducibility, a second smaller series of 1, 2, and 3 mg/L dicyclomine spiked serum samples when compared to this calibration curve showed signal ratios on the order of $91.2 \pm 3.0\%$, $88.0 \pm 5.3\%$, and $86.2 \pm 7.8\%$ ($n = 3$), respectively.

4. Conclusion

The pre-concentration effect of mini-SPE with nano-ESI MS was studied for the quantitative determination of dicyclomine with cyclopentolate as the internal standard. Both 1 cm and 0.5 cm of C18 and C4 reversed phase SPE cartridges were compared. The fraction after elution of about 17 μL of MeOH from the 1 cm C18 SPE cartridge had the lowest detection limit of 10 μg/L (16.1 fmole). This was lowered 100 times as compared to standard or nano-ESI MS and a calibration curve starting below 1 mg/L could be established from 0.05 to 5 mg/L. Although the spiked dicyclomine samples in synthetic serum were likely not pre-concentrated but were likely cleaned-up with the 1 cm C18 cartridge, it is expected that reversed phase mini-SPE could facilitate the quantitation of these and other pharmaceuticals, particularly in simpler sample matrices, before nano-ESI MS detection.

Acknowledgements

Support for purchase of the ESI MS instrument and nano-ESI MS accessory through grants from the Hayes Foundation and Miami University to establish the Ohio Mass Spectrometry Consortium is greatly appreciated.

References

[1] M. Wilm, M. Mann, Anal. Chem. 68 (1996) 1–8.
[2] M. Karas, U. Bahr, T. Dülcks, Fresenius J. Anal. Chem. 366 (2000) 669–676.
[3] W.J. Griffiths, S. Liu, Y. Yang, R.H. Purdy, J. Sjövall, J. Rapid Commun. Mass Spectrom. 13 (1999) 1595–1610.
[4] R. Sandhoff, B. Brügger, D. Jackel, W.D. Lehmann, F.T. Wieland, J. Lipid Res. 40 (1999) 126–132.
[5] B. Brügger, G. Erben, R. Sandhoff, F.T. Wieland, W.D. Lehmann, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 1339–1344.
[6] U. Bahr, A. Pfenninger, M. Karas, Anal. Chem. 69 (1997) 4530–4535.
[7] M. Wilm, A. Shevchenko, T. Houthaeve, S. Breit, L. Schweigerer, T. Fotsis, M. Mann, Nature 379 (1996) 466–468.
[8] H. Zhang, P.E. Andren, R.M. Caprioli, J. Mass Spectrom. 30 (1995) 1768–1771.
[9] H. Erdjument-Bromage, M. Lui, L. Lacomis, A. Grewal, R.S. Annan, D.E. McNulty, S.A. Carr, P. Tempst, J. Chromatogr. A 826 (1998) 167–181.
[10] F. Rusconi, J.-M. Schmitter, J. Rossier, M. le Maire, Anal. Chem. 70 (1998) 3046–3052.
[11] M.G. Pluskal, Nat. Biotechnol. 18 (2000) 104–105.
[12] S. Liu, J. Sjovall, W.J. Griffiths, Anal. Chem. 75 (2003) 5835–5846.
[13] P. Viberg, S. Nilsson, K. Skog, Anal. Bioanal. Chem. 378 (2004) 1729–1734.
[14] M. Frommberger, P. Schmitt-Kopplin, G. Ping, H. Frisch, M. Schmid, Y. Zhang, A. Hartmann, A. Kettrup, Anal. Bioanal. Chem. 378 (2004) 1014–1020.
[15] Y. Shen, N. Tolic, C. Masselon, L. Pasa-Tolic, D.G. Camp, K.K. Hixson, R. Zhao, G.A. Anderson, R.D. Smith, Anal. Chem. 76 (2004) 144–154.
[16] J.C. Garriott, R. Rodriguez, L.E. Norton, Clin. Toxicol. 22 (1984) 455–462.
[17] P.J. Meffin, G. Morre, J. Thomas, Anal. Chem. 45 (1973) 1964–1966.
[18] E. Beretta, G. Vanazzi, J. Chromatogr. 308 (1984) 341–344.
[19] J.A. Holeman, N.D. Danielson, J. Chromatogr. Sci. 33 (1995) 297–302.
[20] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019–3030.