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Using on-line solid phase extraction for determination of amiloride in human urine by sequential injection technique

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Abstract

This presented paper deals with a new methodology for the direct determination of amiloride in human urine. The methodology described is based on the sequential injection analysis technique (SIA) coupled with solid phase extraction (SPE) microcolumn. SPE microcolumn was used for selective retention of amiloride, while the urine matrix components were eluted with water carrier flow to the waste. Due to the acid-basic and polarity properties of amiloride molecule and principles of ion-exchange chromatography, it was possible to retain amiloride on the ion-exchange sorbent (SPE BAKER WCX—carboxy group). Eluting solution was 0.01 M HNO_3 + 0.1 M KCl, flow rate 20 $\mu l\,s^{-1}$. The fluorescence detection of amiloride was performed at λ_{em} 385 nm (secondary filter). Recovery was found in the range 96.8–99.4% for 10 times diluted urine, linearity of determination in the range 0.5–100 $\mu g\,ml^{-1}$ ($r = 0.998$), and 3σ limit of detection (LOD) was 0.05 $\mu g\,ml^{-1}$. The whole procedure comprising raw sample pre-treatment, analyte detection and column reconditioning took 8 min. The proposed SIA–SPE method has been applied for direct determination of amiloride in human urine.

Keywords: Sequential injection analysis (SIA); Solid phase extraction (SPE); Amiloride; Urine; Sample preparation

1. Introduction

Sequential injection analysis (SIA) was introduced by Ruzicka and Marshall in 1990 [1] as a following generation in the development of flow injection technique. The principles upon which SIA is based are similar to those of flow injection analysis (FIA), namely controlled partial dispersion and reproducible sample handling. Characteristic advantages of SIA include its versatility, full computer compatibility, high sample throughput, and low sample and reagent consumption.

However, in recent years, it has become apparent that the scope of SIA can be extended to encompass a variety of more complex, on-line, sample-manipulation and pre-treatment procedures. Then, the ports of the multi-position selection valve are coupled to various units (e.g., reservoirs, detectors, pumps, reactors, separators, special cells and other manifolds) [2]. One of the most widely used sample-pre-treatment procedure in SIA is automated solid phase extraction (SPE), which employs an appropriate solid- or liquid-extraction material attached on a suitable support for which SIA is ideally suited. The extraction procedure accomplishes two goals: separation of the analyte from interfering species in the sample and preconcentration of the analyte to increase the sensitivity. The great advantage of SPE is that both organic compounds and inorganic species can be extracted. Depending on the nature of the analyte and on the required retention mechanism, different extraction materials can be used (e.g., hydrophilic ion-exchange resins, surface-modified beads, molecularly imprinted polymers, restricted-access materials and different types of hydrophobic polymers) [2].

SIA–SPE can be applied in analysis of such complex samples as bio-fluids. Few SIA–SPE determinations in biological material were found in literature [3–6]. Removing of interfering sample matrix was achieved with urine samples while the SIA–SPE system based on modified silica gel sorbents was found to be limited in case of serum analysis [5].

The aim of the presented work was to examine further possibilities of the SIA–SPE system for direct analysis of amiloride in human urine with fluorescence detection. Amiloride, structure of which is shown in Fig. 1, was chosen as analyte possessing

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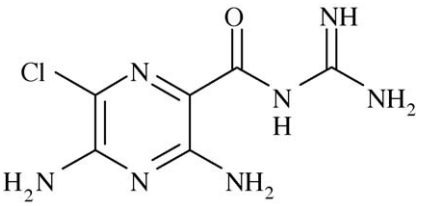

Fig. 1. The structure of amiloride.

fluorescence capability and representing polar substances from the group of diuretic pharmaceuticals. Being a mild diuretic, amiloride is widely used in clinical practice in the treatment of hypertension and different kinds of oedema. Monitoring of amiloride in bio-fluids is important not only in the clinical practice by treatment of patience but also in the field of doping control [7].

Amiloride has been individually determined in pharmaceutical preparations and biological fluids like urine and plasma using several methods including, spectrophotometry [8–11], high performance liquid chromatography [12–14], flow injection analysis [15], fluorimetry [16,17], capillary isotachophoresis [18], chemiluminescence oxidation [19], differential-pulse polarography [20] and differential-pulse stripping voltammetry [21].

No SIA–SPE procedure has been reported to date for the determination of amiloride. Method for direct determination of amiloride in urine comprising on-line sample preparation based on SIA–SPE hybrid technique has been proposed and advantages and disadvantages of such a connection are reported in the presented paper.

2. Experimental

2.1. Sequential injection system and SPE column

A commercially available instrument FIAlab® 3500 system (FIAlab® Instruments Inc., Bellevue, USA) with a syringe pump (syringe reservoir 5.0 ml) and an eight-port selection Cheminert valve (Valco Instrument Co., Houston, USA) was used. The manifold was equipped with fiber-optic fluorimetric detector PMT-FL (Ocean Optics Inc., Dunedin, USA) with UV light source D-1000-CE (Analytical Instrument Systems Inc., Flemington, USA). The fluorescence signal was adopted by secondary filter (385 nm, Edmund Industrie Optik, GmbH, Karlsruhe, Germany). The whole SIA system was controlled by the version of program FIAlab® for Windows 5.0. Flow lines were made of 0.75 mm i.d. PTFE tubing. On-line sample preparation was performed on SPE column BAKER–carboxy group. For the lab-made preparation of the microcolumn, silica gel chemically modified with WCX–carboxylic acid (BAKER), the grain size 40–60 μm, was packed into PTFE tubing with 2 mm i.d. and 10 mm length. Both ends were closed with glass wool. The SPE column was placed between the selection valve and flow cell of the detector. An overall schematic view of SIA–SPE coupling is shown in Fig. 2.

2.2. Reagents and SPE sorbents

All chemicals used were of analytical grade quality. Amiloride and organic solvents were obtained from Sigma–Aldrich. Chemicals for buffer preparation were obtained from Merck, Germany. Millipore Milli-Q RG (Millipore s.r.o.,

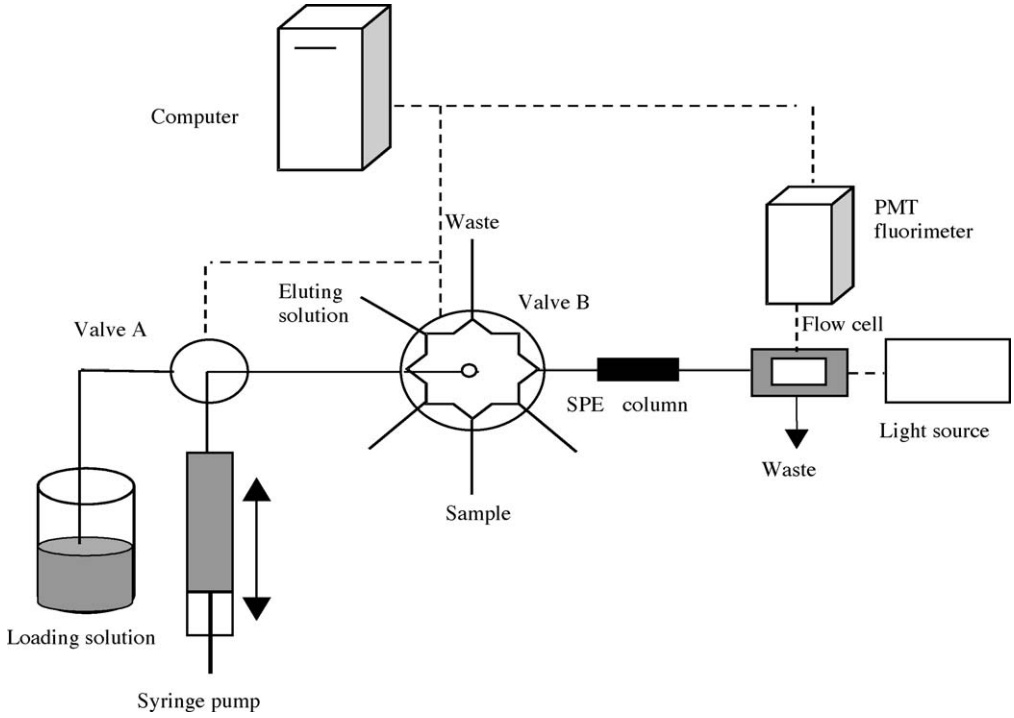

Fig. 2. The schematic view of SIA–SPE coupling.

Table 1
The sequence of the particular steps of SIA–SPE programme for the amiloride extraction and determination

Step number	Step	Flow rate ($\mu l\,s^{-1}$)	Volume (μl)	Type of reagent
1	Carrier stream aspiration	100	3500	Water
2	Amiloride injection	20	40	Spiked urine solution
3	Sample propelling through the column/removing urine matrix to the waste	20	3500	Water
4	Amiloride elution/peak recording	20	1300	0.01 M HNO_3 with 0.1 M KCl
5	Washing the system	40	2500	Water

Prague, Czech Republic) ultra pure water was used for preparing the solutions. Eluting solutions were degassed by helium before use. The sorbents used in study were BAKERBOND C-18 and BAKERBOND WCX (carboxy group) from JT Baker (Netherlands), LiChrolut® TSC, LiChrolut® EN and LiChrospher® RP-18 ADS from Merck (Germany) and CIM® SCX (aromatic sulphuric acid) monolithic disk from BIA Separations (Slovenia).

2.3. Preparation of spiked urine and standard samples

A stock solution of amiloride ($100\,\mu g\,ml^{-1}$) was prepared by dissolving the substance in water. The flask was stored in the refrigerator protected to light for 2 weeks without stability problems. Fresh working standard solutions were prepared daily by appropriate dilution of the stock solution in water to the concentration $20\,\mu g\,ml^{-1}$. The sample of human urine was diluted 10 times and spiked with stock solution of amiloride to get the final concentrations of amiloride (5, 10, $20\,\mu g\,ml^{-1}$) in 10 times diluted urine. The samples were spiked just before analysis.

2.4. SIA–SPE procedure

The following procedure was carried out in the proposed SIA–SPE system. The carrier stream (ultra pure Millipore Milli-Q water) and the appropriate defined volume of sample were aspirated into the system. The sample was dispensed to the microcolumn; amiloride from the sample was adsorbed onto the sorbent in the microcolumn, which was successively washed with the carrier stream and thus the interfering substances were removed to the waste. In the next step, the eluent was aspirated and dispensed through the microcolumn to the fluorescence detector. The design of the proposed SIA–SPE procedure including syringe pump steps, volumes and flow rates of reagents is mentioned in Table 1. The resulting signal was recorded in the form of peaks; the peak heights were calculated automatically by FIAlab® software and the data were stored by the PC for subsequent processing. Each measuring cycle was carried out in triplicate and the mean peak height values were used for data evaluation. All measurements were performed at ambient temperature.

3. Results and discussion

3.1. Optimization of the steps of the sequential injection extraction

A procedure based on sequential aspiration of different washing and eluting reagents and their propelling through the column was proposed. The priority of the study was to optimize the composition of eluting reagents to obtain the peak of the analyte that is differentiated from the matrix of urine. The washing and eluting reagents used in the particular steps influenced retention of the analyte and matrix during the whole procedure and the optimization of their composition was a complex process. The optimization of the single steps discussed in the following text was based on finding the optimum composition of the eluting reagents for different sorbents and adapting the composition of the other eluting reagents to these conditions. The analytical cycle involved three main steps: (1) loading the sample onto the column and removing the urine ballast material, (2) washing the column, removing the salts and residual interfering substances complicating the detection and (3) elution and detection of the analyte. The composition of eluting reagents used for particular steps was optimized for each sorbent separately.

The experiments concerning the design of SIA–SPE system including the elution reagents and sorbents has been started by searching the optimal sorbent. A brief description of SPE optimization steps is showed in Fig. 3. First experiments were carried out with C-18 modified silica gel sorbents BAKERBOND C-18 (particle size 40–60 μm) and special restricted-access material sorbent LiChrospher® RP-18 ADS column (particle size 25 μm). These sorbents were not found suitable due to the polar character or the analyte. Amiloride has shown insufficient retention and elution of analyte was observed during the washing of the column by water carrier stream.

The next tested sorbent was LiChrolut® EN (particle size 40–60 μm) usually used for extraction of polar analytes in environmental analysis. The composition of this sorbent is styrendivinylbenzene resin, pH stability 1–13. The molecule of amiloride showed total adsorption and insufficient elution. The tested elution reagents were 100% methanol, methanol at pH 2.5 adjusted with H_3PO_4, methanol at pH 11.4 adjusted with NaOH, mixture of methanol with 0.01 M NaOH (50:50, v/v) and alkalized mixture of methanol:acetone (3:2, v/v), pH 12.6. None of these solutions were suitable for quantitative amiloride elution.

LiChrolut® TSC (particle size 40–60 μm) was chosen as a sorbent with mixed mode phase for extraction of cations and neutral compounds. The extraction of amiloride on this sorbent was sufficient and quantitative elution was achieved with alkaline reagent—mixture of 0.01 M NaOH and 0.01 M NaCl. On the other hand, the sorbent was not possible for long term use due to its poor stability in alkaline conditions. This effect resulted in poor reproducibility of amiloride extraction and determination in standard solutions. The elution with organic solutions –

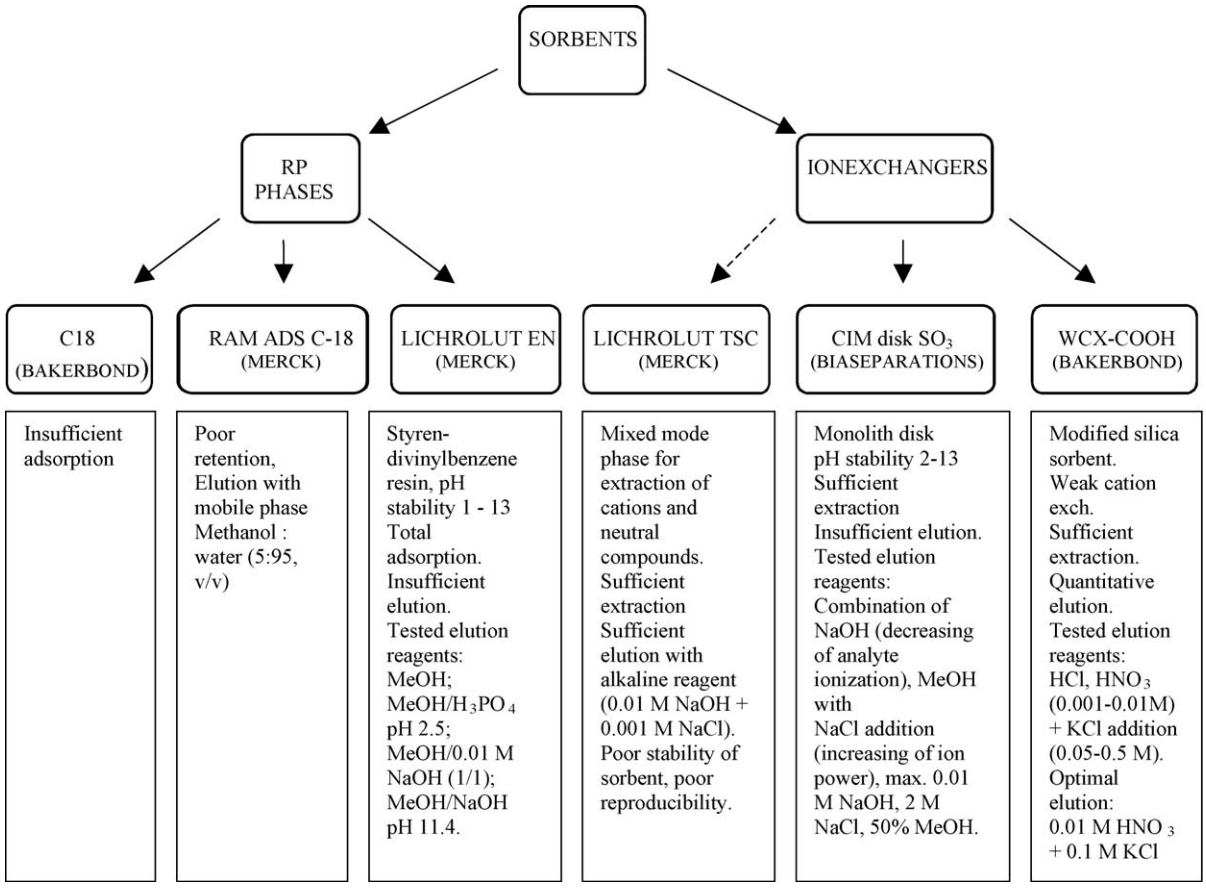

Fig. 3. The diagram of SPE optimization steps.

100% methanol, 100% acetonitrile or alkalized methanol – was not achieved on this sorbent.

Monolithic disk CIM® SCX (aromatic sulphuric acid) was chosen as a strong cation exchange sorbent with wide range pH 2–13 stability. The extraction of amiloride on the sorbent was sufficient and quantitative, but sufficient elution was not achieved in this case. The tested elution reagents were methanol with 0.01 M NaOH in different ratios and mixture of methanol—0.01 M NaOH (50:50, v/v) with 2 M NaCl addition. None of these combinations – decreasing of analyte ionization, or increasing of ion power of elution reagent – was effective for quantitative amiloride elution.

The last tested sorbent was BAKERBOND WCX (carboxy group—weak cation exchange). Testing of this sorbent was chosen after the experience with the strong cation exchange behaviour. Due to the amiloride structure there was high probability of sufficient retention of amiloride on WCX sorbent following quantitative elution. The extraction of amiloride from water solution on WCX sorbent was sufficient in this case. The strategy to achieve quantitative elution of amiloride was based on decreasing of ionization of carboxy group on the modified silica sorbent surface using strong mineral acids as elution reagent.

Tested elution reagents were HCl and HNO_3 in the concentration range 0.001–0.01 M. The addition of KCl into acid solutions for increasing of ion power in concentration range 0.05–0.5 M was tested too. The optimal elution reagent for quantitative elution of amiloride from WCX sorbent was found as a mixture of 0.01 M HNO_3 with 0.1 M KCl. Under these conditions the peak of amiloride was recorded during the elution with 1.3 ml of elution reagent. No peak was observed after next blank sample injection. Removing of the urine matrix interferences was achieved washing the column with water. Nevertheless, sample of urine need for 10 times dilution before application onto the column, to total avoid urine interference signal. No peak from residue of urine matrix was observed after blank sample urine injection under these conditions. Amiloride from the urine sample was adsorbed on the column while the interfering fluorescence substances were removed to the waste (Fig. 4). The steps of proposed SIA–SPE procedure are mentioned in Table 1. The system should be washed with water between the single determinations to remove the rest of eluting reagent from the piston pump.

3.2. pH conditions and fluorescence

Due to the structure of WCX SPE sorbent, it was necessary to decrease the pH of the eluting reagent to values at which ionization of free carboxy group on sorbent surface is suppressed and the analyte can be eluted from the column. Sufficient

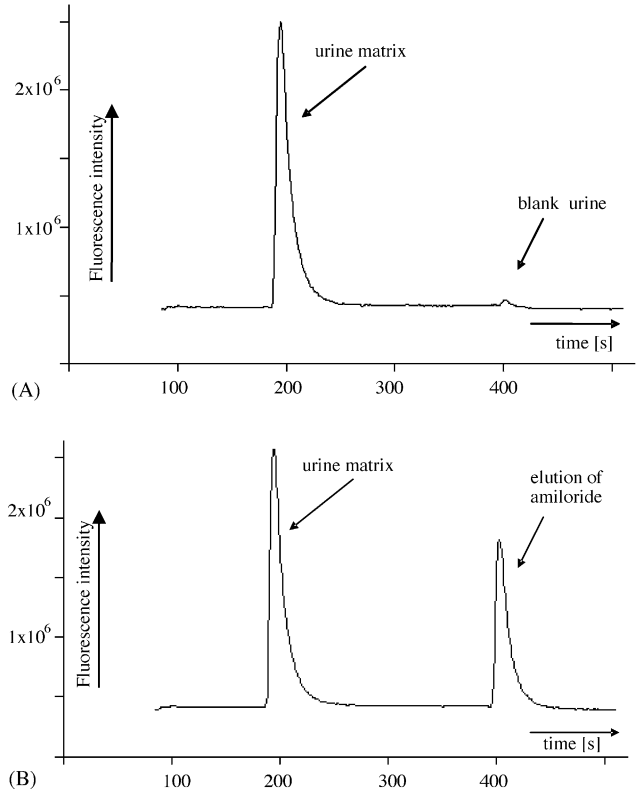

Fig. 4. The record of blank urine (A) compared to the urine spiked with 20 μg l^{-1} of amiloride (B). The interfering substances are washed out with water (the first peak), while amiloride is retained on the column. Amiloride is eluted with mixture of 0.01 M HNO_3 + 0.1 M KCl (the second peak) and detected in the next step.

extraction of amiloride was achieved with eluting reagent 0.01 M HNO_3 with 0.1 M KCl at pH 2.0. Low pH was crucial for the quantitative elution of the amiloride. The effect of pH value on the fluorescence intensity was tested without SPE column in the wide range pH values 2–10. The testing solutions were 0.01 M HNO_3, phosphate buffers (0.05 M KH_2PO_4 and 0.05 M K_2HPO_4) and 0.01 M NaOH. These solutions were mixed with the zone of amiloride directly in the system and they were transferred through the fluorescence detector. The increasing of the fluorescence signal of amiloride was observed in acid area. Changing pH to alkaline area resulted in weak decreasing of the fluorescence signal about 10%. Combination of 0.01 M HNO_3 with 0.1 M KCl as elution reagent did not decrease fluorescence signal and did not influence the sensitivity of determination.

3.3. Flow rates and volumes of the eluting reagents in particular steps of the procedure

SIA is generally characterized by short time of the analysis and high sample throughput. Depending on the principal of the determination sample throughput going over 60 samples h^{-1} can be achieved. However, in case of SIA–SPE the whole analytical procedure is more complex comprising sample pre-treatment and analyte quantitation and thus sample throughput is substantially reduced. Flow rates used with this technique are limited by back-pressure of the SPE column especially by using longer columns or lower particles size.

The first step of the procedure (loading of the sample and removing the urine matrix from the column) was considered to finish when the detector had reached the baseline. The fluorescence signal after injection of 40 μl of undiluted urine did not reach the baseline after using of full reservoir of the piston pump (5.0 ml of water washing solution). This aspect indicated presence of some low amount of fluorescence interfering substances that increased the noise of the baseline during the elution time of amiloride. This effect did not succeed to remove without losing of the analyte during the washing out step. Hence 10 times dilution of urine sample was carried out to avoid the disturbing effect of residual interferences. A total volume of 3.5 ml of the water carrier stream (flow rate of 20 μl s^{-1}) corresponding to time 175 s after injection was found to be sufficient for complete removing of the urine matrix after injection of 40 μl of 10 times diluted urine. Elution profiles of the urine matrix interferences using fluorescence detection are presented in Fig. 4A and B, respectively.

The second step—elution reagent 0.01 M HNO_3 with 0.1 M KCl was propelled through the column at the flow rate of 20 μl s^{-1}. The lowest volume ensuring the complete elution of the analyte peak was 1.3 ml. The optimization of elution flow rates was carried out in the range 10–30 μl s^{-1}. The maximum peak height was observed at flow rate 20 μl s^{-1}.

The last step of the analytical cycle was washing the system with 2.5 ml of water at flow rate 40 μl s^{-1}. This step was necessary to include to the cycle due to removing of elution reagent from the pump, column and tubes.

3.4. Adsorption capacity of the BAKERBOND WCX column

The adsorption capacity of the BAKERBOND WCX column (2 mm i.d. and 10 mm length) was studied. Standard solution of amiloride (40 μl, 20 μg ml^{-1}) was successively injected onto the column using the water as carrier. After six injections corresponding to total amount of 4.8 μg of amiloride the analyte started to leak from the column. The effect of the injected volume (10, 25, 50 and 100 μl) of amiloride solution (20 μg ml^{-1}) on the fluorescence intensity was studied and a linear relation characterized by calibration equation $I_F = (85{,}878.0 \pm 91.1)v + (42{,}893.8 \pm 5235.9)$ (I_F is the intensity of fluorescence and v is the volume of amiloride solution) was found. The extraction capacity of the column enables the increase of the injected sample volume and thus decreases of the limit of detection. However, bigger sample volume would probably require higher volume of loading and washing reagent for removing the interfering matrix, which would be time consuming.

3.5. Validation

The method was validated with respect to the linearity, precision, accuracy, selectivity and sensitivity in order to evaluate the reliability of the results provided by the method.

3.5.1. Calibration and sensitivity

The calibration curve was established by measuring the fluorescence signal of seven solutions of various concentrations of amiloride in urine. The linear relation between the fluorescence intensity and concentration of amiloride was found in the range 0.5–100 μg ml^{-1} and was described by the following equation: $I_F = (8131 \pm 217)c + (42{,}167 \pm 9482)$ (I_F means the intensity of fluorescence and c is the concentration of amiloride), the correlation coefficient being 0.9982.

The limit of detection and limit of quantitation were calculated by the comparison of the 3-fold (3σ) and 10-fold (10σ) variation, respectively, of baseline noise and signals of urine samples spiked with known concentrations of amiloride. The detection limit was 0.05 μg ml^{-1}; the limit of quantitation was estimated to be 0.17 μg ml^{-1} for 40 μl 10 times diluted urine injection. The sensitivity of the system is sufficient for real drug level monitoring of amiloride in the human urine of patient.

3.5.2. Precision

Precision of the proposed procedure was characterized by parameter of repeatability, which was calculated for 10 consecutive measurements at concentration level 20 μg ml^{-1} of amiloride in spiked urine samples. The relative standard deviation (R.S.D.) was 5.4%. The inter-day repeatability was determined at three concentration levels (0.5, 5 and 20 μg ml^{-1}) of the amiloride standard water solution for 3 consecutive days. The results in form of R.S.D. were determined less than 3.2% ($n = 10$) including all concentration levels.

3.5.3. Accuracy

Accuracy of the method was estimated using the parameter of recovery. The recovery was calculated at three concentration levels (5, 10 and 20 μg ml^{-1}) by comparison of the responses of amiloride from spiked urine samples with those found by injection of the standard solutions at the same concentrations. The mean absolute recovery lay between 96.8 and 99.4% for amiloride and extraction efficiency was relatively constant over the range mentioned above (R.S.D. = 1.59%). The SPE column can be used repeatedly in the SIA system, but decreasing of recovery and extraction efficiency was observed after using more than 70 injections of sample urine.

3.5.4. Selectivity

The absence of interfering endogenous components of urine during the elution of amiloride is demonstrated in Fig. 4. Amiloride from the urine sample was adsorbed on the column while the interfering fluorescence substances were removed to the waste. The analyte was then eluted from the column separately and provided fluorescence signal, which could be used for the determination of amiloride. Fig. 4 shows typical record obtained during washing and eluting step of blank urine (A), and washing and elution step by analysis of urine spiked with amiloride (20 μg ml^{-1}) (B). Human urine is 10 times diluted in both cases. Washing of interfering components from the column was achieved with 3500 μl of water, flow rate 20 μl s^{-1} and thus it was possible to achieve a sample throughput of 8 samples h^{-1}.

Table 2
Influence of interfering substances on SIA–SPE determination of amiloride (concentration of amiloride: 5 μg ml^{-1})

Tested compound	Concentration of added compound (μg ml^{-1})	Found amount of amiloride (%)[a]
Metoprolol	200	98.8
Atenolol	100	96.9
Betaxolol	20	100.2
Acebutolol	400	126.5
Furosemide	60	102.3
Hydrochlorthiazide	90	100.8
Chlorthalidone	90	98.2
Losartan	100	96.5
Verapamil	360	100.1

[a] Mean value of three determinations.

The next interference studies were carried out for determination of amiloride in the presence of different pharmaceuticals. The pharmaceuticals were chosen from the group of pharmaceutical preparations that is usual combined with amiloride in the treatment of hypertension and different kinds of oedema. The tested compounds were from the group of cardioselective β-blockers (metoprolol, atenolol, betaxolol and acebutolol) and from the group of diuretics (furosemide, verapamil, losartan, hydrochlorothiazide and chlorthalidone). The interference influence on amiloride signal was tested for amiloride solution (5 μg ml^{-1}) with addition of one tested compound. The concentrations of tested compounds were chosen accordance with usual drug dosage. The results of interference studies are mentioned in Table 2. Only acebutolol from the group of tested compounds showed increasing of amiloride signal about 26%. On the other side, concentration of acebutolol was 80 times higher than amiloride concentration. The next compounds showed no significant interference.

4. Conclusions

A hybrid SIA–SPE technique with fluorescence detection of amiloride has been proposed. The SPE column can be used repeatedly and enables one to remove the interfering substances and to determine amiloride directly in human urine. The proposed SIA–SPE method involving sample preparation can be simply automated and shows the possibility of restriction of manual sample handling. Repeated injections of untreated urine are possible; the procedure ensures quantitative removal of urine matrix and on-column enrichment of the analyte. Minimum manipulation of the biological sample results in improved precision and accuracy, shorter analysis time and lower costs per analysis. In contrast to the HPLC with off-line SPE technique, the SIA–SPE technique is based on non-continuous flow, which enables reduction of volume of consumed reagents and produced waste. Method exhibits similar sensitivity as conventional separation techniques and it is sufficient for real drug level monitoring of amiloride in the urine. Moreover, the method requires less expensive instrumentation than usual separation techniques.

Although SIA–SPE method enables to remove the interfering substances, it is still a non-separative technique. In the case of

analysis of biological material it mostly enables to determine only the total amount of the original analyte and its metabolites, not the precise amounts of particular substances. Nevertheless, the proposed method is sufficient enough for screening of patient compliance or doping control.

Acknowledgements

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