On-line coupling of sample pretreatment with chromatography or mass spectrometry for high-throughput analysis of biological samples

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COUPLING OF SOLID-PHASE EXTRACTION AND MASS SPECTROMETRY
**On-line coupling of solid-phase extraction with mass spectrometry for the analysis of biological samples. Determination of clenbuterol in urine using multiple-stage mass spectrometry in an ion-trap mass spectrometer**

**Summary**

Solid-phase extraction (SPE) was coupled to ion-trap mass spectrometry to determine clenbuterol in urine. For SPE a cartridge exchanger was used, and after extraction, the eluate was directly introduced into the mass spectrometer (MS). For two types of cartridges, *i.e.*, C$_{18}$ and polydivinylbenzene (PDVB), the total SPE procedure (including injection of 1 ml urine, washing, and desorption) has been optimised. The total analysis, including SPE, elution, and detection, took 8.5 min with PDVB cartridges, while an analysis time of 11.5 min was obtained with C$_{18}$ cartridges. A considerable amount of matrix was present after extraction of urine over C$_{18}$ cartridges, resulting in significant ion suppression. With PDVB cartridges, the matrix was less prominent, and less ion suppression was observed. For single MS, a detection limit (LOD) of about 25 ng/ml was found with PDVB cartridges. With C$_{18}$ cartridges an LOD of only about 50 ng/ml could be obtained. Applying tandem mass spectrometry (MS/MS) did not lead to an improved LOD, because of an interfering compound. However, a considerable improvement in the LOD could be obtained with MS$^3$. The selectivity and sensitivity were increased by the combination of efficient fragmentation of clenbuterol and reduction of the noise. Detection limits of 2 and 0.5 ng/ml were obtained with C$_{18}$ and PDVB cartridges, respectively. The ion suppression was 4 to 45% (concentration range: 250 to 1.0 ng/ml) after extraction of urine using PDVB cartridges, and up to 70% ion suppression was observed using C$_{18}$ cartridges. With MS$^4$, no further improvement in selectivity and sensitivity was achieved, due to inefficient fragmentation of clenbuterol and no further reduction of the noise.

4.1.1 Introduction

The necessity of high-throughput analysis of biological samples is increasing, since, due to stricter policies for the registration and use of drugs, more samples have to be analysed in less time. Moreover, the development of more potent drugs results in lower concentrations of these drugs in biological samples. Thus, fast and sensitive analytical techniques are required. A solution for these seemingly incompatible requirements is presented by systems based on on-line liquid chromatography-mass spectrometry (LC-MS) with atmospheric pressure ionisation interfaces. Using the specificity of the MS, analytical systems have been developed in which only short LC columns (25 to 50 mm) are required for the analysis of biological samples [1-3], thus leading to short analysis times. However, the time-limiting step is usually the sample preparation, especially if carried out manually. A powerful technique for clean-up and preconcentration of biological samples is solid-phase extraction (SPE) [4,5]. With increasing numbers of samples, automation of SPE is needed. Consequently, there is a growing interest for on-line and at-line (including 96-well SPE [6,7]) coupling of SPE to LC [8-11] and, more recently, to gas chromatography (GC) [12-18]. On-line SPE is a very attractive sample pretreatment technique since the entire process of activation, conditioning, sampling, washing, and elution takes place in an enclosed circuit, which eliminates error-prone steps like evaporation and reconstitution. Therefore, in general, better precision and sensitivity are observed when compared with off-line SPE.

A very rapid system for the analysis of complex samples is obtained by direct coupling of automated SPE (“short-column LC”) to MS [19-26]. A true chromatographic separation step is not performed, which enhances speed and reduces costs. However, as a result, the SPE procedure must be performed very efficiently to ensure that clean extracts are obtained, which can be difficult if complex biological samples have to be analysed. The presence of matrix compounds in the extract can cause ion suppression [27-30], resulting in loss of reliability and accuracy of the analytical data obtained with an SPE-MS system. Multiple MS (MS<sup>n</sup> with n≥2) may give higher selectivity and thus higher sensitivity, but the effect of ion suppression will persist.

Inspection for the abuse of illegal compounds, like most growthpromoters, requires high-throughput analysis of biological samples, and detection of those compounds at low concentrations. The combination of SPE and MS, as described above, shows high potential for such applications. A popular growth promoter is clenbuterol, which is administered to cattle to increase the deposition of protein while reducing fat accretion. The effect is growth of muscle tissue and a weight gain [31]. Consumption of meat that contains clenbuterol can cause acute poisoning. Due to the severe effects on
human health [32-33], the use of clenbuterol as a growth promoter is prohibited in most countries. Previous efforts to develop a fast and cost-effective screening method for clenbuterol in urine resulted in a method with a three-step sample pretreatment, including use of immobilised antibodies, followed by chromatographic separation and electrochemical detection [34]. The limit of detection (LOD) was about 4 ng/ml.

Another system coupled SPE directly to a triple-quadrupole mass spectrometer using C\textsubscript{18} and mixed mode cartridges for sample pretreatment [19]. About 50\% ion suppression was observed, as well as the formation of adducts of clenbuterol and creatinine if mixed mode cartridges and electrospray ionisation (ESI) were used. Experiments with C\textsubscript{18} cartridges and atmospheric pressure chemical ionisation (APCI) showed no interference from creatinine. Selected reaction monitoring (SRM) resulted in an increase of the signal-to-noise ratio (S/N) by a factor of 5, compared to MS/MS full-scan detection with extraction of the \(m/z\) 203 signal. The LOD was about 20 and 2 ng/ml for the ESI source and APCI source, respectively. The total analysis time was still long, about 13.5 min, including sampling (4 min) and sample pretreatment.

In this paper we present the continuation of our research [19] concerning the potential of SPE-MS as a screening method for clenbuterol in urine. Instead of a triple-quadrupole mass spectrometer, a relatively cheap ion-trap instrument was used. A gain in sensitivity had to be achieved by adjusting the MS mode, i.e., single MS vs. MS\textsuperscript{n} (n\geq 2). The clean-up is more critical with an ion-trap mass spectrometer than with a triple-quadrupole one. Besides ion suppression [27-30] a possible loss of signal can be caused by the limited capacity for ions of the ion-trap. Only an APCI source was used, since the best results had been obtained with this source in previous experiments [19]. The purpose was to produce cleaner extracts by use of another stationary phase in the SPE cartridges. Furthermore, ways to reduce the analysis time were investigated.

### 4.1.2 Experimental

#### 4.1.2.1 Chemicals and instrumentation

All on-line experiments were performed with a Prospekt (Spark, Emmen, The Netherlands) using one six-port valve, the cartridge-switching device, and a solvent delivery unit (SDU). Activation, conditioning, sampling, trapping, and washing were done using the SDU. The effluent was connected to waste during these steps. All steps of the SPE procedure were carried out using a forward-flush mode, by which some chromatographic separation was obtained. It was possible to replace a cartridge with a new cartridge in the cartridge holder after single or multiple use. The elution was performed using a Hewlett-Packard
gradient pump Series 1100 (Hewlett-Packard, Waldbronn, Germany), which
was connected to an LCQ ion-trap mass spectrometer (Thermoquest, San Jose, CA, USA). The mass spectrometer was equipped with an APCI source. Experiments with UV detection were carried out with a Hewlett-Packard diode array detector, Series 1100. The Prospekt cartridges used in this work were HySphere–9 (C\textsubscript{18}, 10×2 mm, particle size 7 µm) and HySphere Resin GP (10×2 mm, particle size 10 µm), a spherical polymeric phase of polydivinylbenzene (PDVB).

Methanol was of HPLC grade (Lab Scan, Dublin, Ireland). The ammonium acetate was of pro-analysis quality (Merck, Darmstadt, Germany). The 5 mM ammonium acetate buffer was adjusted to pH 8 using 2.5% ammonia (pro-analysis quality). Pure water was obtained from an Elgastat maxima system (ultra pure water, Salm and Kipp, Breukelen, The Netherlands). Aqueous solutions were filtered over a 0.45-µm RC 55 membrane filter (Schleicher & Schuell, Dassel, Germany) prior to use. Clenbuterol (Sigma Aldrich, Dorset, United Kingdom) was dissolved in methanol (1 mg/ml) and stored in the dark at -20ºC. Samples were prepared by diluting the clenbuterol stock solution with 5 mM ammonium acetate buffer (pH 8), or with human urine.

4.1.2.2 SPE procedure

The SPE procedure for C\textsubscript{18} cartridges was as follows: activation was performed with 2.5 ml methanol, and conditioning with 2.5 ml 5 mM ammonium acetate buffer (pH 8) at a flow-rate of 2.5 ml/min. The 1-ml sample was loaded onto the cartridge at 1.0 ml/min, followed by a washing step with 4.0 ml ammonium acetate buffer (5 mM, pH 8) at a flow-rate of 2.0 ml/min. Elution was performed with a gradient of ammonium acetate buffer (5 mM, pH 8) and methanol. In 1.0 min the methanol percentage was increased from 0 to 10 %, which was followed by a subsequent increase to 40% in 2.5 min. This percentage was maintained for 2.5 min. A flow-rate of 1.0 ml/min was used during elution.

For the polymeric cartridges, activation, conditioning, and sampling were performed with the same procedure as for C\textsubscript{18} cartridges. The cartridge was washed with 4.0 ml of a mixture of ammonium acetate buffer (5 mM, pH 8) and methanol (1:1 v/v) at a flow-rate of 2.0 ml/min. Clenbuterol was eluted from the cartridge using a gradient of ammonium acetate buffer (5 mM, pH 8) and methanol. The methanol percentage was increased from 50 to 70% in 0.5 min. This percentage was maintained for 2.0 min. A flow-rate of 1.0 ml/min was used during elution.
4.1.2.3 Mass spectrometry

Using the ion-trap mass spectrometer with an APCI source, the vaporiser temperature was set at 450°C. The sheath gas and auxiliary gas (both nitrogen) were 30 and 3 (arbitrary units), respectively. The discharge current was set at 3.00 μA and the capillary voltage was 8.00 V. The temperature of the heated capillary was 200°C, and the tube lens offset was set at 20.00 V. All scans were recorded in full-scan mode with 3 microscans over the range of m/z 185 to 285 using positive-ion mode. The maximum injection time was set at 200 ms. Helium was applied as cooling gas and collision gas. Extracted ion chromatograms in all MS modes were obtained for [M+H]+ or fragment ions ± 0.5 Th. The isolation width during MSn experiments was 2.5 Th. Collision energies were optimised for individual MSn steps.

4.1.3 Results and discussion

4.1.3.1 Mass spectrometry

During the optimisation of the settings of the mass spectrometer, which can be performed automatedly or manually, the temperature of the heated capillary proved to be a critical factor. The highest response was found with the heated capillary set at 170°C. However, at this temperature a significant memory effect was observed for clenbuterol. Therefore, the heated capillary was set at 200°C, which resulted in a loss in sensitivity of about 25%, but no memory effect was observed.

The fragmentation of protonated clenbuterol ([M+H]+) could be monitored very precisely. In MS/MS mode hardly any fragments other than m/z 259 were formed from the precursor ion m/z 277 (loss of H2O). Consecutive fragmentation of m/z 259 (MS3) produced an ion with m/z 203 as the only fragment (loss of the isobutene group). In MS4 experiments the ion with m/z 203 was fragmented even further. This resulted in the formation of five fragments: m/z 186 (loss of NH3), m/z 168 (loss of Cl•), m/z 167 (loss of HCl), m/z 132 (loss of Cl• and HCl), and m/z 131 (loss of two HCl) [35]. The complete fragmentation pathway is depicted in Fig. 1.

The collision energies required for the formation of fragments (Fig. 1) could be optimised in such a way that the precursor ion was almost completely fragmented to just one daughter ion with the exception of MS4. The optimum collision energy (arbitrary units) was 24, 30, and 35% for MS2, MS3, and MS4, respectively. The overall collision induced dissociation (CID) efficiency (E_{CID}), which includes both fragmentation efficiency (E_F) and collection efficiency (E_C), was about 90, 70, and still 33% for MS2, MS3, and MS4, respectively. This
means that the overall $E_{\text{CID}}$ is 63% for MS$^3$ and 21% for MS$^4$. The overall $E_{\text{CID}}$ in the ion-trap mass spectrometer is very high in comparison to that of a triple-quadrupole one. The $E_F$ is somewhat higher in the ion-trap mass spectrometer. However, the main difference is due to the $E_C$, since this efficiency is up to 100% in the ion-trap and only 10-50% in a triple-quadrupole [36-37]. This difference can be explained by the fact that MS/MS in the ion-trap mass spectrometer is “tandem-in-time”, whereas with a triple-quadrupole mass spectrometer “tandem-in-space” is performed. With a triple-quadrupole, the parent ion must be transmitted through a collision cell (a quadrupole and associated lenses) with mass resolution. Scattering of ions in the collision cell results in rather low transmission efficiency. With the ion-trap mass spectrometer, all MS$^n$ processes occur in the same spatial region. Therefore, no transmission losses can occur [36].

![Fragmentation pathway of clenbuterol](image)

Fig. 1: Fragmentation pathway of clenbuterol. Numbers in parentheses indicate the collision energy required for consecutive fragmentation.
Ion-trap MS offers the possibility to perform MS\textsuperscript{3} using wide-band activation. This technique involves the fragmentation of a precursor ion with a chosen \(m/z\) value, and the \((m/z-18)\) ion will automatically be fragmented as well. For clenbuterol this implied the simultaneous fragmentation of \(m/z\) 277 and 259 to form the ion with \(m/z\) 203, which is comparable to the fragmentation under multiple collision conditions in a triple-quadrupole mass spectrometer [19]. The required collision energy (40\%) was higher than for conventional MS/MS using the ion-trap (24\%). The \(E_{\text{CID}}\) using wide-band activation was about 60\%. This efficiency provides no improvement as compared to MS\textsuperscript{3} without wide-band activation. Therefore, wide-band activation was considered to be advantageous only if the ions \([\text{M+H}]^+\) and \([\text{M+H-18}]^+\) were both present at similar abundances. In general, wide-band activation is 10-40\% less effective than single frequency resonant excitation, i.e. the fragmentation of a single \(m/z\) value [38]. Since the efficiency of MS\textsuperscript{3} analysis was rather high in this case, wide-band activation was not further investigated.

4.1.3.2 Optimisation of SPE

\textit{C\textsubscript{18} cartridges}

Chromatograms obtained with diode array detection (DAD) and single MS in the total ion current mode (TIC) showed a significant amount of urine matrix (Fig. 2A) if C\textsubscript{18} cartridges were used. With C\textsubscript{18} cartridges, it was not possible to wash the cartridge with a small percentage of methanol without eluting clenbuterol [19]. Clenbuterol is a relatively polar compound with a low retention on a C\textsubscript{18} stationary phase. This is very unfavourable for separating clenbuterol from polar matrix compounds. As a result, efficient removal of the urine matrix could not be achieved. The absence of a proper wash procedure may be unfavourable for the detection limit, since co-eluted compounds can interfere, for example, via ion suppression [27-30] (see below), during analysis by MS.

Using the optimised conditions it was not possible to determine clenbuterol in urine below about 1 \(\mu\)g/ml with DAD (Fig. 2B), which corresponds to earlier published results [19]. Using the C\textsubscript{18} cartridges the total analysis time (including sample pretreatment) was about 11.5 min. This procedure was slightly shorter than previous experiments with C\textsubscript{18} cartridges [19], in which about 13.5 min were required for a single analysis. The gain in time was mainly caused by an increase of the sampling flow to 1 ml/min. The increase in flow did not influence the peak shape or the sorption of clenbuterol.

The use of C\textsubscript{18} cartridges also led to a deposit of matrix compounds on the spray shield and (the front of) the heated capillary of the ion-trap mass spectrometer. Also, a decrease of the response of clenbuterol was observed over
one day. The system (from spray shield to skimmer) had to be cleaned at least once per day, which is time-consuming in routine analysis.

![Absorbance vs Time Graph](image)

**Fig. 2:** UV detection at 247 nm of SPE eluate after extraction with a C₁₈ cartridge of 1.0 ml of (A) blank urine and (B) urine spiked with 0.9 µg/ml clenbuterol. The chromatograms are shown from the start of the sampling step (elution starts after 4 min).

An interesting phenomenon was observed when C₁₈ cartridges were used. Clenbuterol eluted about 0.5 min later after extraction from urine, as compared to extraction from buffer. Furthermore, after extraction of clenbuterol from buffer the peak width (at the base) was about 0.8 min, while extraction from urine produced a peak with a width of only about 0.5 min. A possible explanation for this difference in peak broadening and elution time might be that the urine matrix interacts with the C₁₈ stationary phase, thus changing the properties of the cartridge.

**Polydivinylbenzene cartridges**

In order to improve the selectivity a polymeric phase was studied. Using cartridges with the polymeric phase, it proved to be possible to decrease the amount of matrix observed in the chromatogram. The polymeric phase chosen was polydivinylbenzene. Apart from its more hydrophobic characteristics, the aromatic rings of the stationary phase also permit π-π interactions between the sorbent and the analyte and, therefore, an increase in retention. As a result, the polymeric phase was expected to be more retentive in comparison with the C₁₈ cartridges [39]. It was possible to wash more rigorously, using a mixture of ammonium acetate buffer and a relatively high percentage of methanol, without eluting clenbuterol from the cartridge. Nevertheless, if the polymeric phase is used there is still some matrix present after extraction of blank urine (Fig. 3A).
Fig. 3: UV detection at 247 nm of SPE eluate after extraction with a PDVB cartridge of 1.0 ml of (A) blank urine and (B) urine spiked with 0.9 µg/ml clenbuterol. The chromatograms are shown from the start of the sampling step (elution starts after 4 min).

For the cartridges with the polymeric phase, a mixture of ammonium acetate buffer and methanol could be used during the wash step. If a mixture of 60:40 v/v ammonium acetate buffer (5 mM, pH 8)/methanol was used for washing the cartridge, after injection of 1 ml of urine, a significant amount of matrix was still present in the eluate. As with C<sub>18</sub> cartridges, this resulted in a deposit of matrix compounds on the spray shield and (the front of) the heated capillary and a decrease in response of clenbuterol. The system had to be cleaned at least once per day. Furthermore, the response of clenbuterol was significantly higher when standard solutions were analysed, as compared with urine samples. This indicates that washing with 40% methanol could not prevent ion suppression when urine was analysed.

During the analysis of standard solutions, washing with a mixture of 50:50 v/v ammonium acetate buffer (5 mM, pH 8)/methanol was found to lead to a similar response to washing with 40% methanol. This indicates that no breakthrough occurred. In contrast, analysis of urine samples washed with 50% methanol produced responses that were twice as high (concentration range 50-250 ng/ml) as those observed when washing urine-sampled cartridges with 40% methanol, indicating that less interference of matrix, i.e. ion suppression, occurred. Moreover, by loading the cartridge with urine and consecutive washing with 50% methanol, no deposit of matrix on the spray shield and the heated capillary was observed. For precautionary reasons the system was cleaned once per week. However, no decrease of response was observed during a week.

For further experiments 50% methanol was used during the wash procedure whenever the cartridges with the polymeric phase were used. Using
an even higher percentage of methanol during the wash step resulted in breakthrough of clenbuterol. The elution of clenbuterol occurred using a gradient of methanol starting with 50:50 v/v ammonium acetate buffer (5 mM, pH 8)/methanol (i.e. the mixture of the wash step), and increasing the percentage methanol to 70% within 0.5 min. A good peak shape was obtained, but it was not possible to determine clenbuterol in urine below about 1 µg/ml with DAD (Fig. 3B). However, it should be noted that the total analysis time was now reduced to 8.5 min including sample pretreatment. This is mainly due to the fact that it was possible to wash with a relatively high percentage of methanol. As a consequence, a faster gradient with a smaller range could be used during elution, and only about 2.5 min data acquisition was required.

4.1.3.3 Use of the total system

Comparison of ion-suppression effects

The reliability of quantitative results provided by an LC-MS system may not be absolute. Co-eluting ‘unseen’ endogenous compounds may interfere with the accurate determination of the analyte of interest. The undetected matrix components may reduce the ion intensity of the analyte and affect the reproducibility and accuracy of the LC-MS method [27-30]. Ion suppression is inherently independent of the MS mode, as ionisation and MS analysis are separated in time and space [27]. Under routine analysis conditions, using a deuterated version of the analyte, or a close homologue, as an internal standard, suppression effects can be corrected for. However, even then it is necessary to study the cause and amount of suppression.

Quantitative information about ion suppression can be obtained by comparing the response of the analyte in the biological matrix with the response in the mobile phase. It is assumed that the difference in response is caused by components of the extract that are not present in the mobile phase. Another useful experiment is to extract a blank sample, which gives qualitative information about the interfering compounds. A post-column infusion of analyte combined with extraction of a blank sample will also provide quantitative information about the ion suppression [28]. Polar compounds are more sensitive to ion suppression than non-polar components [28]. Using an SPE-MS system to determine a relatively polar compound, e.g. clenbuterol, ion suppression should be carefully monitored. A comparison of ion-suppression effects, using external calibration, at different concentrations with C18 cartridges and PDVB cartridges is presented in Table 1. The ion suppression was studied using the MS3 mode, since this mode offered the best selectivity and sensitivity (see below).
Table 1: Ion suppression (%) of different concentrations of clenbuterol extracted from urine using a PDVB cartridge and a C\textsubscript{18} cartridge measured with MS\textsuperscript{3}.

<table>
<thead>
<tr>
<th></th>
<th>93 ng/ml</th>
<th>45 ng/ml</th>
<th>9 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDVB</td>
<td>4</td>
<td>27</td>
<td>32</td>
</tr>
<tr>
<td>C\textsubscript{18}</td>
<td>37</td>
<td>69</td>
<td>No data</td>
</tr>
</tbody>
</table>

As can be observed from the data in Table 1, the ion suppression was higher at lower concentrations with both cartridges, which can be explained by the less favourable matrix/analyte ratio. Also, ion suppression was more prominent after extraction of clenbuterol from urine with C\textsubscript{18} cartridges, which is due to the fact that the cartridge could not be washed with methanol without eluting clenbuterol. Therefore, many matrix compounds were still present in the eluate, causing ion suppression. Despite high ion suppression, only a small relative standard deviation (<6%) was obtained. This indicates that the ion suppression is almost constant at a certain concentration in the set-up used during these experiments. However, using other urine samples will most probably show dissimilar ion suppression, as other interfering compounds can be present and at different concentration levels.

**Analytical data**

Using both the C\textsubscript{18} and the PDVB cartridges, linearities and LODs were determined for the extraction of clenbuterol from buffer and urine. Several MS modes were used. The results are presented in Table 2.

Table 2: Detection limit, linear range and regression coefficient (R) using PDVB cartridges and C\textsubscript{18} cartridges and the MS, MS\textsuperscript{2} and MS\textsuperscript{3} mode.

<table>
<thead>
<tr>
<th></th>
<th>LOD (ng/ml)</th>
<th>PDVB Range (ng/ml)</th>
<th>R\textsuperscript{a}</th>
<th>C\textsubscript{18} LOD (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>2.5</td>
<td>5-250</td>
<td>0.9950 (n=6)</td>
<td>2.5</td>
</tr>
<tr>
<td>Urine</td>
<td>25</td>
<td>50-250</td>
<td>0.9910 (n=3)</td>
<td>50</td>
</tr>
<tr>
<td>MS\textsuperscript{2}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>50</td>
<td>---\textsuperscript{b}</td>
<td>---\textsuperscript{b}</td>
<td>---\textsuperscript{b}</td>
</tr>
<tr>
<td>MS\textsuperscript{3}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>0.5</td>
<td>1.0-250</td>
<td>0.9988 (n=8)</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* a: Weighted regression (1/x)
  b: Not measured
The use of single MS did not provide the required selectivity to determine low levels of clenbuterol in urine (Table 2). A large amount of matrix was observed in all TIC chromatograms. Extracting the molecular ion \((m/z \ 277)\) gave a reduction of the visible interfering matrix (Fig. 4), but total elimination of the matrix effect by extracting any specific \(m/z\) value proved to be impossible.

Fig. 4: Extracted ion chromatograms at \(m/z \ 277\) after extraction of 50 ng/ml clenbuterol in urine with SPE-single MS using (A) a C\(_{18}\) cartridge and (B) a PDVB cartridge.

The matrix interference is probably due to endogenous compounds, normally present in urine, e.g. amines, urea, lipids, and proteins. Alternatively, these interferences may also result from compounds originating from digested food and drinks, or administered drugs and their metabolites. It should be noted that the difference in selectivity of the stationary phases also affected the presence of the interfering compounds. With C\(_{18}\) cartridges \(m/z 248, 300\) and 414 were the most prominent ions, whereas with PDVB cartridges \(m/z 248, 274\) and 276 appeared to interfere the most with the accurate determination of clenbuterol. None of the interfering compounds could be positively identified.

Generally, sensitivity can be improved by operating the mass analyser in a higher MS mode (MS\(^n\), where \(n\geq2\)), leading to a more significant reduction of the noise than the signal. However, with clenbuterol, MS\(^2\) led to a loss of sensitivity (Table 2). This is due to the presence of an interfering compound that also produces a fragment with \(m/z 259\) (Fig. 5). Most probably, the precursor ion of this fragment \((m/z \ 276)\) is an ammonium adduct, which loses \(\text{NH}_3\) in the first fragmentation step. Alternatively, as many endogenous compounds present in urine contain an amine group, the fragment may originate from a primary amine, also losing \(\text{NH}_3\) in the first fragmentation step.
Fig. 5: Extracted ion chromatogram at \( m/z \) 259 after extraction of 50 ng/ml clenbuterol in urine with a PDVB cartridge and detection by MS/MS.

Fig. 6: Extracted ion chromatogram at \( m/z \) 203 after extraction of 1.0 ng/ml clenbuterol in urine with a PDVB cartridge and detection by MS$^3$. 
In contrast, for MS$^3$, the noise could be reduced more than the clenbuterol fragment signal at $m/z$ 203. This resulted in an associated increase in sensitivity. The LOD was 0.5 ng/ml and 2.0 ng/ml with PDVB and C$_{18}$ cartridges, respectively (Table 2). A representative MS$^3$ chromatogram is depicted in Fig. 6. Applying MS$^4$ did not lead to any further improvement in sensitivity, *i.e.* the LOD was 10 ng/ml clenbuterol in urine. This is mainly due to inefficient fragmentation of the ion at $m/z$ 203 to a total of five fragments (Fig. 7).

In all MS modes presented in Table 2, linearity (R) was always better than 0.99. As expected, the LODs for standard solutions in buffer were about the same in all the MS modes.

**Fig. 7:** Mass spectrum of clenbuterol after SPE-MS$^3$ analysis

($m/z$ 277 $\rightarrow$ $m/z$ 259 $\rightarrow$ $m/z$ 203 $\rightarrow$ full scan [$m/z$ 185-285]).

## 4.1.4 Conclusions

Direct coupling of SPE with ion-trap MS offers the possibility to determine drugs in biological samples with a short analysis time. Ion-trap MS offers the possibility of very selective and sensitive detection, especially if MS$^n$ ($n \geq 2$) is used. However, special attention must also be paid to the SPE procedure to ensure that clean extracts are obtained. This is essential for reliable analysis, since the presence of matrix compounds can increase the background signal and also leads to reduction of the signal of the analyte due to ion suppression during the formation of the ions in the LC-MS interface. Summarising, development of an SPE-MS method implies considering the
required selectivity at the front of the analysis system (SPE) as well as at the end (MS).

At the front of the system, the stationary phase material proves to be an important parameter. Using C\textsubscript{18} cartridges, an LOD of 2 ng/ml was obtained for SPE-MS\textsuperscript{3} using an ion-trap mass spectrometer. The use of PDVB cartridges resulted in cleaner extracts, a lower detection limit (0.5 ng/ml), and a shorter analysis time (8.5 min). The cleaner extracts are probably caused by π-π interactions between the phenyl ring of clenbuterol and the phenyl groups of the stationary phase, so that more efficient washing of the cartridge can be realised. The use of this polymeric phase can possibly be expanded to screening for other drugs with an aromatic functionality, e.g. fingerprinting of cocaine or amphetamines. In the future, more selective SPE, e.g. using molecular imprints and immobilised antibodies, will be investigated to obtain even cleaner extracts.

The use of ion-trap MS is relatively new for applications in bioanalysis. To our knowledge, this type of MS was not previously used in an SPE-MS system. From the results in this work it can be concluded that the ion-trap MS can be used for analysis without a real chromatographic separation step. If clean extracts can be obtained, the use of an SPE-(ion-trap) MS system offers the possibility to perform an analysis, including sample pretreatment, in less than 10 min. Thus, high sample throughput can be achieved; however, the SPE process needs to be further optimised in order to obtain sufficient compatibility with the speed of the MS step. The SPE might be shortened even further by faster activation, conditioning, and washing of the cartridge or by reduction of the volumes in each step in the sample pretreatment procedure. However, this can also negatively influence the sorption and desorption characteristics of the stationary phase. A set-up using two cartridges parallel to each other [20] will also help to increase the sample throughput.

In conclusion, on-line SPE-(ion-trap) MS\textsuperscript{n} is very promising and the system will be tested for other compounds and matrices in the near future.

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4.1.5  References

Ion suppression in the determination of clenbuterol in urine by solid-phase extraction – mass spectrometry*

Summary

Ion suppression effects were observed during the determination of clenbuterol in urine with solid-phase extraction – multiple-stage mass spectrometry (SPE-MS\(^3\)), in which a polymeric stationary phase (polydivinylbenzene) was applied. Post-cartridge infusion of analyte to the SPE eluate after the extraction of blank urine was performed to obtain a profile of the suppression. Single and multiple-stage MS was performed to get insight in the suppressing compounds. The ion suppression was mainly ascribed to two \(m/z\) values, but still no identification of the compounds was achieved from the multiple-stage MS data. No ionisable and non-ionisable complexes and/or precipitation of clenbuterol with matrix compounds were observed. A concentration dependence of the percentage of suppression was observed. Up to 70% of the signal was suppressed upon post-cartridge infusion of 0.22 µg/ml (at 5 µl/min) clenbuterol into the eluate, and this decreased to about 4% at infusion of 22 µg/ml clenbuterol. Molecularly imprinted polymers were used to enhance the selectivity of the extraction. Although matrix components were still present after extraction, no interference of these compounds with the analyte was observed. However, the bleeding of the imprint from the polymer (brombuterol) caused significant ion suppression.

4.2.1 Introduction

In the biopharmaceutical field, fast, selective and sensitive analytical systems are required. These seemingly incompatible requirements may be met by systems based on on-line liquid chromatography (LC) and mass spectrometry (MS) with atmospheric interfaces [1,2]. A key factor in bioanalysis is, however, the sample pretreatment. A powerful technique for clean-up and preconcentration of biological samples is solid-phase extraction (SPE). When carried out off-line, the extraction procedure can be time-limiting. Automation of SPE and on-line coupling with LC-MS implies that the separation step becomes the most time-consuming step. Consequently, short LC columns (25 to 50 mm) are applied [3-5], or the actual LC separation was omitted, i.e. SPE directly coupled to MS [6-12]. Both approaches resulted in systems in which the extraction was again limiting the sample throughput.

The application of an SPE-MS system for bioanalytical purposes implies that the SPE procedure must be performed very rapidly for high-throughput aims, whereas it should also be efficient to ensure that clean extracts are obtained. The selectivity of the MS can eliminate the signals of co-eluting (matrix) compounds, but their presence in the eluate may still cause ion suppression [13-17], resulting in the loss of reliability and accuracy of the obtained data.

The effect of ion suppression in LC-MS systems has been recognised lately and a number of reports can be found in literature [10,11,18-32]. The causes of ion suppression with electrospray ionisation (ESI) can be a decrease in evaporation of the solution or an increase in surface tension due to (high concentrations of) matrix compounds [16,17]. Another pathway is binding to and (co-)precipitation of the analyte with non-volatile materials [17]. Alternatively, a competition between compounds for the charge in the liquid phase may result in ion suppression as well [18]. A final possible cause is gas-phase neutralisation processes [17]. The latter, as well as the co-precipitation, is of particular importance with atmospheric pressure chemical ionisation (APCI). With APCI, gas-phase basicity is the key. Only the strongest gas-phase base (positive mode) will be ionised [19].

A general trend can be observed in LC-MS applications. Particularly ESI is prone to ion suppression [10,18-20,23-32], although ion suppression with APCI has also been described [11,22]. The stronger effect for ESI can be partly explained by the higher tendency towards adduct formation with ESI [28]. Furthermore, with ESI an upper limit of the total number of ions (typically $10^{-5}$ M) is observed, which depends on the surface area of all droplets being formed during ESI [33]. Thus, a saturation of the ionisation can occur. In all applications, problems are observed when analysing relatively polar compounds, i.e., compounds with a high gas phase acidity or basicity, in the
presence of even more polar interfering matrix compounds [33]. Thus, for the elimination of the ion suppression, one should attempt to decrease the amount of polar matrix compounds present after the sample preparation step.

Various reports describe the presence of ion suppression after SPE with a C_{18} stationary phase [11,23,28,29,32], but the effects are also observed after extraction with an ion-exchange phase [30] or with liquid-liquid extraction [20]. Various solutions have been presented to correct for and/or minimise the ion suppression. The use of a suitable internal standard, e.g. a deuterated analogue or a structural analogue of the analyte, may be used, provided that a similar suppression effect is observed for the internal standard [24,26,28,30]. Extracting a smaller volume of the sample [20] may also be useful to reduce the ion suppression due to reduction of the amount of interfering compounds. Other approaches for reducing or eliminating ion suppression can be the use of LC/LC [20,22,23] or a change in sample preparation [19,25,29,32]. The latter is preferred to differ in selectivity from the separation step (if applied) [25]. An additional step in the extraction, such as SPE followed by preparative LC and subsequent LC-MS analysis has also been applied [32]. All reports only mention the presence of ion suppression, but no identification of the suppressing compounds was described.

A few methods have been proposed for determining interferences of matrix components [15]. Extracting a blank sample with subsequent separation and/or detection will give qualitative information about the interfering compounds. A post-column infusion of analyte to the SPE eluate after the extraction of a blank sample will provide quantitative information about the ion suppression. With this system, a time-profile of the suppressing compounds can also be obtained. Another method that provides quantitative information is comparing the response of the analyte after extraction from the biological matrix with the response after extraction from a standard solution. It is assumed that the difference in response is caused by components of the extract that are not present in the standard solution, but one should be aware that any losses in the extraction procedure are also included in this approach. With off-line SPE, this can be encountered by spiking the eluate after extraction. However, in an on-line system spiking the eluate is not possible.

From a previous study [11], it was observed that the best results for the determination of clenbuterol in urine with an SPE-MS system with an APCI interface were obtained using polydivinylbenzene (PDVB) cartridges. However, some ion suppression (about 4%) was observed at high concentrations of clenbuterol, and more ion suppression (up to 40%) was observed at lower levels (<10 ng/ml) of clenbuterol. These results were the basis for the current study. The ion suppression will be studied with regard to the origin and the amount of the suppression. More selective extraction, by using molecularly imprinted polymers (MIPs), will be applied in order to investigate the possibilities of
MIPs in an SPE-MS system with respect to the extraction selectivity and the ion suppression.

4.2.2 Experimental

4.2.2.1 Chemicals and instrumentation

All on-line SPE-MS experiments were performed with a Prospekt sample handler (Spark, Emmen, The Netherlands) using one six-port valve, the cartridge-switching device, and a solvent delivery unit (SDU). Activation, conditioning, sampling, trapping, and washing were done using the SDU. The effluent was connected to waste during these steps. All steps of the SPE procedure were carried out using a forward-flush mode. Cartridges were automatically replaced after single use. A second flow stream was used for the elution applying a gradient pump Series 1100 (Hewlett-Packard, Waldbronn, Germany), which was connected to an LCQ ion-trap mass spectrometer (Thermoquest, San Jose, CA, USA) via the cartridge. The mass spectrometer was equipped with an APCI source. Experiments with UV detection were carried out with a Hewlett-Packard diode array detector Series 1100. The Prospekt cartridges were HySphere Resin GP (Spark, 10×2 mm, particle size 10 µm), a spherical polymeric phase of polydivinylbenzene (PDVB), or MIPs (MIP Technologies, Lund, Sweden, brombuterol imprinted, 10×2 mm, polymer particle size 25-38 µm).

Methanol and acetonitrile were of HPLC grade (Lab Scan, Dublin, Ireland). Ammonium acetate and acetic acid were of analytical-reagent grade (Merck, Darmstadt, Germany). Trifluoroacetic acid (TFA, spectrophotometric grade 99+%%) was obtained from Aldrich Chemical (Milwaukee, WI, USA). The 5 mM ammonium acetate buffer was adjusted to pH 8 using 2.5% ammonia (analytical-reagent grade). Water was obtained from an Elgastat maxima system (ultra pure water, Salm and Kipp, Breukelen, The Netherlands). Aqueous solutions were filtered over a 0.45-µm RC 55 membrane filter (Schleicher & Schuell, Dassel, Germany) prior to use. Clenbuterol.HCl (Sigma Aldrich, Dorset, United Kingdom) was dissolved in methanol (1 mg/ml) and stored in the dark at -20°C. Human male urine (samples #1 and #2) and female urine (sample #3), as well as calf (sample #4) and bovine (sample #5) urine were used. Spiking of the urine samples was performed by addition of a small volume of the stock solution that is adequately diluted with buffer to urine.
4.2.2.2 Procedures

The SPE procedure with PDVB cartridges was based on a previously described method [11], but higher flow-rates were used during the various steps of the extraction procedure in order to obtain a rapid SPE-MS system. The following procedure was used: conditioning was performed with 2.5 ml 5 mM ammonium acetate buffer (pH 8) at a flow-rate of 5.0 ml/min. The 1-ml sample was then loaded onto the cartridge with an excessive amount of buffer during 1 min at 3.0 ml/min, followed by a washing step with 5.0 ml buffer/methanol (1:1 v/v) at a flow-rate of 5.0 ml/min. The elution was performed at a flow-rate of 1.0 ml/min with a gradient of ammonium acetate buffer (5 mM, pH 8) and methanol. In 0.25 min the methanol percentage was increased from 50 to 70%. The latter percentage was maintained for 1.75 min.

Based upon a previously described method [34], the following extraction procedure was used with the MIP cartridges. Conditioning with 1.25 ml water was applied. The 1-ml sample was loaded onto the cartridge within 1 min at a flow-rate of 3.0 ml/min. The cartridge was then washed at 5.0 ml/min with 5.0 ml acetonitrile containing 1% acetic acid. The elution (1 ml/min) was started with a mixture of water/methanol (60/40 v/v) containing 5 mM TFA. Within 1 min this was replaced by acetonitrile/methanol (80/20 v/v, 5 mM TFA). This mixture was maintained for 2 min.

The continuous-infusion method was applied to determine the profile of the ion suppression. Unless stated otherwise, clenbuterol (0.5 µg/ml in methanol) was infused post-cartridge at 5 µl/min. Data acquisition with the MS was always started 0.5 min prior to the start of the elution.

4.2.2.3 Mass spectrometry

During the experiments with the ion-trap mass spectrometer with an APCI source, the vaporiser temperature was set at 450°C. The sheath gas and auxiliary gas (both nitrogen) were 65 and 10 (arbitrary units), respectively. The discharge current was set at 5.00 µA and the capillary voltage was 24.00 V. The temperature of the heated capillary was 200°C, and the tube lens offset was set at 25.00 V. All scans were recorded in the full-scan mode with 3 microscans over the range of m/z 70 to 500 using the positive-ion mode. The maximum injection time was set at 200 ms. Protonated clenbuterol ([M+H]+, m/z 277) was monitored during the single MS mode, whereas m/z 259 and 203 were monitored in the MS2 and MS3 mode, respectively. Extracted ion chromatograms in all MS modes were obtained for [M+H]+ or fragment ions ± 0.5 Th. The isolation width during MSn experiments was 1.8 Th. Helium was applied as cooling gas and collision gas. The collision energies were 25, 28 and 35% during MS2, MS3 and MS4 experiments, respectively.
4.2.3 Results and Discussion

4.2.3.1 Extraction with PDVB

**Suppression profile**

The changes in the SPE procedure (see Section 4.2.2.2) in comparison to a previously described method [11] implied that the total analysis time was decreased to 4 min. Analysis of blank urine with single MS showed a large matrix peak (Fig. 1A).

![Suppression profile graphs](image)

Fig. 1: (A) SPE-MS of blank urine applying PDVB cartridges (total ion chromatogram). (B) SPE-MS\(^3\) of blank urine with post-cartridge infusion of clenbuterol (0.5 µg/ml, 5 µl/min); extracted ion chromatogram (XIC) \(m/z\) 203. (C) SPE-MS\(^3\) of clenbuterol in urine (100 ng/ml); XIC \(m/z\) 203.
Numerous $m/z$ values with varying abundances and elution times were found in this large peak originating from sample #1. Four other urine samples of human (male and female), bovine and calf origin were also monitored using single MS, and similar results were obtained with regard to elution times and $m/z$ values found in blank urine. Upon post-cartridge continuous infusion of clenbuterol while extracting blank urine, two suppression regions, that is from 0.4-1.2 and from 1.6-2.0 min, were observed (Fig. 1B). Only the first region interfered with the determination of clenbuterol, since the elution window of clenbuterol (0.9-1.5 min, Fig. 1C) and the first suppression region partially overlap. Thus, the focus was on the matrix compounds in this overlapping elution window (0.9-1.2 min).

Elimination of the ion suppression may be accomplished by changing the pH in the wash step. When increasing the pH from 8.0 to 9.5, no differences in the presence of matrix compounds and the elution of clenbuterol was observed. Applying an acidic wash step, at pH 4.5, clenbuterol eluted from the cartridge during the wash step. Thus, in this approach, changing the pH was not useful. Therefore, the cause of the ion suppression was further investigated for the original system.

**Complex formation / precipitation**

The ion suppression can be due to various processes. One of those is the formation of a (non-ionisable) complex of the matrix component and the analyte [10], which subsequently deposits [17] on the surface of the spray shield and heated capillary. Another possibility of a (ionised) complex of clenbuterol with creatinine has been observed after extraction on an ion-exchange cartridge and analysis with ESI/MS [10], but in the present set-up no such complex was seen. The formation of such a complex after extraction on PDVB cartridges and/or precipitation was checked by extraction of about 100 ng/ml clenbuterol from buffer, buffer/urine (4:1 and 1:1) and urine. After five extractions of one of the four solutions mentioned above the spray shield and heated capillary were washed, prior to extraction of another solution, with 5 ml methanol and 5 ml buffer pH 8, hereby cleaning the MS and breaking a possible complex of matrix compounds with clenbuterol. All fluids were collected, and subsequently triplicate injections into the MS were performed. About 1% of the summed amount of clenbuterol of the five extractions was found in the wash solvents regardless of the samples being extracted. Thus, the ion suppression was not caused by the formation of a non-ionisable complex and subsequent precipitation. This suggests that the suppression is caused by a competition of the analyte and the matrix components for the charge in the gas phase.
Concentration dependence

In the previous study [11] a concentration dependence in the ion suppression was observed upon the comparison of the extractions from spiked standard solutions and from spiked urine. Below 10 ng/ml (or <6 ng/min with a peak width of 0.6 min and injecting 1 ml), the suppression was about 40% (absolute) and this gradually decreased to only 4% at higher concentrations (>100 ng/ml, or >60 ng/min). The continuous infusion method with varying concentrations was now also used to study this effect. The observed suppression, averaged over the elution window of clenbuterol, was about 70% with the infusion of 0.22 µg/ml clenbuterol into the eluate at 5 µl/min (i.e. 1.1 ng/min) and decreased to about 4% during the infusion of 22 µg/ml at 5 µl/min (110 ng/min). The decreasing percentage of ion suppression with increasing concentrations is probably due to a more favourable analyte/matrix ratio at higher concentrations. The absolute amount that was suppressed increased with increasing infusion concentrations (0.22 to 1.0 µg/ml). With higher concentrations, the suppressed amount reached a plateau and remained almost constant at about 3 ng. This indicates that there is indeed a competition for the charge in the gas phase between the analyte and the matrix molecules. The latter can suppress the analyte until the matrix components are fully charged. This explains the increase and subsequent stabilisation of the suppressed amount of clenbuterol if the infused concentration is increased.

Structural information about suppressing compounds

Three m/z values, i.e., m/z 257, 274 and 276, were considered to be possibly linked to the ion suppression effects of clenbuterol, since compounds giving these m/z values were present in the elution window of interest. However, m/z 257 was related to m/z 274 by the loss of NH₃, thus the number of m/z values of interest was reduced to only two. The m/z values 274 and 276 were present in all urine samples, and are thus considered to be common urine components. More structural information of the compounds at m/z 274 and 276 was obtained by performing MS², MS³ and MS⁴. A scheme of the fragmentation pattern of these compounds is presented in Fig. 2. Despite the fact that many urine compounds are known [35], and even though some functional groups of the components could be specified, so far no positive identification could be made. This is partly due to the complexity of the urine matrix, since many substances can be present in this sample type, which originate from various exogenous and endogenous sources. Furthermore, several metabolism processes, e.g. hydroxylation, methylation, acetylation, glucuronidation, sulfonation and combinations of these reactions, can result in the presence of many compounds besides the parent molecules. The unknown compounds are most likely not sulfonated, since no isotope peaks of sulfur were observed.
Furthermore, glucuronidation and methylation are not likely either, as these groups would immediately be lost in MS/MS analysis [36-39].

**Fragmentation of m/z 274**

MS: 274

$\rightarrow$

MS$^2$: 257 (-17: NH$_3$)

$\rightarrow$

MS$^3$: 229 (-28: -CO or -C$_2$H$_4$) $\rightarrow$ MS$^4$ of 229: 211 (229-18: -H$_2$O)

197 (-60: -CH$_3$COOH) 193 (211-18: -H$_2$O)

179 (197-18: -H$_2$O) 183 (211-28: -CO or -C$_2$H$_4$)

161 (179-18: -H$_2$O) 169 (211-42: -C$_3$H$_6$)

145 (-66) 159 (229-70: -C$_4$H$_{10}$)

137 (197-60: -CH$_3$COOH) 145 (229-84: -C$_4$H$_{12}$)

127 (145-18: -H$_2$O) 127 (145-18: -H$_2$O)

121 (137-18: -H$_2$O) 119 (179-28: -CO or -C$_2$H$_4$)

137 (179-42: -C$_3$H$_6$) 121 (-58)

105 (-74 or 119-14-14: -CH$_2$) 95 (-84)

83 (-96)

81 (-98)

**Fragmentation of m/z 276**

MS: 276

$\rightarrow$

MS$^2$: 259 (-17: NH$_3$)

$\rightarrow$

MS$^3$: 230.5

199 (-60: -CH$_3$COOH) 181 (199-18: -H$_2$O) $\rightarrow$ MS$^4$ of 181: 163 (181-18: -H$_2$O)

171 (199-28: -CO or -C$_2$H$_4$) 135 (163-28: -CO or -C$_2$H$_4$)

163 (199-36: -2 H$_2$O) 121 (135-14: -CH$_2$)

143 (171-28: -CO or -C$_2$H$_4$) 107 (121-14: -CH$_2$)

129 (143-14: -CH$_2$) 97 (-84)

81 (-100)

Fig. 2: Fragmentation pattern and possible functional groups of two main interfering m/z values.
4.2.3.2 Extraction with MIPs

More selective extraction of clenbuterol could possibly be obtained with (brombuterol) MIP cartridges. A representative chromatogram is presented in Fig. 3A. The extraction of blank buffer already showed severe interference of the brombuterol imprint (peak #1, 0.5-1.2 min). Upon the extraction of urine, a second peak (peak #2, 1.2-2.0 min) was observed, which originated from the urine matrix. The \( m/z \) values of the matrix compounds (peak #2) present after extraction with the MIP cartridges were different from those present after extraction with the PDVB cartridges, and \( m/z \) 274 and 276 were not present.

**Fig. 3:** (A) SPE-MS of blank urine applying (brombuterol) MIP cartridges (total ion chromatogram). (B) SPE-MS\(^3\) of blank urine with post-cartridge infusion of clenbuterol (0.5 µg/ml, 5 µl/min); XIC \( m/z \) 203. (C) SPE-MS\(^3\) of clenbuterol in urine (100 ng/ml); XIC \( m/z \) 203.
Continuous infusion of clenbuterol (0.5 µg/ml at 5 µl/min) showed that no ion suppression was observed at peak #2. Hardly any ionisation of clenbuterol was observed from 0.5-0.7 min at the infused analyte concentration (Fig. 3B). Peak #1 was confirmed to be due to bleeding of brombuterol from the polymer. Thus, the significant amount of brombuterol, which co-eluted with clenbuterol (Fig. 3C), caused the ion suppression. More extensive washing of the polymer before use may reduce this effect. However, due to time-limitations, no such experiments were conducted.

When comparing the results of the extraction of spiked urine with a PDVB or a MIP cartridge (Figs. 1B and 1C versus 3B and 3C, respectively), it can be concluded that the suppression (percentage) of clenbuterol is more prominent due to the bleeding of brombuterol from the polymer (MIP extraction) than due to matrix compounds (PDVB extraction), since the decrease of the clenbuterol signal is larger (Figs. 1B and 3B) and the peak area (or abundance) is much higher after extraction with PDVB cartridges (Figs. 1C and 3C).

4.2.4 Conclusions

Ion suppression is a critical aspect in SPE-LC-MS systems for bioanalysis, and it becomes even more important to monitor this effect when the LC column length is reduced or the LC separation step is omitted. Post-column continuous infusion of analyte to the SPE eluate after the extraction of a blank sample can be used to get insight into the extent of the ion suppression. Subsequent single and multiple MS analysis of blank urine will then provide information about the interfering compounds. However, the use of an LC separation of the interfering compounds (which should also be capable of handling a large polarity range) as well as high-resolution MS may be useful to increase the chance for identification of the interfering compounds. In an ion suppression study, samples from different sources should be used. However, the current study showed that common urine compounds seem to be the suppressing components.

Ion suppression should be an important consideration during the validation of a method. If ion suppression is observed, the selectivity of the extraction should be optimised by changing the washing or elution step in the procedure of the extraction or by the choice of another stationary phase. MIPs were considered to be very suitable in order to prevent ion suppression, but the bleeding of the imprint from the polymer was shown to be significant and severely suppressed the ionisation of clenbuterol. The bleeding of the MIPs needs to be minimised, otherwise this type of extraction might not be suitable for direct coupling to MS. One should thus always be alert that any compound,
matrix and non-matrix related (e.g. the internal standard), can cause ion suppression when present in a relatively high concentration. However, in combination with a real separation step the selectivity obtained with the MIP extraction principle is often different from the LC separation, thus this type of extraction combined with a short LC column and MS detection can more easily result in the prevention of ion suppression than the combination of a common reversed-phase extraction and a similar separation step. Furthermore, other selective SPE materials, such as immobilised antibodies, seem very promising in this respect.

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4.2.5 References

Ion suppression in the determination of clenbuterol in urine by SPE-MS

On-line coupling of solid-phase extraction with mass spectrometry for the analysis of biological samples. Determination of prednisolone in serum*

Summary

Solid-phase extraction (SPE) was directly coupled to mass spectrometry (MS) for the rapid determination of prednisolone in serum. A C\textsubscript{18} stationary phase allowed washing of the cartridge with 25\% methanol. A rapid increase of the percentage of methanol (25 to 50\% within 0.1 min) was applied during the elution. The high flow-rates during the extraction (5.0 ml/min) combined with MS detection resulted in a total analysis time of 4 min. Some matrix interference was still observed with a triple-quadrupole MS, even in the multiple reaction monitoring mode. This resulted in a detection limit (LOD) of about 10 ng/ml. The matrix interference and the LOD were similar for atmospheric pressure chemical ionisation and atmospheric pressure photo ionisation. Applying an ion-trap MS in the MS/MS mode resulted in cleaner chromatograms. Due to extensive fragmentation of prednisolone, the LOD was not lower than about 5 ng/ml prednisolone in serum, and a limit of quantitation of about 10 ng/ml (relative standard deviation <15\%) was observed.

4.3.1 Introduction

Prednisolone is a glucocorticoid derived from hydrocortisone. Its main therapeutic application is due to its immunosuppressive effects [1]. For this reason, prednisolone is often administered prior to, during and after organ transplants in order to decrease the risk of organ rejection. Prednisolone is a hormone-like compound and its side-effects are therefore also hormone-related. A balance must be found between the side effects and the chance of a successful organ transplant. Thus, the concentration of prednisolone should be carefully monitored. Since immediate action should be undertaken upon blood levels that are too high or too low, the rapid determination of prednisolone in serum at concentrations down to the low ng/ml level is required. Most methods used for the determination of prednisolone in serum or plasma apply liquid-liquid extraction [2-5] or off-line solid-phase extraction (SPE) [6,7]. Such techniques are time-consuming and error-prone steps such as evaporation and reconstitution of the eluate are required. Modern developments in the coupling of liquid chromatography with mass spectrometry (MS) [8,9] have offered tremendous potential for high-throughput analysis. On-line coupling of SPE with LC-MS is well established [10-13]. Furthermore, the potential of SPE coupled directly with MS was also shown [14,15]. For the determination of clenbuterol in urine, good sensitivity and selectivity were obtained by applying MS³, whereas the total analysis time was about 8.5 min.

In this study, we investigated the potential of SPE-MSⁿ for the rapid analysis of prednisolone in serum down to the low ng/ml level. Steroids are easily fragmented to various fragments simultaneously by thermal degradation as well as by collision-induced dissociation (CID) [16-21], which may result in limited sensitivity, and the latter aspect should thus be carefully investigated. To achieve low-ng/ml levels, an ion-trap MS and a triple-quadrupole MS were compared. The latter type of MS was used applying atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photo ionisation (APPI). A detailed description of the APPI mechanism can be found in literature [22]. Basically, a dopant is ionised through photoionisation and reacts with solvent molecules, after which proton transfer to the analytes takes place. APPI may ionise analytes, and in particular hormones, more efficiently than APCI [22-24], thus potentially allowing the determination of lower concentrations of prednisolone.
4.3.2 Experimental

4.3.2.1 Chemicals and instrumentation

All on-line SPE-MS experiments were performed with a Prospekt sample handler (Spark, Emmen, The Netherlands) using one six-port valve, the cartridge-switching device, and a solvent delivery unit (SDU). Activation, conditioning, sampling, trapping, and washing were done using the SDU. The effluent was connected to waste during these steps. All steps of the SPE procedure were carried out using a forward-flush mode. A cartridge was replaced after single use. A second flow stream from a Series 1100 gradient pump (Hewlett-Packard, Waldbronn, Germany) was used for the elution, which was connected to the mass spectrometer via the cartridge. During the optimisation of the SPE procedure two polymeric (PLRP-S and Resin GP) and a C\textsubscript{18} stationary phase (all from Spark-Holland, Emmen, The Netherlands) were used. After optimisation, HySphere–9 (C\textsubscript{18}, 10×2 mm, particle size 7 µm) cartridges were applied.

Methanol was of HPLC grade (Lab Scan, Dublin, Ireland). Glacial acetic acid was of analytical-reagent grade (Merck, Darmstadt, Germany). Water was obtained from an Elgastat Maxima system (Salm and Kipp, Breukelen, The Netherlands). Aqueous solutions were passed through a 0.45-µm RC 55 membrane filter (Schleicher & Schuell, Dassel, Germany) prior to use. Prednisolone (Ph. Eur., Genfarma, Maarssen, The Netherlands) was dissolved in methanol (1 mg/ml) and stored in the dark at -20ºC. Spiking of samples was performed by addition of a small volume of the stock solution that is adequately diluted with buffer to foetal calf serum (PAA Laboratories, Linz, Austria).

4.3.2.2 SPE procedure

The final SPE procedure for the C\textsubscript{18} cartridges was as follows: activation was performed with 2.5 ml methanol, and conditioning with 3.75 ml diluted acetic acid (0.5%, pH about 3) at a flow-rate of 5.0 ml/min. A 500-µl sample was loaded onto the cartridge with diluted acetic acid (0.75 min at 2.0 ml/min) to minimise possible carry-over, followed by a washing step with 3.75 ml of 25:75 methanol:diluted acetic acid (0.5%) at a flow-rate of 5.0 ml/min. The elution was started with 25:75 methanol:acetic acid (0.5%), and subsequently within 0.1 min the methanol percentage was increased to 50%. This percentage was maintained for 1.15 min. A flow-rate of 1.0 ml/min was used during elution. Quantitation was performed by the use of external calibration.
4.3.2.3 Mass spectrometry

An LCQ Classic ion-trap MS (Thermoquest, San Jose, CA, USA) equipped with an APCI source was used. The vaporiser temperature was set at 350°C. The sheath gas and auxiliary gas settings (both nitrogen) were 34 and 3 (arbitrary units), respectively. The discharge current was set at 5.00 µA and the capillary voltage was 15.00 V. The temperature of the heated capillary was 170°C, and the tube lens offset was set at 40.00 V. All scans were recorded in the full-scan mode with 3 microscans over the range of $m/z$ 295 to 370 using the positive-ion mode. The maximum injection time was set at 300 ms. Helium was applied as cooling gas and collision gas. Extracted ion chromatograms in all MS modes were obtained for $[\text{M}+\text{H}]^+$ ($m/z$ 361) or fragment ions ± 0.5 Th. The isolation width during MS$^n$ experiments was 2.0 Th. The collision energy applied during MS/MS experiments was 20%.

An API3000 triple-quadrupole MS (MDS-Sciex, Concord, Ontario, Canada) was used with both an APCI and an APPI [22] source. The settings used during single-MS analysis and multiple-reaction monitoring (MRM) are presented in Table 1. In the single-MS mode a Q1-scan was performed from $m/z$ 100 to 400. In the MRM mode $m/z$ 361.1 was fragmented and the products at $m/z$ 307.0 and 325.0 were monitored. When applying APCI, nitrogen was used as curtain gas and auxiliary gas, and zero air was used as nebulising gas. During APPI experiments, only nitrogen was used. The lamp protection gas was set at about 1 l/min, and a lamp current of 0.75 mA was used. Toluene was used as dopant, which was added to the auxiliary gas line via a T-piece. A flow-rate of 50 µl/min was used.

Table 1: Settings of the triple-quadrupole MS during Q1-scan and MRM experiments.

<table>
<thead>
<tr>
<th>Nebulizer gas (arb)</th>
<th>Q0 (V)</th>
<th>Curtain gas (arb)</th>
<th>IQ1 (V)</th>
<th>CAD (arb)</th>
<th>IQ2 (V)</th>
<th>Needle current (µA)</th>
<th>RO1 (V)</th>
<th>Temperature (°C)</th>
<th>IQ2 (V)</th>
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<tr>
<td>15</td>
<td>-10.00</td>
<td>10</td>
<td>-11.00</td>
<td>0 (4°)</td>
<td>-16.00</td>
<td>2.00$^b$</td>
<td>-11.0</td>
<td>450</td>
<td>-18.0</td>
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<tr>
<td>Orifice (V)</td>
<td>20</td>
<td>RO2 (V)</td>
<td>-100 (-25.0°)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ring (V)</td>
<td>50</td>
<td>ST3 (V)</td>
<td>-120 (-45.0°)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEM</td>
<td>2500</td>
<td>RO3 (V)</td>
<td>-102 (-27.0°)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (V)</td>
<td>-100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a: Numbers between brackets are settings for MRM experiments
b: For APPI experiments – source offset voltage = 2000 V.
4.3.3 Results and Discussion

4.3.3.1 Optimisation of the SPE procedure

When applying SPE, steroid- or hormone-like compounds are commonly extracted from the sample by use of an apolar extraction phase [25-27]. Therefore, the apolar C\textsubscript{18} phase and two polymeric phases (PLRP-S and Resin GP) were investigated for the extraction of prednisolone. The sample was loaded onto the cartridge after which the stationary phase was washed with buffer at pH 8.5, 7 or 3. Subsequently, a 10-min gradient from 0 to 100% methanol, buffered at the same pH, was used for the elution. Diode-array detection was applied during the optimisation of the SPE procedure. The peak shape and the position for the analyte with regard to the matrix were the criteria for the selection of an appropriate stationary phase. With the polymeric phases, hardly any separation between the analyte and the matrix could be obtained.

With the C\textsubscript{18} stationary phase, no separation was observed at pH 8.5. Decreasing the pH to 7 or 3 did not move the prednisolone peak due to the fact that prednisolone is a neutral species. However, the retention of the co-extracted matrix components was increased, resulting in more distinction between prednisolone and the matrix compounds. With pH 3, the best results were obtained, since most of the matrix compounds eluted at more than 50% methanol. Some matrix compounds eluted in front of prednisolone and some co-elution was observed. It was possible to wash the cartridge with 25:75 methanol:diluted acetic acid (0.5%) at a flow-rate of 5.0 ml/min without breakthrough of the analyte from the extraction phase. This ensured that the early eluting matrix compounds, i.e. polar components, were removed and a 100% recovery was obtained for prednisolone.

Table 2: Optimised SPE procedure for C\textsubscript{18} cartridges; injected sample volume is 500 µl.

<table>
<thead>
<tr>
<th></th>
<th>Flow-rate (ml/min)</th>
<th>Volume (ml)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>5.0</td>
<td>2.5</td>
<td>0:00 – 0:50</td>
</tr>
<tr>
<td>Conditioning</td>
<td>5.0</td>
<td>3.75</td>
<td>0:50 – 1:25</td>
</tr>
<tr>
<td>Sampling</td>
<td>2.0</td>
<td>1.5</td>
<td>1:25 – 2:00</td>
</tr>
<tr>
<td>Washing</td>
<td>5.0</td>
<td>3.75</td>
<td>2:00 – 2:75</td>
</tr>
<tr>
<td>Elution</td>
<td>1.0</td>
<td>1.25</td>
<td>2:75 – 4:00</td>
</tr>
</tbody>
</table>

Subsequently, the elution was performed starting at 25% methanol and increasing this to 50% within 0.1 min. No further increase in the percentage of methanol was used so that most of the co-extracted and apolar matrix components that were not removed during the wash step were retained by the stationary phase, and could therefore not interfere with the MS detection. After
each analysis the cartridge was discarded. The final extraction procedure is presented in Table 2. The total analysis time was about 4 min.

### 4.3.3.2 SPE-MS system

**Use of ion-trap MS**

An ion-trap MS was applied for the SPE-MS system. The vaporiser temperature was set at 350°C, which gave adequate evaporation and no memory effect was observed. However, extensive fragmentation was observed due to the thermlability of the analyte and/or the easy CID of prednisolone. The fragmentation pattern and a mass spectrum in the MS mode are shown in Figs. 1A and B. The fragmentation of prednisolone implied that the parent ion

![Fragmentation Pattern and Mass Spectra](image)

**Fig. 1:** (A) Structure and fragmentation pathway of prednisolone in the ion-trap MS; (B) mass spectrum (MS mode); (C) mass spectrum (MS/MS mode, fragmentation of $m/z$ 361).
m/z 361 was only about 54% of the total abundance. With MS/MS experiments, no formation of the fragments with m/z 329 and 301 was observed, suggesting that these ions were indeed formed by thermal degradation. Three fragments, i.e., m/z 343, 325 and 307, were formed after CID of the parent ion [M+H]+ (Fig. 1C). Fragment m/z 343 gave the highest signal, but the summation of the extracted ions of m/z 307 and 325 resulted in the best signal-to-noise (S/N) ratio, even though only about 15% of the signal of the product ion was converted into these fragments.

The use of the ion-trap MS in the single-MS mode (Fig. 2A) showed severe matrix interferences and a rather high limit of detection (LOD; 50 ng/ml; three times the blank level) was obtained. The application of MS/MS resulted in clean chromatograms after extraction of blank serum (Fig. 2B) and an improved LOD (5 ng/ml; determined as three times the level of spikes in the chromatogram) was observed (Table 3). A good reproducibility and linearity were obtained. A representative chromatogram of spiked serum is depicted in Fig. 2C. Good linearity and reproducibilities were observed over the investigated concentration range. The limit of quantitation was about 10 ng/ml (relative standard deviation <15%). Comparing the signals after extraction from buffer and from serum showed higher signals for the latter. At 10 ng/ml, the signal after extraction from serum was about 2 times as high as the signal after extraction from buffer. This decreased to a factor of about 1.5 at 20 ng/ml and was about 1.2 at higher concentrations (30-550 ng/ml). A post-cartridge continuous infusion of the analyte while extracting blank serum [28] showed a similar ion enhancement effect.

Table 3: Analytical data of the SPE-MS2 systems for the determination of prednisolone in serum using an ion-trap MS (MS/MS mode) and a triple-quadrupole MS (MRM mode).

<table>
<thead>
<tr>
<th></th>
<th>Ion-trap MS</th>
<th></th>
<th>Triple-quadrupole MS</th>
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<tbody>
<tr>
<td></td>
<td>APCI</td>
<td></td>
<td>APCI</td>
</tr>
<tr>
<td>LOD (ng/ml)</td>
<td>5</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Linearity* (R)</td>
<td>0.9944</td>
<td>0.9962</td>
<td>0.9959</td>
</tr>
<tr>
<td>Range (ng/ml)</td>
<td>10-550*</td>
<td>10-275*</td>
<td>10-275*</td>
</tr>
<tr>
<td>RSD (%)±</td>
<td>8.8</td>
<td>9.9</td>
<td>10.2</td>
</tr>
</tbody>
</table>

*: weighted regression (1/x).
#: maximum concentration investigated.
±: at 20 ng/ml, n=6.
Fig. 2: (A) SPE-MS with an ion-trap MS using the APCI interface for extraction of blank serum (total ion count, $m/z$ 295-370); (B) SPE-MS/MS of blank serum and summation of extracted ions $m/z$ 307 and 325, (C) SPE-MS/MS of serum spiked with 10 ng/ml prednisolone.
Use of triple-quadrupole MS

Another type of MS, a triple-quadrupole instrument, was used with an APCI and APPI source. The vaporiser temperature was set at 450°C for adequate evaporation of the eluate. With lower temperatures, memory effects were observed. The high temperature caused severe fragmentation of prednisolone due to thermal degradation. Similar chromatograms as with the ion-trap MS were obtained in the single-MS mode (Q1 scan) upon analysis of serum samples (Fig. 3A). However, applying MS/MS (MRM mode), monitoring the same fragments as with the ion-trap MS (that is m/z 325 and 307), did not completely eliminate the matrix interference (Fig. 3B). The LOD was now about 10 ng/ml (Table 3). A representative chromatogram is depicted in Fig. 3C. With APPI, similar results were observed as with APCI with respect to sensitivity, linearity and reproducibility (Table 3). The matrix interference was at the same level and no improved sensitivity was observed. An increase in sensitivity can probably only be obtained if electronic noise is limiting the sensitivity. In case of chemical noise, i.e. background caused by matrix compounds, such an increase is less likely to be obtained. This was confirmed by comparing the LODs of APCI and APPI in the MRM mode for the extraction of prednisolone from buffer, in which no chemical noise interfered with the determination. Then, about a factor of five more sensitivity was observed with APPI than with APCI. These results were in accordance with the results of other studies [22,23].

The determination of the LOD (three times the blank level) after extraction from serum and detection with the triple-quadrupole MS was rather ambiguous due to the presence of the matrix. In this study, constant signals were obtained with the blank serum. However, with real-life samples, often no blank sample is available, thus making detection at such low levels more unpredictable. The presence of matrix interference from blank serum may be due to similar fragmentation pathways of co-extracted compounds, since many endogenous compounds such as steroid hormones have similar fragmentation patterns [16,17]. The loss of H₂O is rather easy to establish from any hydroxylated compound. With hormone-like compounds, the loss of two or more H₂O molecules is very common [16,20].

The difference in matrix interference between the ion-trap MS (MS/MS mode) and the triple-quadrupole MS (MRM mode) is probably due to the difference of the MS/MS principles. In the ion-trap MS, only the precursor ion is accelerated sufficiently to be fragmented. Fragments are readily stabilised in the centre of the trap, hereby decreasing the potential for further fragmentation. In the triple-quadrupole MS, more fragmentation is commonly observed [17,18], since all ions are accelerated. This implies that an ion that is already fragmented will still be accelerated towards the end of the second and the third quadrupole. Thus, a formed fragment (product ion) may thereby collide and
fragment further in the second and the third quadrupole. This has the consequence that not only the analyte is more easily fragmented (as observed during MS/MS experiments), but also the matrix components. In a triple-quadrupole MS, the higher level of interference can thus be due to product ions (ions should already be formed in the source or in the first quadrupole to $m/z$ 361) and their consecutive fragments, which is less common in an ion-trap MS.

Fig. 3: (A) SPE-MS with a triple-quadrupole MS using the APCI interface for extraction of blank serum (Q1 scan $m/z$ 100-400); (B) SPE-MS/MS of blank serum (MRM mode, transition of $m/z$ 361 to $m/z$ 325 and 307); (C) SPE-MS/MS of serum spiked with 10 ng/ml prednisolone (offset 500 cps).
4.3.4 Conclusions

The separation of prednisolone from endogenous compounds present in serum was difficult to obtain, and some matrix interference was still observed in the MRM mode. The present SPE-MS/MS system applying the ion-trap MS showed an increase in selectivity with regard to the triple-quadrupole MS. The LOD is in the low-ng/ml range, but is still rather high due to the thermal degradation and easy CID of prednisolone. The total analysis time was about 4 min, which was due to high flow-rates (up to 5 ml/min, except for the elution) during the SPE procedure. The use of even higher flow-rates (up to 10 ml/min), as well as the application of two cartridges in parallel may further enhance the throughput.

In general, SPE-MS\textsuperscript{n} has shown good potential for high-throughput bioanalysis. The development of a rapid SPE-MS system implies rapid extraction, while obtaining good selectivity at the front of the analysis (SPE) as well as at the end (MS). One should always carefully monitor the effects of matrix, and in particular those compounds that co-elute with the analyte of interest. The use of an internal standard may help to improve the reproducibility when required.

Acknowledgements

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4.3.5 References

Chapter 4 – Coupling of solid-phase extraction and mass spectrometry