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On-line coupling of solid-phase extraction with mass spectrometry for the analysis of biological samples I. Determination of clenbuterol in urine

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Abstract

The potential of the direct coupling of solid-phase extraction (SPE) with mass spectrometry (MS) for the analysis of biological samples is demonstrated. For SPE a cartridge exchanger is used and the eluate is directly introduced into the mass spectrometer. This system has been investigated for the determination of clenbuterol in urine. With mixed-mode cartridges, a considerable ion suppression has been obtained. The mass spectrum at the elution time of clenbuterol is dominated by that of creatinine and adduct formation of clenbuterol and creatinine has been observed. The whole procedure including injection of 1 ml urine, washing and desorption has been developed with cartridges containing 8-μm C_{18}-bonded silica. If only a single MS step is used, the selectivity and, therefore, the sensitivity are insufficient. The detection limit is about 100 ng/ml. However, with atmospheric pressure chemical ionisation and the tandem MS mode the detection limit has been decreased to about 2 ng/ml and the ion suppression is only about 10%. For the electrospray ionisation the detection limit is about 10-times higher and the ion suppression is less favourable. The repeatability for the SPE–MS–MS procedure was 6.5% at 10 ng/ml ($n=5$) and the difference between the response factors at 10 ng/ml and 100 ng/ml was only 2.5%. The MS behaviour of clenbuterol and the matrix under the present conditions is discussed.

Keywords: Solid-phase extraction–mass spectrometry; Mass spectrometry; Interfaces, SPE–MS; Clenbuterol; Creatinine

1. Introduction

In pharmaceutical industries and related institutes high-throughput analysis of biological samples is becoming more and more important. Moreover, due to the development of more potent drugs, drug concentrations in biological samples are often present at much lower levels than before. Therefore, fast analytical techniques with much higher sensitivity and selectivity are needed. For this purpose, the recent development of atmospheric pressure ionisation interfaces for on-line liquid chromatography–mass spectrometry (LC–MS) was very important [1,2]. The mass specificity of LC–MS led to the development of various analytical assays for biofluids with less sample preparation and short chromatographic run times of 2–5 min [3–5]. The sample throughput with LC–MS has become so large that manual extraction techniques have become the bottleneck for the total analytical procedure. Solid-

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phase extraction (SPE) has been developed to a very strong technique for clean-up and preconcentration of biological samples. A large variety of sorbents and embodiments (columns, discs) is now available and the technique can easily be automated. Automation of off-line SPE is possible and a promising recent development is ''96-well SPE'' [6]. The on-line coupling of SPE to LC is now routine for many years and, more recently, SPE has also been coupled to gas chromatography (GC) [7]. On-line SPE appears to be attractive because the entire process of conditioning, sample application, washing and elution takes place with constant flows yielding better precision of quantitative methods in comparison with off-line vacuum driven extraction procedures. Another important advantage of on-line SPE procedures is that no sample transfers are made and that the transfer of analyte is complete which cannot be achieved with off-line SPE.

A very elegant system for rapid analysis of complex samples can be obtained by the on-line coupling of SPE or short-column LC to MS detection [8–10]. In this way the sample is directly injected into the SPE–MS system and a real efficient separation step is eliminated. Although the advantages are numerous, i.e., SPE–MS is fast and very cost-effective, there are also restrictions which should be carefully considered before a robust method is developed. Body fluids are complex biological matrices containing numerous amounts of compounds, ranging from simple inorganic salts to large proteins, which all can interfere in the different stages of the procedure. Since an analytical column is not present, a much higher clean-up efficiency is required from the SPE procedure. If the sample is not sufficiently cleaned prior to introduction into the mass spectrometer, co-eluting endogenous compounds are able to cause ion-suppression effects [11], resulting in low precision and accuracy. Also in the tandem MS mode such effects can give a strong limitation. It is therefore essential to optimise both the SPE procedure and the MS detection in an integrated approach in order to avoid that (un)detected compounds interfere with detection of the analyte(s).

In this study on-line SPE–MS(–MS) has been evaluated for application in bioanalysis. SPE will be applied for clean-up, preconcentration and separation. In order to obtain insight in the viability of SPE–MS(–MS) systems for the analysis of biological samples the determination of low concentrations of a relatively polar analyte with poor UV characteristics, i.e., the determination of the β-agonist clenbuterol in urine was selected. Our research group is involved in the development of fast and cost-effective screening methods for illegal growth hormones. Attempts to develop a simple and highly sensitive assay for clenbuterol in urine yielded a method with a limit of detection of 4 ng/ml despite a three-step sample pretreatment procedure including immobilised antibodies followed by a chromatographic separation with electrochemical detection [12]. As SPE–MS interface both atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) were used. The possibilities and restrictions with respect to selectivity and sensitivity of the SPE–MS(–MS) system have been investigated.

2. Experimental

2.1. SPE procedure

All on-line SPE experiments were performed on a Prospekt (Spark, Emmen, The Netherlands), which consists of a cartridge switching device with three six-port valves and a low-pressure solvent delivering system, capable of delivering six different solvents. As Prospekt cartridges Bond Elut Certify (10×2 mm, particle size 40 μm), a combination of C_8 and cation exchanger, and Hysphere-9 (C_{18}, 10×2 mm, particle size 7 μm) were applied. Activation, conditioning, trapping and cleaning were done with the solvent delivery unit and the effluent was connected to waste. Desorption was done with a gradient HPLC pump system (Kratos SF400), which was coupled to the mass spectrometer. The UV detection experiments were performed with a Hewlett-Packard diode array detector HP series 1100 (Hewlett-Packard, Waldbronn, Germany).

For the Bond Elut Certify cartridges the SPE procedure was as follows: activation was performed with 2.0 ml methanol at a flow-rate of 1.0 ml/min and conditioning with 1.0 ml acetic acid (0.007 *M*) at a flow-rate of 1.0 ml/min. The sample was flushed over the cartridge by 0.75 ml acetic acid (0.007 *M*) at a flow-rate of 0.5 ml/min. The applied sample volume was 200 μl (Midas autosampler, Spark).

Elution was performed with of a mixture of 25% ammonia–methanol (2:98, v/v) at a flow-rate of 1.0 ml/min.

For the C_{18} cartridges the SPE procedure was as follows: activation was performed with 2.5 ml methanol and conditioning with 2.5 ml ammonium acetate (0.025 *M*, adjusted to pH 8 with 2.5% ammonia) at a flow-rate of 2.5 ml/min. The 1.0 ml sample was flushed over the cartridge at a flow-rate of 0.5 ml/min. Elution was performed with a linear gradient made up of ammonium acetate (0.025 *M*, pH 8) and methanol (0% to 35% in 2.5 min) at a flow-rate of 1.0 ml/min during about 9 min.

2.2. Mass spectrometry

The MS experiments were performed with a Nermag R 3010 triple quadrupole mass spectrometer (Nermag, Argeteuil, France) equipped with a laboratory-built atmospheric pressure ionisation source [13] with APCI and ESI interfaces. The MS–MS performance of the Nermag was improved in our laboratory [14]. The Perkin-Elmer Sciex API 3 data system with a laboratory-built analogue and digital interface was used for data acquisition and data processing. The heated nebuliser temperature during the APCI experiments was set at 500°C. The 1.0 ml/min effluent of the HPLC gradient pump system was directly introduced into the APCI interface. For the ESI experiments a capillary voltage of 3.5 kV was applied. Nitrogen was used as nebuliser gas. The 1.0 ml/min effluent of the HPLC system was split 1:20 prior to introduction into the ESI interface.

The extracted ion chromatogram was obtained by monitoring the $[M+H]^+$ ion of clenbuterol at m/z 277. MS–MS detection was performed by monitoring the decay of the $[M+H]^+$ ion of clenbuterol at m/z 277 to its most intense fragment ion at m/z 203 in the selected reaction monitoring (SRM) mode. Argon was applied as collision gas and the collision energy was set at 40 eV.

3. Results and discussion

3.1. Optimisation of SPE

For on-line SPE the Prospekt with automated cartridge exchanger and a solvent delivery unit for the selection of conditioning, washing and eluting solvents was used. After single or multiple use the cartridge is discarded and replaced by the next cartridge in a cartridge holder. Since SPE is applied as sample clean-up and separation device the cartridges were eluted in the forward-flush mode to obtain some chromatographic separation. The packing material was optimised with respect to trapping capacity of the relatively polar clenbuterol and efficient removal of urine matrix compounds.

A very efficient sample handling procedure based on a mixed mode SPE procedure which was developed for a large series of drugs [15] was tested for the clenbuterol assay. A mixed mode stationary phase contains C_8 and cation-exchange groups and therefore compounds with different physicochemical properties can be retained and selectively eluted. Clenbuterol can be sorbed in its protonated form at low pH values. The ion-exchange properties of the mixed mode phase make it possible to apply for some analytes relatively high concentrations of organic modifier during the washing step and, therefore, it is expected to give the required high selectivity. However, during analysis of a spiked urine sample using SPE with the mixed mode cartridge, a reduction of 50% of clenbuterol precursor ion ($[M+H]^+$) signal was observed in comparison to aqueous solutions. The ion chromatogram at m/z 114 indicates that a large creatinine (molecular mass 113) band co-eluted with clenbuterol (Fig. 1) and in the mass spectrum taken at the maximum of the elution profile of clenbuterol concentration adduct formation $[M+\text{creatinine}+H]^+$ was observed (Fig. 2). Moreover, the first part of this spectrum was strongly dominated by the spectrum of creatinine. Creatinine is excreted in human urine at concentrations of approximately 0.8 mg/ml. Attempts to optimise the separation of clenbuterol and creatinine were not successful. The sorption and elution behaviour of both compounds were similar on the mixed mode material (cation-exchange).

C_{18} cartridges were found to have more potential for this assay, since no significant reduction in the precursor ion signal was observed if APCI was used as SPE–MS interface, and the selectivity for creatinine was much higher. Because creatinine is significantly more polar than clenbuterol, it is washed away before the elution of clenbuterol to the MS system. However, clenbuterol itself is also a

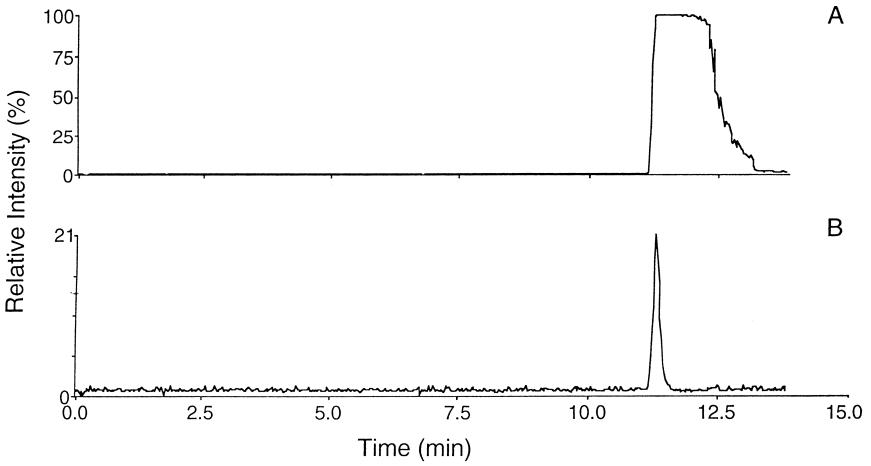

Fig. 1. Extracted ion chromatograms in the ESI mode at m/z 114 of protonated creatinine (A) and at m/z 277 of protonated clenbuterol (B). Monitoring of the SPE eluate using a Bond Elut Certify cartridge after injection of 200 μl urine spiked with 2 μg/ml clenbuterol.

relatively polar compound with a low retention on a C_{18} stationary phase, which is unfavourable for separation of clenbuterol from polar matrix compounds. The SPE procedure using C_{18} sorbent was further investigated for the SPE–MS–MS determination of clenbuterol. A small particle size of the cartridge packing material combined with gradient elution was found to be favourable with respect to maximum clenbuterol recovery and separation from a large part of the endogenous compounds excreted in urine. The use of a short washing step with a solvent containing a low percentage of a modifier did not give an improvement of the sample clean-up. The selected solvents and buffer salts used for the elution are volatile in order to avoid compatibility problems with the vacuum of the mass spectrometer.

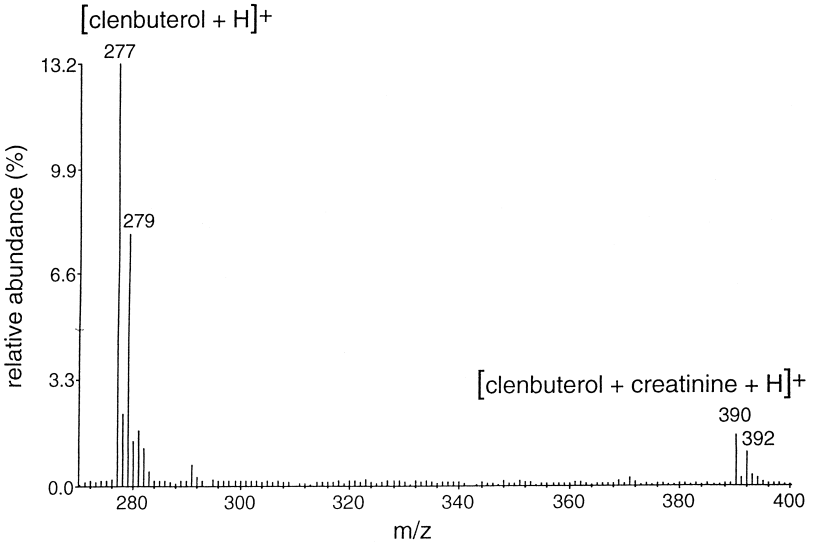

Fig. 2. Partial mass spectrum recorded at the maximum clenbuterol concentration (see Fig. 1).

Generally, the cartridge was replaced after three injections of 1 ml urine, in most cases this was not necessary.

An increase of the flow-rates of the different steps is required since the total analysis time is relatively long (about 15 min). The use of a relatively high flow-rate of 2.5 ml/min during activation and conditioning of the cartridge did not have a negative influence on the separation, and on the form and height of the clenbuterol peak. The flow-rates of sample loading, washing and desorption steps were found to be more critical because of the effect on the recovery and peak broadening, and, therefore, these flow-rates were still not increased.

3.2. Mass spectrometry

Fig. 3 demonstrates that mass selective detection is also required during method development, since the photometric detection in the UV range is dominated by matrix compounds and cannot discriminate between the matrix compounds and clenbuterol at the required sensitivity. The detection limit could not be decreased by use of an other UV wavelength. LC–MS interfaces based on atmospheric pressure ionisation such as ESI and APCI allow the sensitive determination of β-agonists [16–18]. Both ESI and APCI were used in combination with the optimised SPE procedure (with C_{18} cartridges). A single MS step did not give the required selectivity for the determination of clenbuterol in urine with the described SPE–MS technology using either ESI or APCI (see Fig. 4). As expected, increasing the sample volume from 200 μl to 1.0 ml did not result in a higher signal-to-noise (S/N) ratio. Apparently a number of co-eluting endogenous compounds also gave a signal at m/z 277. Background ESI spectra around the elution time of clenbuterol give significant peaks at m/z values 310 and 312, whereas APCI mass spectra around clenbuterol revealed several relatively large peaks at m/z values above 400. The ions can originate from single or multiple protonated or sodiated co-eluting endogenous compounds, e.g., different carbohydrate structures, amines, urea, lipids, proteins, etc., possibly present at much higher concentrations than the analyte. The quality of the spectra were insufficient to deduce structural information, and therefore the identity of these compounds could not be retrieved.

In the SRM mode the decay of the $[M+H]^+$ ion of clenbuterol at m/z 277 to its most intense fragment ion at m/z 203 was monitored. The frag-

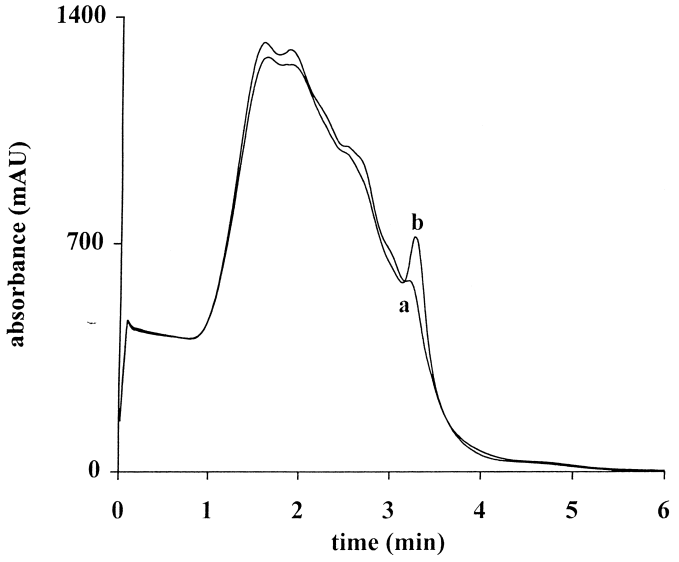

Fig. 3. Monitoring of the SPE eluate using a C_{18} cartridge with UV detection at 210 nm of 1.0 ml of (a) blank urine and (b) urine spiked with 1.0 μg/ml clenbuterol. The chromatograms are shown from the start of the elution step.

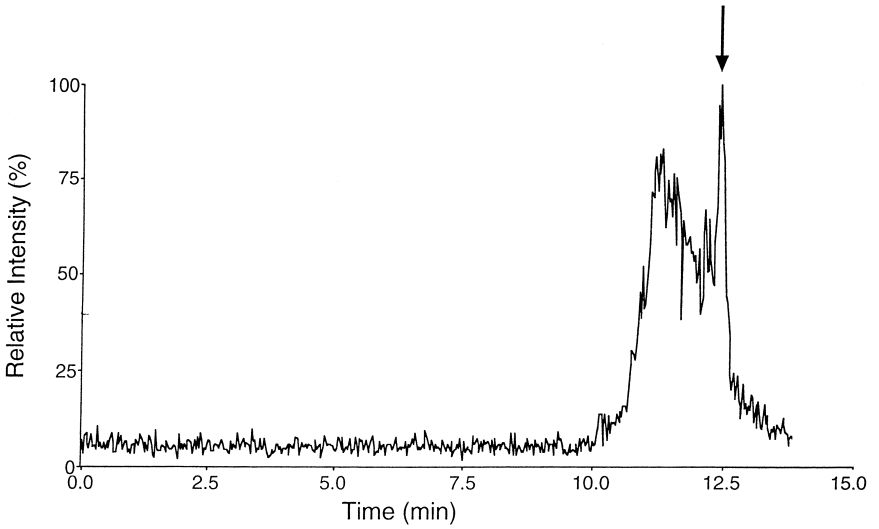

Fig. 4. Extracted ion chromatogram in the APCI mode at m/z 277. Monitoring of the SPE eluate using a C_{18} cartridge after injection of 1 ml urine spiked with 100 ng/ml clenbuterol (arrow marks clenbuterol peak).

ment ion at m/z 203 is formed after loss of H_2O followed by the loss of isobutene from protonated clenbuterol. The SRM mode provided the required selectivity for both ESI and APCI, as is presented in Fig. 5. Although the absolute signal decreased with a factor 5 (only determined for APCI) the S/N ratio increased considerably compared to the extracted-ion trace. Another indication for the increased specificity using SRM is that increasing of the sample volume from 200 μl to 1.0 ml did result in a proportional higher S/N ratio. With the APCI interface an endogenous compound was observed which eluted just before clenbuterol. Compounds can give a peak at the m/z value of the selected precursor ion mass in different manners, e.g., not only their most abundant natural isotopes, but also its other natural isotopes (^{13}C, ^{15}N, ^{18}O) either as single or multiple charged species.

As stressed before, co-elution of other compounds can influence the ionisation efficiency, and although they do not give rise to peaks in the MS windows used in this study, they can have a detrimental effect on the quantitation. Ion suppression was assessed by comparing the SRM signal of spiked urine samples and aqueous clenbuterol solutions. The recovery of the total procedure for standard solutions was 100%. For ESI and APCI a signal suppression of approximately 40% and 10%, respectively, was observed for urine samples. The phenomenon that APCI gives rise to less ion suppression than ESI for the same combination of analyte and matrix was observed earlier [11] and can be explained by differences in ion formation processes of the two interfaces. In ESI the compounds are ionised in the liquid phase and ions are released from charged droplets. The applied electric field between the capillary tube and the counter electrode results in accumulation of charged compounds at the droplet surface. The upper limit of the total number of ions which can be formed during ESI is related to the total surface area of all droplets and this upper limit is usually reached at 10^{-5} M sample ion concentration [19]. If many compounds are co-eluting, their relative liquid phase basicities and surface activities will determine the ionisation efficiency of the individual compounds. Since the endogenous compounds are present in relatively high concentrations and their relative basicities and surface activities can be comparable or higher than those of the analyte, the limit of 10^{-5} M of ions is rapidly exceeded leading to ion suppression. APCI is less prone to ionisation saturation since the maximum number of ions formed in the gas-phase ionisation process is much higher. The reagent ions generated from the vaporised mobile phase by

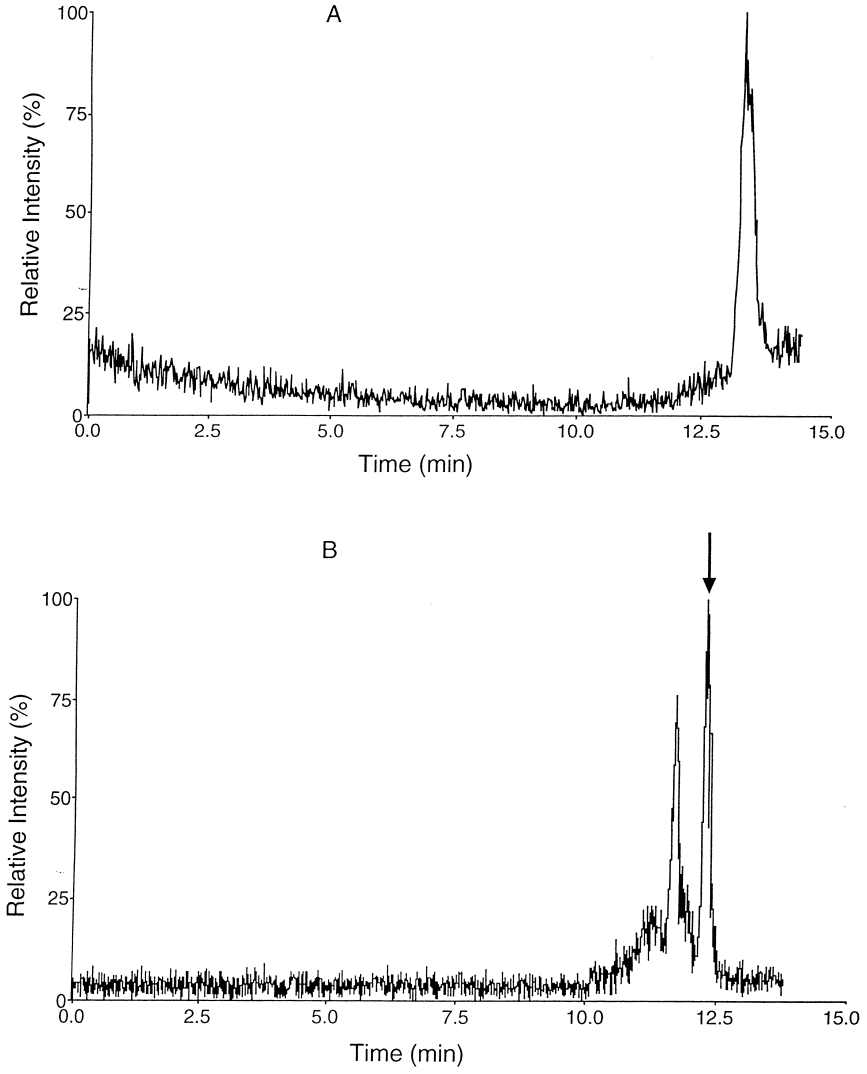

Fig. 5. Selected reaction monitoring (decay of m/z 277 to m/z 203) of the SPE eluate using a C_{18} cartridge: (A) 1 ml urine spiked with 100 ng/ml clenbuterol measured with ESI, (B) 1 ml urine spiked with 10 ng/ml clenbuterol measured with APCI (arrow marks clenbuterol peak).

corona discharge are redundantly formed and, therefore, signal saturation or ion suppression is expected to occur at higher ion concentrations compared to ESI.

Fig. 5 shows that the limits of detection for ESI and APCI are about 20 ng/ml and 2.0 ng/ml, respectively for injection of a 1.0 ml urine sample. The repeatability for the analysis of urine spiked with 10 ng/ml using APCI was 6.5% (relative standard deviation, $n=5$) and the difference in response factor between 100 ng/ml and 10 ng/ml was only 2.5%. The higher sensitivity of APCI indicates that the ionisation efficiency in the gas phase is higher than in the liquid phase under the present conditions. A further decrease of the detection limits seems possible by the use of larger sample volumes. The detection limit obtained for APCI makes this system very useful for screening

purposes in the low ng/ml range. It should be noted that Figs. 4 and 5 show the total time of the procedure from the activation of the cartridge and also including the injection (i.e., 4 min for 1 ml urine).

4. Conclusions

A system consisting of SPE on-line coupled to MS seems very powerful for the direct analysis of biological samples. The analysis time is short and a further decrease can probably be obtained by optimisation of the various steps in the SPE procedure and reduction of the volumes of the SPE system. Development of such an SPE–MS method is not easy because matrix components can interfere the MS detection, especially also by ion-suppression effects. If a hydrophobic non-selective cartridge is used, tandem MS is needed for sufficient selectivity. The use of tandem MS provided a 5–50-times lower detection limit than single MS. For the determination of clenbuterol in urine, a detection limit of about 2 ng/ml has been found with an injection of 1 ml urine and an APCI interface between SPE and MS–MS. With ESI, the detection limit is about 10-times higher and a significant ion suppression occurs. A further improvement of the sensitivity seems possible by use of modern types of MS apparatus. The main goal of this study was the evaluation of SPE–MS(–MS) for bioanalysis. Therefore, the method for clenbuterol in urine was not well validated. Moreover, generally for a screening method less validation is needed.

In the future, other model components and matrices will be tested and the influence of the main matrix components on the MS behaviour of analytes will be studied. Special attention will be given to more selective packing materials as molecular imprints and immobilised antibodies. The usefulness of single and tandem MS will be compared continuously.

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