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New trends in sample preparation: on-line microextraction in packed syringe (MEPS) for LC and GC applications Part III: Determination and validation of local anaesthetics in human plasma samples using a cation-exchange sorbent, and MEPS–LC–MS–MS

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Abstract

The need for on-line sample preparation for high-throughput applications in bioanalysis has increased during the past decade. In this paper a robust and on-line sample preparation technique, micro extraction in packed syringe (MEPS) has been developed and validated. The method is a miniaturized, fully automated, solid-phase extraction (SPE) technique that can be connected on-line to GC or LC without any modification of the chromatographs. The performance of MEPS as sample preparation method is illustrated by the determination of local anaesthetics in human plasma samples on-line with high performance liquid chromatography (HPLC) and tandem mass spectrometry. The sampling sorbent was 1 mg silica based benzenesulphonic acid cation exchanger that was inserted in a 250 μl syringe. Ropicavine and two of its metabolites (PPX and 3-OH-ropivacine), lidocaine and bupivacine were used as model substances. The accuracy values of quality control samples (QC) were between 95% and 109%, and precision (relative standard deviation, R.S.D.) had a maximum deviation of 9% for the analytes.

Keywords: Microextraction in packed syringe; Sample preparation; Cation exchange sorbent; Local anaesthetics; Fully automated; High throughput; HPLC/MS/MS

1. Introduction

In pharmaceutical industry, the measurement of drug levels in biological fluids gives information that is needed during drug discovery and development. The more rapidly these measurements can be done the more quickly drugs may progress toward regulatory approval. It may thus be important to minimize sample preparation and analysis times where possible. Sample preparation is frequently done off-line and in fact, this is often a limiting step to get fast bioanalysis; the introduction of on-line sample pretreatment would greatly speed up the analyses. Further, as the number of samples grows high-throughput and fully automated analytical techniques becomes required. Current developments of sample handling techniques are directed toward automatization and on-line coupling of sample preparation units and detection systems. In addition, there is a trend toward development of more selective sorbents for sample clean-up and enrichment.

The sample preparation is an important step in the pharmaceutical analysis process because of the difficulties that follow with complex sample matrices, e.g. plasma and urine.

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Proteins and other interferences may result in deterioration of the performance of separation columns and in increased column backpressure. Typical demands on sample preparation are automation, miniaturization, on-line coupling, consumption of small solvent volumes, and short extraction times [1]. The procedure should be highly reproducible, with high recovery, it should be fully automated, and involve a minimum number of steps [2]. Also enrichment of the compounds is among the reasons for the use of a well-designed sample preparation.

Solid-phase extraction (SPE) is today the most commonly used sample preparation method [3]. SPE is used to extract, concentrate and clean-up compounds of interest from a sample matrix using a solid support. Here, the analytes are adsorbed on the packing bed and this is followed by the elution or thermal desorption for recovery.

There are two different formats for solid-phase extraction, cartridges and SPE discs [3]. Cartridges were initially developed to provide the possibilities for higher sample throughput [4–8]. The use of SPE discs was the first attempt to miniaturize SPE [9]. Further miniaturization in SPE has, for instance, resulted in pipette tips [9–10], column switching systems [1,11–14], and multi-well plates [15]. Solid-phase microextraction (SPME) is another commonly used sample preparation method [16]. The method is based on partitioning of the analytes between the sample matrix and a fused-silica fiber that is coated with an appropriate stationary phase [16–17]. The extraction efficiency of SPME depends on a number of factors such as extraction time, agitation, sample pH, salt concentration and temperature [18].

In a number of cases, SPE has been used in connection with high performance liquid chromatography (HPLC) for extraction of local anaesthetics in plasma samples [19–24]. SPE usually gives recoveries above 70%. However, most of the SPE methods use relatively high sorbent amounts (>100 mg) and high sample loading (>1 ml). Further, SPE cartridges are relatively expensive and the systems are difficult to automate. Also SPME has been used for extraction of local anaesthetics in plasma samples [25–27]. Large sample volumes ($\geq$1000 μl), long sample preparation time (> 40 min), low recovery and limited life time of the fiber were some of the drawbacks with this technique. Micro extraction in packed syringe (MEPS) is a new technique for miniaturized, solid phase extraction that can be connected on-line to GC or LC without any modification of the chromatograph [2,28–29]. MEPS differs from commercial solid-phase extraction in that solid support is inserted into a syringe (100–250 μl) as a plug. Thus, there is no need for a separate robot to apply the sample into the solid-phase as with conventional SPE. Further, the packed syringe can be used several times, more than 100 times with plasma, whereas a conventional SPE column is used only once.

Compared to SPE or liquid–liquid extraction (LLE), MEPS will reduce sample preparation time and organic solvent consumption. MEPS is fully automated and takes only 1 min for each sample. Compared to solid-phase micro extraction, MEPS reduces sample preparation time (~1 min), sample volume (10–1000 μl) and a much higher recovery (>50%) can be obtained.

A method based on MEPS on-line with gas chromatography and mass spectrometry was recently developed and validated [2]. In this paper, a method for on-line MEPS using a cation-exchanger as sorbent with HPLC–MS–MS has been developed and validated. Local anaesthetics in human plasma samples were used as model compounds for the evaluation. A simple sample preparation, a low quantification limit, high recovery and the possibility to automate were considered.

2. Experimental

2.1. Chemicals

Ropivacaine, PPX (pipecoloxilidide), 3-OH-ropivacaine (3-hydroxyropivacaine), lidocaine, bupivacaine and $^{2}H_{7}$-ropivacaine (internal standard) in hydrochloride form, Fig. 1, were supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje, Sweden). Acetonitrile, methanol, formic acid and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

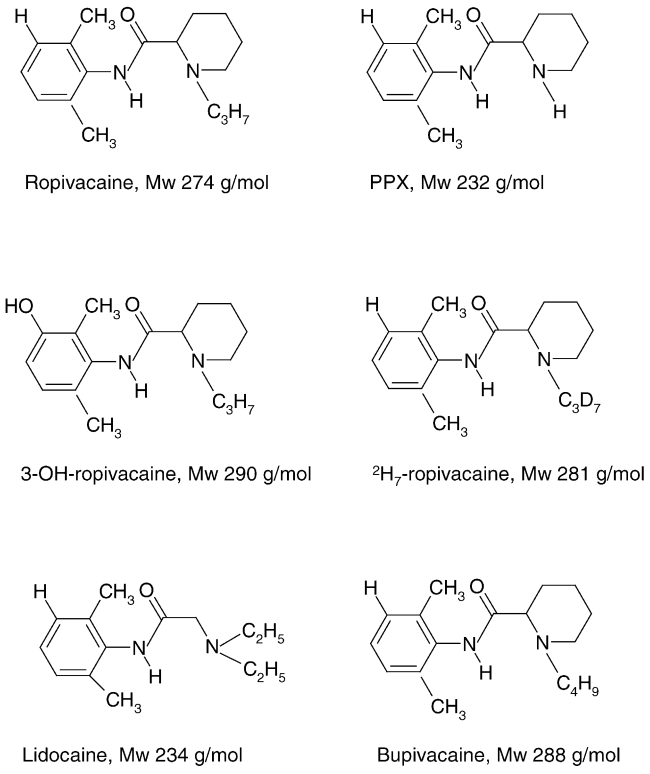

Fig. 1. Chemical structure and molecular weight of ropivacaine and its metabolites (PPX and 3-OH-ropivacaine), lidocaine, bupivacaine and internal standard, $^{2}H_{7}$-ropivacaine.

2.2. Apparatus

The high performance liquid chromatography instrument included two pumps, Shimadzu (Kyoto, Japan), an autosampler, CTC-Pal, Crelab (Knivsta, Sweden) and a 50 μl sample loop. The mass spectrometric instrument was a QII Z-spray mass spectrometer from Micromass (Manchester, UK) and the chromatographic data system was MassLynx version 3.4. The guard column, Optiguard (C_8, 10 mm × 1 mm) was from Optimize Technologies (Oregon City, USA). A Valco C4W valve, Valco Instruments (Houston, USA) was used as gate valve between the liquid chromatograph and the mass spectrometer. The Milli-Q water was obtained using a Reagent Grade Milli-Q Plus water purification system from Millipore Corporation (Bedford, USA). A centrifuge, Hettich Rotanta/AP (Tuttlingen, Germany), was used for plasma centrifugation.

2.3. Preparation of samples

Plasma samples were stored at −20 °C. Before use, the plasma was thawed at room temperature and centrifuged at 3500 rpm for 10 min. Stock solutions of ropivacaine, PPX, 3-OH-ropivacaine, lidocaine, bupivacaine and $^2H_7$-ropivacaine (internal standard) in methanol/water 1:1 (v/v) (0.1% formic acid) were prepared (300 μM). Spiked plasma samples were prepared by adding a few microliters of the analytes to 1.0 ml of centrifuged plasma. Then 25 μl of $^2H_7$-ropivacaine was added. After vortexing the samples were extracted and analyzed. The concentration range of the standard curve was between 2 and 2000 nM. All standard solutions were stored at 4 °C.

2.4. Analytical procedure

2.4.1. Chromatographic system

Isocratic HPLC was used. Mobile phase was methanol/water 1:1 (v/v) (0.1% formic acid). The flow rate was 0.20 ml/min and sample volume (loading) was 25 μl. A guard column was used between the valve and the ionization source.

All experiments were conducted using a triple quadrupole mass spectrometric instrument (Micromass) equipped with a Z-electrospray interface operated in positive ion mode. The parameter settings used were: capillary voltage at 3.1 kV, cone voltage at 38 V, extractor at 5 V, RF lens at 0.2 V, source block and desolvation temperatures at 150 and 320 °C, respectively. Nitrogen was used both as drying (400 L h^{-1}) and nebulizing gases (20 L h^{-1}), the vacuum was 2×10^{-5} in the mass analyzer and 2×10^{-3} in the collision cell. Argon was used as collision gas and collision energy was 25 eV. The gases were from AGA (Lidingö, Sweden). The eluate from the syringe was introduced into the valve before introducing into the ionization source. The data were collected and processed using MassLynx version 3.4, and all calculations were based on peak area ratios.

The scan mode was multiple reaction monitoring (MRM) using precursor ion at (M + 1) *m*/*z* (*m*/*z*: 235, 289, 233, 275, 291 and 282) and after collisional dissociation the product ions 86, 140, 84, 126, 126 and 133 were used for quantification of lidocaine, bupivacaine, PPX, ropivacaine, 3-OH-ropivacaine and the internal standard.

2.5. MEPS conditions

MEPS was performed using a 250 μl gas-tight syringe. The sorbent used was a benzenesulphonic acid cation exchange silica based sorbent, having cation exchange and significant non-polar secondary interactions. This sorbent has irregular particles with average size of 50 μm and nominal 60 Å porosity. One milligram of the solid material was manually inserted inside the syringe as a plug. The sorbent material is tightened by filters to avoid moving inside the syringe.

Before using for the first time, the sorbent was manually conditioned with 50 μl methanol followed by 50 μl of water (0.1% formic acid). After that, the syringe was connected to the autosampler and spiked plasma sample (25 μl) was drawn onto the syringe by the autosampler. It is important that plasma samples are drawn slowly (20 μl s^{-1}) and with caution to obtain good percolation between sample and solid support. The sorbent was then washed once with 100 μl of water (0.1% formic acid) to remove protein and other interferences. The analytes were then desorbed by 50 μl methanol/water 95:5 (v/v) (0.25% ammonium hydroxide) directly into a gate valve, which was situated between the liquid chromatograph and the tandem mass spectrometer. Cleaning of the sorbent was done with 5 × 50 μl elution solution followed by 5 × 50 μl of the washing solution between every extraction. This step decreased memory effects, but also functioned as conditioning step before the next extraction. The same packing bed was used for about 100 extractions before it was discarded.

2.6. Validation

Each calibration curve consisted of nine calibration points covering 2–2000 nM for the test compounds in plasma and a zero concentration. The plasma used was collected from different objects (at least six). The peak area ratios for substances and internal standard were measured and a standard curve without zero concentration was constructed. The calibration curves were described by the equation:

$$y = ax^2 + bx + c$$

where *y* is peak area ratio, *x* is the concentration, *a* is the curvature, *b* is the slope and *c* is the intercept. The calibration curves were quadric and the weight was 1/*x*. The quality control (QC) samples were prepared with the concentration of 40, 400 and 1500 nM for ropivacaine and its metabolites (PPX and 3-OH-ropivacaine), and for lidocaine and bupivacaine in human plasma. The accuracy and precision were calculated for the QC samples, both within and between days. The ex-

periments were done three times during three different days. The method was validated at optimized conditions.

3. Results and discussion

3.1. Method development

Ropivacaine, lidocaine and bupivacaine are widely used amide type local anaesthetics that belong to the pipeloxilidide group [24]. Lidocaine is widely used in anaesthesiology. Lidocaine also has antiarrhytmic effects and is used as a therapeutic agent in the treatment of cardiac disorders. Ropivacaine is a relatively new amide-type local anaesthetics drug, mainly used for surgery and post-operative pain relief. Also, it has a lower central nervous and cardiotoxic potential than bupivacaine [30]. The major metabolites of ropivacaine are PPX and 3-OH-ropivacaine. To optimize micro extraction in packed syringe, factors affecting the recovery such as amount of sorbent, the composition and volume of washing solution, and the composition and volume of the elution solution were studied.

3.1.1. Washing

The studied substances have pK_a-values between 7.0 and 8.1. This means that at low pH the analytes are positively charged. Using a strong cation exchange silica based sorbent such as benzenesulphonic acid the substances were readily adsorbed. The sample was then cleaned from interferences by washing with a solvent having a low ionic strength. Different mixtures of methanol and water (0.1% formic acid) were tested. After that the sample (25 μl) was drawn onto the syringe, it was washed once with 100 μl of the washing solution which was injected directly onto the HPLC (no column). The recovery was measured as the response of a processed spiked plasma sample expressed as percentage of pure standard solution. The use of methanol in the washing mixture increased the leakage and decreased the recovery. The lowest amount leakage (<0.4%), with no interferences and highest recovery was obtained with 100 μl water (0.1% formic acid) as washing solution. Thus, 100 μl water (0.1% formic acid) was selected as washing solution for the method.

3.1.2. Elution solvent

Because a cation exchange silica based sorbent was used for extraction, the pH of the elution solution had a large influence on the recovery. Therefore, eluents with higher pH (>8) would be preferred as elution solution. To study the recovery, solutions containing different amounts of methanol, water and ammonium hydroxide were investigated. After introduction of the sample (25 μl) onto the syringe and washing with 100 μl of water (0.1% formic acid), the elution efficiency was measured and compared to that of pure standard solution. The eluting efficiency increased as the methanol content in the eluent increased. Acceptable recovery (∼50%) was obtained when using 50 μl of a solution of methanol/water 95:5 (v/v) (0.25% ammonium hydroxide) at high pH, the amines then were in uncharged form. Fig. 2 shows a mass chromatogram at the optimum conditions. This solution was selected as elution solution for the method.

3.1.3. Amount of sorbent

To achieve selective extraction of target substances with acceptable recovery from plasma samples, the amount sorbent material was optimized in relation to the volume of elution solution. When spiked plasma samples were extracted using 0.5 mg of the sorbent material, with the above mentioned washing and elution solution, the recovery was decreased by about 10%. Probably, the adsorption capacity was insufficient here. Also when using 2 mg of packing bed the recovery decreased. The reason for this was that a larger volume elution solution was needed for desorption of the substances. The smallest amount sorbent by which acceptable recovery (∼50%) was obtained was 1 mg when 25 μl of sample was used with 100 μl water (0.1% formic acid) as washing solution and 50 μl methanol/water 95:5 (v/v) (0.25% ammonium hydroxide) as elution solution, Fig. 3. The relative standard deviation (R.S.D.) was about 5% ($n = 3$).

The packing bed showed good reproducibility and stability for repeated use. It was used for more then 100 extractions

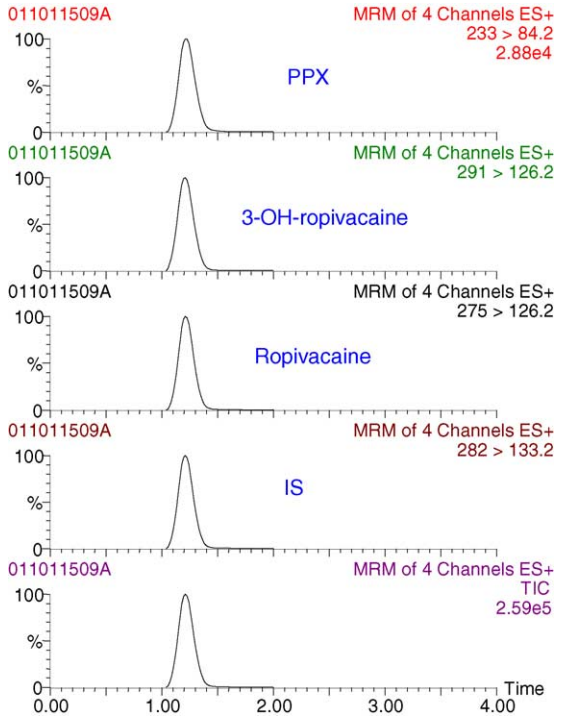

Fig. 2. Mass chromatograms obtained from human plasma spiked with analytes (*m/z*: 291, 282, 275, and 233 for 3-OH-ropivacaine, internal standard, ropivacaine and PPX, respectively). Sample preparation: 25 μl (1.5 μM) of spiked plasma sample was drawn into the syringe, then washed with 100 μl water (0.1% formic acid) and eluted with 50 μl methanol/water 95:5 (v/v) (0.25% ammonium hydroxide) directly into the HPLC. Mobile phase: methanol/water 1:1 (v/v) (0.1% formic acid). Flow rate: 0.20 ml/min.

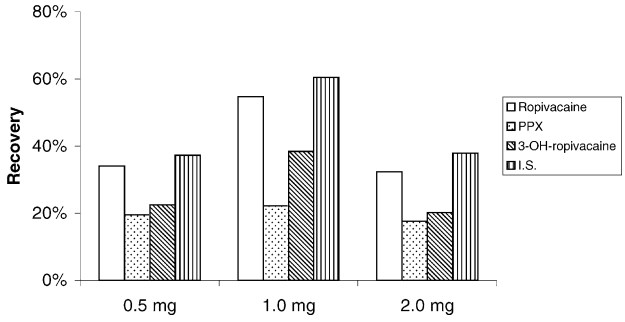

Fig. 3. Extraction yield (%) when amount sorbent was optimized. The recovery of ropivacaine and its metabolites PPX and 3-OH-ropivacaine compared to direct injection of pure standard solutions. The relative standard deviation (R.S.D.) was about 5% ($n = 3$). Experimental conditions as in Fig. 2.

without any significant problems such as loss of extraction power.

3.2. Selectivity

Chromatograms from human plasma samples spiked with ropivacaine, lidocaine and bupivacaine as well as blank chromatograms are shown in Figs. 4 and 5. The blank shows no peaks interfering with those of the target substances.

3.3. Calibrations

The same experimental conditions (precolumn, sample volume, elution volume, washing volume and sorbent) were used for the calibration set. $^{2}H_{7}$-ropivacaine was used as internal standard to validate the method. The constructed calibration curve consisted of nine levels of human plasma samples in the concentration range 2–2000 nM. The regression parameters for all calibration curves are given in Table 1. The correlation coefficients (R^2) values obtained were >0.998 for all runs. The calibration curves indicate that the method is suitable for quantitative analysis.

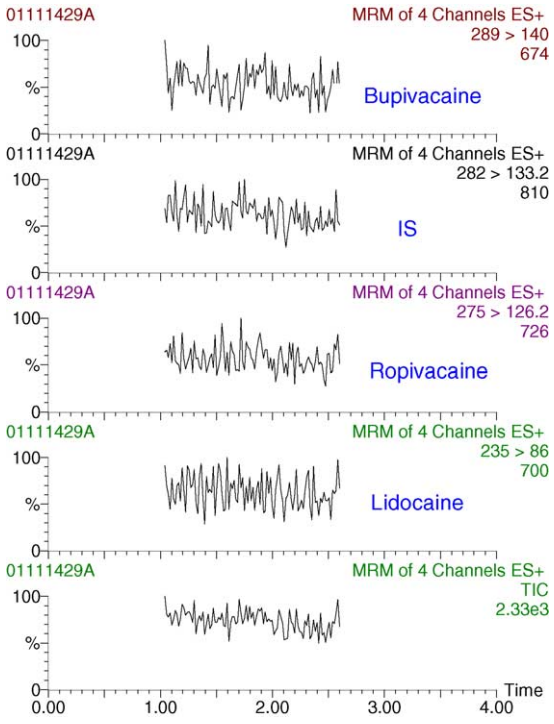

Fig. 4. Chromatogram of human blank plasma after injection of highest concentration (2.0 μM) plasma standard. Experimental conditions as in Fig. 2.

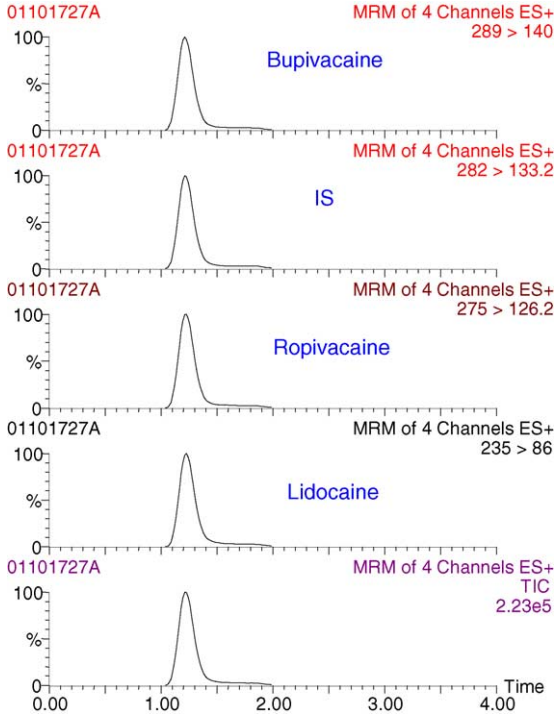

Fig. 5. Mass chromatograms obtained from human plasma spiked with analytes (m/z: 235, 275 and 289 for lidocaine, ropivacaine and bupivacaine, respectively, 1.0 μM) and internal standard $^{2}H_{7}$-ropivacaine (m/z: 282). Other experimental conditions as in Fig. 2.

Table 1
Regression parameters for calibration curves ($n = 3$)

Substance	Curvature, a (10^{-8})	Slope, b	Intercept, c	R^2
Ropivacaine	−10.39	0.0017	0.0056	0.9999
	−8.599	0.0017	0.007	0.9996
	4.962	0.0015	−0.007	0.9998
Lidocaine	−6.788	0.0019	0.0057	0.9999
	−10.243	0.0021	0.0114	0.9999
	13.296	0.0015	0.0071	0.9999
Bupivacaine	−6.374	0.0019	0.0028	0.9999
	−6.025	0.0019	0.0099	0.9998
	10.422	0.0019	0.0071	0.9999
PPX	2.37	0.0005	0.0028	0.9992
	−2.375	0.0017	0.0011	0.9996
	0.256	0.0005	0	0.9992
3-OH-ropivacaine	1.235	0.0009	0.00061	0.9985
	−1.421	0.0009	0.00145	0.9999
	3.588	0.0008	0.00029	0.9995

Table 2
Intra- and inter assay precision and accuracy for ropivacaine and its metabolites (3-OH-ropivacaine and PPX), and lidocaine and bupivacaine

Substance	Accuracy		Precision	
	Concentration (nM)	Accuracy (%)	Intraday ($n = 6$) (R.S.D., %)	Interday ($n = 3$ days) (R.S.D., %)
Ropivacaine	1500	99	4	6
	400	100	4	5
	40	98	7	9
Lidocaine	1500	98	4	6
	400	98	4	8
	40	95	7	8
Bupivacaine	1500	98	4	5
	400	106	4	6
	40	102	5	5
PPX	1500	100	6	5
	400	102	5	4
	40	108	7	8
3-OH-ropivacaine	1500	106	5	5
	400	105	2	4
	40	104	4	8

3.4. Accuracy and precision

The accuracy and precision were determined by analysis of quality control samples at three different concentrations, 40, 400 and 1500 nM. The results are shown in Table 2. The intra-assay precisions (R.S.D.) were between 2% and 7% ($n = 6$) and the inter-assay precisions (R.S.D.) were between 4% and 9% ($n = 18$) for all analytes.

The accuracy was from 95% to 109% for all analytes. The results from this study were in close agreement with earlier published data [2,23–25]. The accuracy and the precision of the method were both within the internationally accepted limits [31].

3.5. Limit of quantification (LOQ) and carry over

The carry-over was investigated by injecting elution solution after the highest standard concentration. Less than 0.5% carry-over was observed and after one additional blank injection no carry over could be detected. For high concentration samples two blanks would be advised.

The limit of quantification was set as the lowest measurable concentration with acceptable accuracy and precision. The LOQ for the analytes studied was set to 2 nmol/l. At this concentration the accuracy of LOQ was between 97% and 105% and the precision had a maximum deviation between 8.2% and 10.6% ($n = 6$).

4. Conclusions

Micro extraction in packed syringe is a new sample preparation method suitable for the fully automated determination of analytes in complex matrices. It was thus shown that only small sample volumes were required, solvent consumption was low and the procedure was fast. Extraction was done in 1 min. The MEPS was easily coupled to an auto sampler and was used for more than 100 extractions before it was discarded.

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