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Ionic liquid-based liquid phase microextraction with direct injection for capillary electrophoresis

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ABSTRACT

Liquid–liquid microextraction using the water immiscible ionic liquid, 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, EMIM $NtfO_2$, for the concentration and cleanup of basic compounds for analysis by CE has been investigated. Using an electrolyte comprising 1 mol/L alanine and 3 mol/L acetic acid, EMIM $NtfO_2$ could be directly injected into the capillary after liquid phase extraction. Using the basic dye chryisoidine, sensitivity enhancements approaching 1000-fold were obtained by mixing 20 μL of EMIM $NtfO_2$ with 1500 μL of aqueous sample, leaving only 5 μL of the undissolved ionic liquid which was used for injection into the CE. Lower more repeatable enhancement factors of 200-fold were obtained with slightly larger initial 25 μL volumes of EMIM $NtfO_2$ due to the larger residual volume of ionic liquid which made handling easier. This could be extended to basic pharmaceuticals, and the extraction of clozapine and its two active metabolites, nor-clozapine and clozapine-N-oxide, was demonstrated from urine with enrichment factors greater than 100 obtained. Handling of potentially more dangerous samples, such as serum, through in-vial extraction of clozapine and its metabolites and direct injection of the ionic liquid layer was also demonstrated with enhancements in sensitivity of 80. Limits of detection from 3 to 11 μg/L and 6 to 55 μg/L were obtained from urine and serum, respectively, which are sufficiently low to be useful for the determination of these pharmaceuticals clinically for therapeutic drug monitoring and for forensic toxicology.

1. Introduction

Since its introduction into mainstream analytical chemistry in the 1980s, capillary electrophoresis (CE) has been recognised as a powerful liquid-phase separation technique that is complementary with high performance liquid chromatography (HPLC). While it offers a number of advantages, one of the greatest disadvantages is its poor concentration detection limits, which are often inferior to HPLC by 1–2 orders of magnitude. A number of on-line strategies have been developed to overcome these issues, which can be considered to be based on chromatography or electrophoresis [1–5]. Those based on electrophoresis include stacking, sweeping, dynamic pH junction, isotachophoresis, etc., and rely on analytes being concentrated at a chemical discontinuity inside the capillary. When combined with a voltage injection, they can provide significant enhancements in sensitivity, with improvements over 1 million reported [6], but they often fail when dealing with complex samples that have received little off-line treatment. Chromatographic approaches on the other hand, are often more compatible with more complex samples because the targets are adsorbed onto or into a distinct chromatographic phase thus they can be simultaneously concentrated and isolated from the sample matrix.

Chromatographic methods for cleanup and concentration, such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE), and their newer miniaturised counterparts, solid-phase microextraction (SPME) and liquid phase microextraction (LPME), can be easily implemented off-line and have been used frequently with CE over the past 2 decades for many applications. The difficulty in using chromatographic methods for on-line concentration lies in introducing the chromatographic phase [7–10]. The first reports of SPE appeared in the early 1990s in which small chromatographic particles were placed in a sleeve at the tip of the capillary [11–13]. While functional, they suffered from compression of the packed bed when used for long periods of time, although this has been overcome by recent work [14–16]. Open tubular columns have also been used, and while simple to make, they suffer from limited capacity [17,18]. More recent interest has focused on the fabrication of short monolithic columns at the capillary inlet because they offer good surface areas for extraction while having excellent permeability allowing high flowrates, but can be difficult to fabricate and have limited surface chemistry at this point in time [19–21]. While impressive results have been obtained using all of these approaches, they

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are often used with standards and 'clean' samples, with very few demonstrations of application to more complicated biological fluids without requiring off-line treatment before in-line extraction [14,22–24].

The implementation of LLE, or more appropriately, LPME, on-line or at-line with CE has been somewhat slower to develop. This is not surprising given that the solvents into which the targets are extracted are often non-polar and are not compatible with direct injection into the CE because they do not solvate ions particularly well, and therefore do not conduct. Nevertheless, this issue can be overcome and the group of Chung have been instrumental in the on-line coupling of LPME with CE. They have reported single drop microextraction (SDME) using both 2- and 3-phase systems in a commercial instrument. In 2 phase SDME, analytes are extracted from an aqueous sample into a drop of pentanol at the tip of the capillary, which is then injected into a pentanol-miscible methanolic buffer [25]. While this works well, there is obvious limitation in the choice of extraction and separation solvents. In 3-phase SDME, a drop of aqueous acceptor solution is covered by a thin film of water immiscible organic solvent, such as octanol, and the contents of the acceptor phase are injected into the capillary and separated in an aqueous BGE [26]. This later approach is much more flexible and can also serve to enhance concentration by trapping analytes in the acceptor phase to enhance extraction from the sample. Both of these approaches can be enhanced even further by injecting large volumes of sample and utilising stacking approaches such as large-volume sample stacking or sweeping to enhance detection limits further. Enhancements from several hundred to tens of thousands have been reported in completely automated systems [27–29]. While SDME is powerful, one of the downsides is the increase in time required to do the extraction on-line and in the reports so far an additional 10 min is required for each sample.

In this work a water immiscible ionic liquid is used for LPME of basic analytes. Ionic liquids offer a number of advantages over conventional solvents used for extraction [30–33], and have been shown to be suitable for a wide and diverse range of targets, from metal ions and amino acids through to proteins and DNA. What makes them attractive from an electrophoresis perspective is the fact that they carry charge and this allows them to be directly injected into the capillary without causing the current instabilities that would occur if more traditional non-polar solvents that are used for extraction were to be injected. This is demonstrated firstly with the extraction and separation of a highly colour cationic dye, chrysoidine, followed by the extraction and separation of clozapine and its two active metabolites, desmethylclozapine (norclozapine) and clozapine-N-oxide, from urine and serum. These extractions can be performed off-line with transfer of the ionic liquid into a sample vial, or more attractively, they can be performed in-vial. In the later case, the sample needs only to be placed in the vial with the EMIM $NtfO_2$ and vortex mixed for 30 s prior to placing the vial in the CE instrument for direct injection thus allowing high throughput sample preparation without the increase in time often required for many other approaches with CE.

2. Experimental

2.1. Apparatus

All experiments were performed with an Agilent $HP^{3D}CE$ (Agilent Technologies, Waldbron, Germany) and polyimide-coated fused-silica capillary (Phoenix, AZ, USA) of 50 μm i.d. with the detector position 8.5 cm from the outlet. For experiments with chrysoidine, a total length of 60.0 cm was used, while for CLO and its metabolites, capillary lengths of 40, 50 and 60 cm were used. Detection was performed using the diode array detector at 254 nm with a bandwidth of 20 nm for experiments with chrysoidine, and 214 nm and a bandwidth of 20 nm for the pharmaceuticals. All separations were performed with the capillary thermostated at 25 °C and a voltage of +20 kV was applied to the inlet vial. Samples were injected using pressure at 50 mbar for 5 s. Prior to the first separation, the capillary was rinsed with 1 M NaOH for 30 min, water for 10 min and BGE for 10 min. Between each separation, the capillary was rinsed with BGE for 2 min at 930 mbar.

2.2. Chemicals

Water was purified using a Millipore (Bedford, MA, USA) Milli-Q water purification system. Background electrolytes were prepared from L-alanine (EGA-Chemie, Steinheim, Germany), glacial acetic acid (Sigma, St. Louis, MO, USA), Tris, DL-histidine and ammonium acetate (all from Aldrich, Milwaukee, WI, USA) and phosphoric acid (BDH, Melbourne, Australia). The final optimum BGE contained 0.96 mol/L alanine and 2.4 mol/L acetic acid and was prepared by mixing 3.2 g of alanine and 3.2 mL of glacial acetic acid with 20 mL of water. Electrolytes comprising ammonium acetate were prepared at a concentration of 0.3 mol/L with 2.1 mol/L acetic acid. Electrolytes with Tris and histidine were prepared at the same concentration as alanine (0.96 mol/L with 2.4 mol/L acetic acid).

The ionic liquid, 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide (EMIM $NtfO_2$), was purchased from Aldrich and used without further purification.

Stock solutions of 1 mg/mL Chrysoidine and light green SF yellowish (both from Fluka, Buchs, Switzerland) in methanol were prepared and diluted as required in water. Clozapine (CLO) and desmethylclozapine (CNO) were purchased from Sigma and norclozapine (NOR) was a gift from Novartis (Basel, Switzerland). These were prepared as a stock solution of 1 mg/mL in 50:50 methanol:water and diluted in water, urine or serum as required. Methanol (HPLC-Grade) was from Ajax Finechem (Seven Hills, Australia).

2.3. Sample preparation

Urine was collected from a healthy volunteer and passed through a 0.45 μm syringe filter. No further sample treatment was performed. This was spiked with chrysoidine at concentrations of 5 and 100 μg/mL and 1 μg/mL of CLO, CNO and NOR. Serum was obtained from a healthy volunteer by the local pathology department and used without further purification. This was also spiked with 1 μg/mL of CLO, CNO and NOR.

2.4. Extraction process

Off-line extractions were performed using 1.5 or 2.0 mL plastic microcentrifuge vials. They were filled with sample (30–1500 μL for the 1.5 mL vials, and 2000 μL in the 2.0 mL vials) followed by EMIM $NtfO_2$ (20–100 μL) and vortex mixed on high using a Ratex vortex mixer for 30 s. After mixing, the vials were centrifuged using an eppendorf 5424 centrifuge at 14,000 RPM for 60 s. The bottom EMIM $NtfO_2$ layer was removed using a 10 μL autopipette and was placed in a reduced volume plastic CE vial.

In-vial extractions were performed by filling the sample vial with 30–200 μL of sample and 5–20 μL of EMIM $NtfO_2$ and again vortex mixed on high for 30 s. These were then placed directly into the CE autosampler for injection.

3. Results and discussion

At the time of this work, there were no reports on the use of ionic liquids for LPME with CE. During preparation of this manuscript, Wang et al. demonstrated SDME with CE for the extraction and

separation of phenols from various water samples [34]. They compared 3 different ionic liquids, 1-butyl-3-methylimidazoium hexafluorophosphate, BMIM PF_6, 1-hexyl-3-methylimidazoium hexafluorophosphate HMIM PF_6, and 1-octyl-3-methylimidazoium hexafluorophosphate OMIM PF_6, with BMIM PF_6 showing the best results. This was attributed to the lower viscosity of this ionic liquid and the fact that the other two ionic liquids required a higher concentration of organic solvent in the BGE to recover and separate the extracted phenols. With a 10 min extraction step, improvements in sensitivity of 100–250 were obtained.

In this work, 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, EMIM $NtfO_2$, was selected as a water immiscible ionic liquid with a low viscosity from the data in Berthod et al. [35]. This ionic liquid has previously been shown to have high partition constants for tryptophan when in its cationic form [36]. Experiments at different pH with fluorescein revealed a strong pH dependence on extraction into the EMIM $NtfO_2$ and it was only extracted from aqueous solution when the pH was <2. At this pH fluorescen becomes slightly positively charged indicating that there is a very strong affinity for cationic compounds with the ionic liquid. Further experiments using the highly coloured cationic dye, chrysoidine, and the anionc dye, light green SF yellowish, again indicated a preference of this ionic liquid to extract basic analytes, and this is consistent with the mechanism of extraction previously discussed [36].

3.1. Extraction of chrysoidine

Experiments to determine the partition coefficient for chrysoidine were undertaken by adding 30 μL of EMIM $NtfO_2$ to volumes of water between 30 and 1500 μL that contained 30 ng of chrysoidine (1.41×10^{-10} moles). In all cases the amount of chrysoidine left in the aqueous phase after vortex mixing for 30 s and centrifugation was minimal, as can be seen from the photograph in Fig. 1(a). When the aqueous phase was directly injected into the CE, no obvious signal for chrysiodine was obtained. Experiments were then undertaken to determine the concentration of chrysoidine in the EMIM $NtfO_2$. This was done by taking 5 μL of the ionic liquid phase and diluting in 400 μL of 1:1 MeOH:H_2O and separating in an alanine/acetic acid BGE. The results are shown in Fig. 1(b). At the lowest volume of aqueous phase (30 μL of H_2O, 30 μl ionic liquid) the concentration of chrysoidine was slightly lower than the theoretical concentration with 92% of the chrysoidine extracted into the ionic liquid, giving a partition coefficient of 12.3. More interesting however is the increase in concentration of the chrysoidine in the EMIM $NtfO_2$ as the aqueous volume is increased, which was contrary to expectation. The highest volume of aqueous phase (1500 μL of water, 30 μL of ionic liquid) yielded a concentration twice the theoretical value. While EMIM $NtfO_2$ is reported to be immiscible with water [35], these results indicate that it is slightly soluble. This can also be seen from the change in volume of the bottom chrysoidine enriched ionic layer in Fig. 1(a). Assuming that there was no change in the recovery of chrysoidine over this volume of H_2O and that the peak area was therefore directly proportional to the volume of ionic liquid left, the data in Fig. 1(b) was analysed using non-linear regression through the solver function in excel. An excellent fit was obtained ($R^2 = 0.9998$), as shown in the insert in Fig. 1(b), indicating that these assumptions are reasonable. From this process, the solubility of EMIM $NtfO_2$ in water was calculated to be 11.0 μL/mL (or 16.7 mg/L) and agreed with approximate measurements made with a 1 μL autopipette. This decrease in volume of ionic liquid is actually very beneficial for extraction as it enhances the phase ratio of the extraction system which ultimately leads to a greater ability to concentrate chrysoidine from aqueous samples, as can be seen from the right axis in Fig. 1(b). When using 1500 μL of aqueous sample and 30 μL of EMIM $NtfO_2$ (a 50-fold reduction in volume) chrysoidine was concentrated by a factor of 99.4.

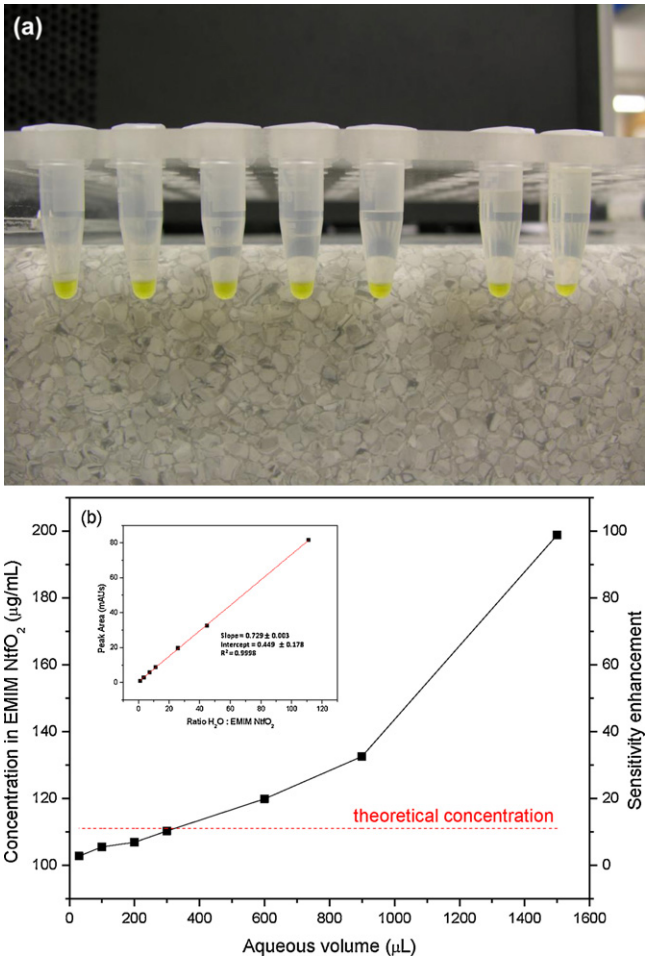

Fig. 1. (a) Photograph of extraction of aqueous chrysoidine into 30 μL of EMIM $NtfO_2$. All solutions initially contained 30 ng of chrysoidine and the aqueous volume was from left to right, 30, 100, 200, 300, 600, 900 and 1500 μL, giving concentrations from 1000 μg/L through to 20 μg/L. Each sample tube was vortex mixed for 30 s followed by centrifugation for 60 s. (b) Experimentally measured concentration of chrysoidine in the EMIM $NtfO_2$ layer (left axis) and the sensitivity enhancement obtained (right axis) as a function of the total aqueous volume. The experimental data points correlate to the sample tubes shown in (a). The theoretical concentration is assuming 100% recovery and no change in volume of either phase. The insert shows the non-linear regression fit used to calculate the solubility of EMIM $NtfO_2$ in water.

3.2. Direct injection of IL after extraction

Ideally, it would be desirable to directly inject the EMIM $NtfO_2$ acceptor phase into the CE. When injected into the capillary, the EMIM cation and $NtfO_2$ anion will move in different directions by electrophoresis and thus compatibility of these ions with BGE ions will be important. Initial experiments with a phosphate BGE did not result in any useful separations as shortly after injection of the ionic liquid the current would quickly decrease and become erratic. Examination of the capillary tip using an optical microscope revealed a small amount of precipitate several mm from the end. It is presumed that migration of the ionic liquid components occurs within the capillary and given the position of the precipitate in the capillary that when EMIM was mixed with phosphate it formed a salt that may not be a liquid at this temperature or it is not soluble in aqueous solution. Better results were obtained using an acetic acid electrolyte with no observable precipitate unless large injections

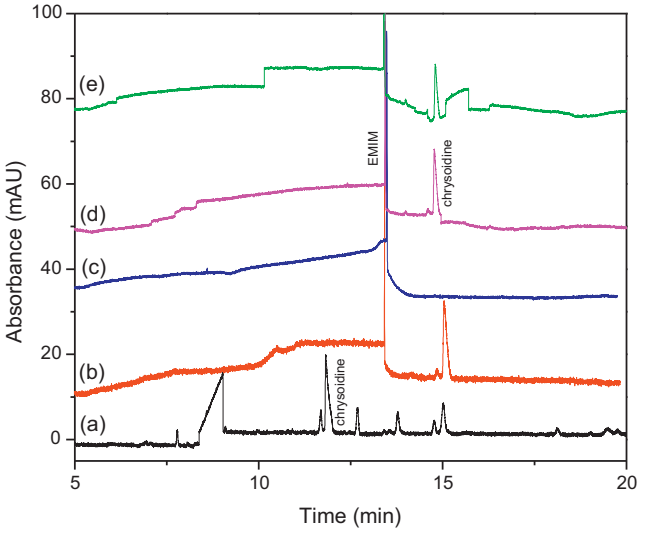

Fig. 2. CE separation of chrysoidine. (a) Direct injection of urine spiked with 100 μg/mL chrysoidine, (b) injection of 100 μg/mL chrysoidine in EMIM $NtfO_2$, (c) direct injection of EMIM $NtfO_2$ layer after extraction of blank urine, (d) direct injection of EMIM $NtfO_2$ layer after extraction of 30 μL urine spiked with 100 μg/mL chrysoidine with 30 μL of EMIM $NtfO_2$, and (e) direct injection of EMIM $NtfO_2$ layer after extraction of 2000 μL of urine spiked with 5 μg/mL chrysoidine. Conditions: 50.0 cm × 50 μm ID, 41.5 cm to detector, +20 kV, injection 5 s @ 50 mbar, detection at 254 nm. BGE: 0.96 mol/L alanine, 2.4 mol/L acetic acid.

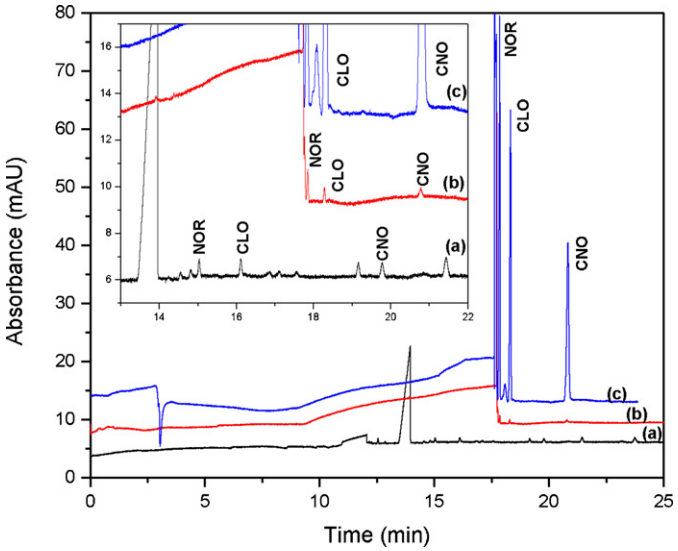

Fig. 3. Extraction of urine spiked with 1 μg/mL of NOR, CLO and CNO. (a) direct injection of spiked urine, (b) 30 μL of spiked urine extracted with 30 μL of EMIM $NtfO_2$, and (c) 1400 μL of spiked urine extracted with 30 μL of EMIM $NtfO_2$. All extractions were performed off-line and the bottom EMIM $NtfO_2$ layer directly injected into the capillary. Conditions: 60.0 cm × 50 μm ID, 51.5 cm to detector, +20 kV, injection 5 s @ 50 mbar, detection at 254 nm. BGE: 0.96 mol/L alanine, 2.4 mol/L acetic acid.

of ionic liquid were made. Experiments using ammonium acetate and acetic acid, pH 4.1, provided stable currents, however the much higher electrophoretic mobility of ammonium resulted in a significantly tailed peak for EMIM which compromised the separation window of many cationic target analytes, such as the dyes and pharmaceuticals of interest in this work. Electrolytes containing a cation with a mobility lower than EMIM, namely with Tris, histidine and alanine were evaluated with the best results obtained using alanine. The second issue that needed to be resolved is related to the high concentration of the EMIM in the ionic liquid (3.90 mol/L) which induced an ITP process with the lower mobility electrolyte cation. High concentrations of electrolyte were therefore required in order to rapidly dissipate the ITP process and allow separations by CZE to be obtained. Concentrations of alanine approaching 1 mol/L were found to provide acceptable results. Higher concentrations could not be used as they rapidly approached the solubility limit of alanine. The optimum electrolyte allowing the direct injection of EMIM $NtfO_2$ into the capillary consisted of 3.2 g of alanine, 3.2 mL of glacial acetic acid and 20 mL of water, giving concentrations of 0.96 mol/L alanine and 2.4 mol/L acetic acid. This gave a large peak for EMIM followed several minutes later by a second much smaller peak for chrysoidine, which can be seen in traces (b), (d) and (e) in Fig. 2. In all of these experiments, the ability to simultaneously detect EMIM in the low UV region (214 nm) and chrysoidine in the visible region (470 nm) made it possible to determine whether chrysoidine had been sufficiently separated from the initial ITP stacking zone greatly assisting electrolyte optimisation.

3.3. Extraction and injection of chrysoidine from urine

To evaluate the performance of EMIM $NtfO_2$ for the extraction of cationic compounds from complex samples, chrysoidine was spiked into urine at a concentration of 100 μg/mL and extracted by vortex mixing for 30 s followed by centrifugation for 60 s. The bottom ionic liquid layer was then removed with an autopipette and placed in a 100 μL sample vial and directly injected into the CE. Trace (a) in Fig. 2 shows the direct injection of the spiked urine with the chrysoidine peak separated from a number of other endogenous peaks. Injection of a 1:1 EMIM $NtfO_2$ extract of the spiked urine is shown in trace (d) where only the chrysoidine peak is observed with all of the endogenous peaks observed with direct injection of the urine being removed as is clear from the extraction of unspiked urine shown in trace (c). Using the injection of 100 μg/mL of chrysoidine in EMIM $NtfO_2$ (b), the recovery of chrysoidine from urine was 86 ± 5% (n = 6), which is marginally lower than that obtained from water. Trace (e) shows the extraction of 2000 μL of urine spiked with 5 μg/mL chrysoidine extracted with 100 μL of EMIM $NtfO_2$. The peak of chrysoidine can be clearly seen indicating the signification preconcentration potential of EMIM $NtfO_2$ to cleanly extract and concentrate basic compounds from complex samples.

3.4. Extraction of clozapine and its metabolites from urine

The ability to concentrate the cationic dye chrysoidine demonstrated above presents the possibility to extract many common pharmaceuticals as these are often cationic. To evaluate this, clozapine (CLO) and its two active metabolites, desmethylclozapine (norclozapine, NOR) and clozapine-N-oxide (CNO) were selected and spiked into urine at a concentration of 1 μg/mL and extracted using a similar protocol to that described above for chrysoidine. Trace (a) in Fig. 3 shows the direct injection of spiked urine, trace (b) shows injection of the ionic liquid extraction of 30 μL of spiked urine with 30 μL of EMIM $NtfO_2$ and (c) is 1500 μL of spiked urine extracted with 30 μL of EMIM $NtfO_2$. It can be seen from the insert that the peaks for CLO, NOR and CNO are just detectable with direct injection of the urine and that there are a number of other peaks present from the urine (confirmed by blank injection, not shown). The peaks are slightly smaller when a 1:1 extraction is performed, but this is primarily due to the different amount of sample injected into the capillary due to the higher viscosity of EMIM $NtfO_2$ than urine. Importantly, many of the endogenous peaks observed from the direct injection of the urine are not extracted providing a much cleaner separation. The peak for NOR migrates just after the peak for EMIM which shows considerable UV absorbance. As anticipated, the peaks for CLO and its metabolites are enhanced when

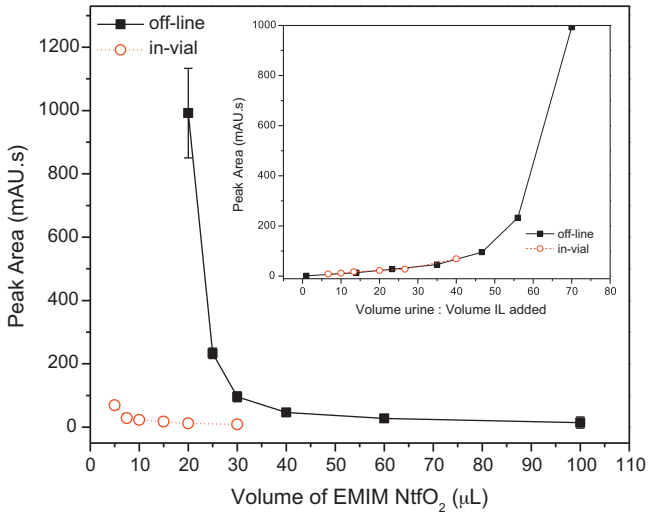

Fig. 4. Change in peak area of CLO as a function of the amount of EMIM $NtfO_2$ added to 1400 μL of spiked serum both off-line in 1.5 mL microcentrifuge tubes (solid line) and in-vial using 200 μL tapered vials. The insert shows the same data represented as the ratio of spiked urine to the volume of EMIM $NtfO_2$ added. All data were collected using the conditions described in Fig. 3 and error bars were constructed from 3 replicate extractions.

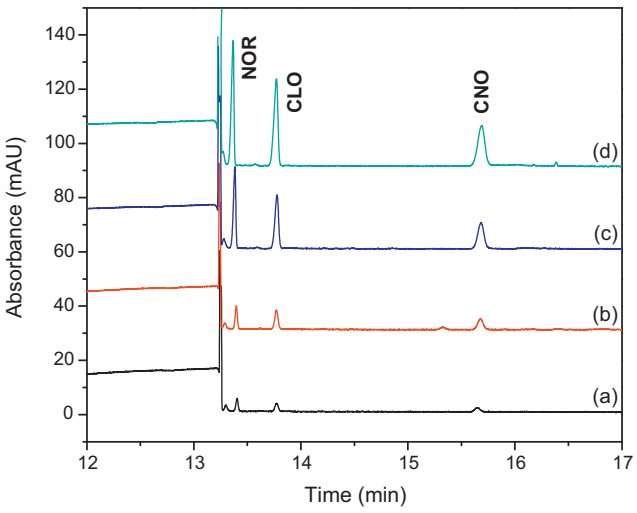

Fig. 5. In-vial extractions of spiked urine with different volumes of EMIM $NtfO_2$. Extraction of 200 μL of urine spiked with 1 μg/mL of NOR, CLO and CNO and extracted in-vial with (a) 30, (b) 20, (c) 7.5 and (d) 5.0 μL of EMIM $NtfO_2$. Extractions were performed by placing solutions in the reduced volume vial and vortex mixing for 30 s followed by direct injection into the CE from the vial. Conditions: 40.0 cm × 50 μm ID, 31.5 cm to detector. All other conditions are in Fig. 3.

1500 μL of spiked urine is used and, like chrysoidine, a sensitivity enhancement of 100–110 was obtained. With the enhanced concentration there is only one small endogenous peak extracted from urine which can be seen just before the peak for CLO.

The above results show the ability to obtain an enrichment of approximately 100. If the volume of ionic liquid can be minimized, then it should be possible to maximize the enrichment effect provided that the analytes are still extracted. Experiments were undertaken using CLO, NOR and CLO using a fixed volume of 1400 μL of spiked urine and reducing the volume of ionic liquid from 100–20 μL. The results for CLO are shown in Fig. 4 where it can be seen that the enhancement factor that can be achieved increases in a non-linear fashion as the volume of ionic liquid is decreased. The maximum enhancement achieved approaches 1000 when a volume of 20 μL of ionic liquid is used. Using the same approach as described above, the solubility of EMIM $NtfO_2$ was calculated using the data for CLO, NOR and CNO (not shown) to be 10.2, 14.1 and 10.6 μl/mL, respectively and is in good agreement with the data obtained for chrysoidine. Given that the average solubility of EMIM $NtfO_2$ in water is 11.4 ± 1.8 μL/mL, the residual volume left when using the smallest volume of 20 μL for extraction is 4.6 μL. Because of this small volume, any minor changes in the initial volume of EMIM $NtfO_2$ added due to the difficulty in pipetting small volumes of the highly viscous ionic liquid will cause a considerable change in the residual volume left and therefore the repeatability of this experiment was very poor as can be seen from the error bars in the figure (n = 3). An initial addition of 25 μL (leaving a residual amount of 9.6 μL) was found to be much more repeatable and provided a reasonable enhancement in sensitivity of 200-fold. Intraday migration time repeatability (n = 6) was within 2% RSD while peak area and heights were within 7% RSD. Interday repeatability (n = 6) for migration times was within 6% RSD while peak heights and areas were within 15%. Recoveries were calculated (n = 6) to be 93 ± 6% for NOR, 89 ± 8% for CLO and 87 ± 8% for CNO giving LODs (based on 3 times signal to noise) of 3.0, 6.5 and 11 μg/L for NOR, CLO and CNO. These LODs are lower than Raggi et al. [37], and are equivalent to those reported by Ho et al. [38], establishing the potential of this method of on-line concentration for application to these samples, but with simpler sample pretreatment requirements than is reported in these two approaches.

3.5. In-vial extraction of clozapine and metabolites from urine

The fact that the EMIM $NtfO_2$ is denser than water and can also be directly injected into the capillary presents the possibility to perform in-vial LLE. 200 μL of spiked urine was placed in a reduced volume sample vial that tapered at the bottom specifically designed for the injection of small volumes. This was vortex mixed with 5–30 μL volumes of EMIM $NtfO_2$ for 30 s and placed directly into the CE for injection. The resultant separations are shown in Fig. 5 with the change in peak area of CLO as a function of the volume of EMIM $NtfO_2$ shown in Fig. 4. Again, clean separations were obtained and there is significant concentration of the analytes, with an improvement in sensitivity of 80 obtained when adding 8 μL of EMIM $NtfO_2$ (trace a). Due to the lower volume of sample that can be placed in the vial and the requirement to leave approximately 5 μL of ionic liquid in the bottom of the vial from which to inject, it was not possible to achieve the larger enhancements in sensitivity that were obtained by performing off-line LLE in these vials. The best enhancement that could be achieved with the in-vial extraction was approximately 80-fold, but it can be seen from the insert in Fig. 3, that the enhancement still followed the same ratio as that obtained off-line. While these improvements are not as great as those that can be achieved off-line, it is important to note that the only sample processing required to achieve this is to pipette the sample and ionic liquid into the vial and to vortex mix for 30 s followed by 60 s centrifugation, thus it is entirely feasible to do the entire sample preparation for 10 samples in 10 min. This is much shorter than that required for other extraction approaches integrated on-line as well as having the advantage of no potential cross contamination between samples.

3.6. In-vial extraction of clozapine and metabolites from serum

In addition to reducing the sample processing, one of the other advantages that in-vial extraction has is that it reduces the need for the operator to handle the sample which not only reduces the possibility of error and mistakes but is also safer as it reduces exposure to dangerous samples. One area in which this is of importance is the routine monitoring of pharmaceuticals in blood, serum and plasma for clinical or forensic purposes. These samples are also much more

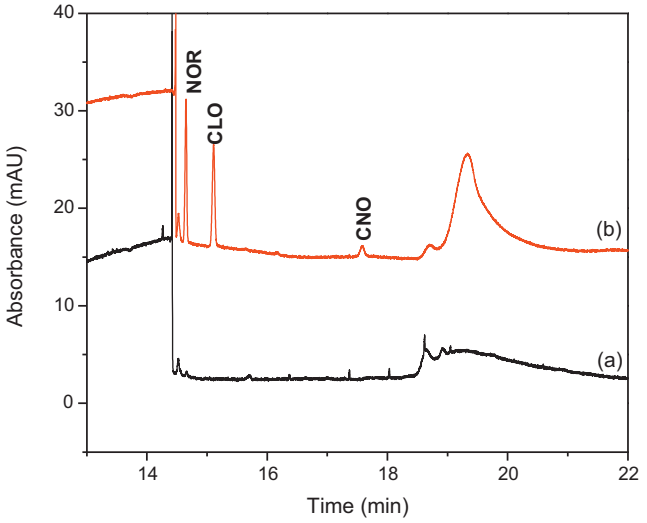

Fig. 6. In-vial extraction of (a) blank serum and (b) blank serum spiked with 1 μg/mL of NOR, CLO and CNO. Conditions: 50.0 cm × 50 μm ID, 41.5 cm to detector. All other conditions are described in Fig. 5.

demanding than urine as there is a large protein content that can potentially interfere with the analytical method. In-vial extractions of serum were performed by placing 200 μL of serum in a reduced volume sample vial with 8 μL of EMIM $NtfO_2$ and extracted as above. Extraction of the blank serum with EMIM $NtfO_2$ provides a relatively clean trace, as seen in Fig. 6(a) although there are some peaks between 18 and 22 min which are believed to be due to proteins. Fig. 6(b) shows the extraction of CLO, NOR and CLO spiked into the serum at a concentration of 1 μg/mL. Good peaks were obtained for NOR and CLO, but the peak for CNO was lower than expected. Recoveries were calculated ($n = 6$) to be 85 ± 7% for NOR, 81 ± 8% for CLO and 20 ± 10% for CNO, giving LODs of 7.0, 13.4 and 55 μg/L. Intraday migration time repeatability ($n = 6$) was within 3% RSD while peak area and heights were within 8% RSD. Interday repeatability ($n = 6$) for migration times was within 5% RSD while peak heights and areas were within 13%. The performance for CLO and NOR is sufficient for determination of these in patient samples [37,38] using similar volumes of serum to previously published methods but with much simplified and safer sample pretreatment. The levels of CNO reported in plasma observed in [38] are higher than the LOD but lower than the LOQ and this method would therefore be unsuitable for the determination of CNO in this matrix. The lower recovery of CNO from this matrix is the main issue and this is currently not understood, but may be due to competition of the proteins with the ionic liquid. This needs to be rectified before the method could be widely applicable.

4. Conclusions

The ability to perform LLE of basic analytes from complex biological samples using the water immiscible ionic liquid EMIM $NtfO_2$ with direct injection for analysis by CE has been demonstrated. Using the basic dye chryisoidine, and off-line LLE with 1500 μL of aqueous sample and 20 μL of EMIM $NtfO_2$, enhancements in sensitivity approaching 1000 were obtained, although more repeatable enrichment factors of 200 were obtained with slightly larger volumes of EMIM $NtfO_2$ due to its partial solubility in water. This could be extended to basic pharmaceuticals, and the extraction of CLO, NOR and CNO was demonstrated from urine with enrichment factors greater than 100 obtained. This could also be performed in-vial and the bottom EMIM $NtfO_2$ layer directly injected into the CE to reduce sample handling and time, and enhancements in sensitivity of 80 were obtained for CLO and NOR from serum, although improvements were worse for CNO due to its lower recovery.

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