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Liquid chromatography–mass spectrometry with on-line solid-phase extraction by a restricted-access C_{18} precolumn for direct plasma and urine injection

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Abstract

In this paper, the on-line coupling of solid-phase extraction, based on a restricted-access support with liquid chromatography–mass spectrometry (LC–MS), for the analysis of biological samples is described. The system was tested with cortisol and prednisolone for plasma analysis and arachidonic acid for urine analysis. A precolumn packed with a 25-μm C_{18} alkyl-diol support is used for direct plasma or urine injection. Using column-switching techniques, the analytes enriched on the precolumn are eluted to the analytical column without transfer loss. An on-line heart-cut technique was employed and only the analyte-containing fraction eluting from the LC column is directed to the MS to protect the LC–MS interface and ion-source from contamination. The whole system is operated in a parallel mode, that is, sample pre-treatment and LC–MS analysis are performed simultaneously to provide the shortest possible analysis time. The only off-line sample pre-treatment step required was centrifugation to remove particulate matter. With the fully automated system, total analysis times of 5 and 9.5 min were achieved for cortisol in serum and arachidonic acid in urine, respectively. Cortisol and related compounds were quantitatively recovered from plasma with a detection limit for prednisolone (direct injection of 100 μl on restricted-access precolumn) of 2 ng/ml.

Keywords: Sample preparation; Restricted access packings; Cortisol; Prednisolone; Arachidonic acid

1. Introduction

In recent years, liquid chromatography–mass spectrometry (LC–MS) has become an important analytical technique for the determination of drugs and their metabolites in biological matrices [1]. The high selectivity of MS, particularly when operated in the MS–MS mode, allows the fast and accurate determination of analytes in complex matrices. Although detection in the MS–MS mode is highly selective, a considerable amount of sample pre-treatment is usually required to remove interfering matrix components. Sample handling is a major bottleneck in bioanalysis using LC–MS, particularly with respect to analysis time. The time required for the removal of particulate matter, proteins and other matrix components that would negatively affect LC separation and MS detection is often considerably

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longer than the entire LC–MS run. Considering the high costs of MS analysis time, it is desirable to develop efficient and reliable sample handling techniques to keep the overall analysis time at a minimum.

Liquid–liquid extraction (LLE) [2–5] and solid-phase extraction (SPE) [6–12] are the most often used sample pre-treatment techniques in bioanalysis. On-line dialysis has been used to remove proteins from plasma prior to SPE [13]. SPE is particularly attractive since it allows the simultaneous removal of matrix components and preconcentration of the analytes. In the majority of bioanalytical LC–MS applications, SPE is performed in the off-line mode using disposable extraction columns [6–12]. The off-line mode has the advantage that a minimum of memory effects can be expected. Moreover, as they are used only once, no special demands concerning reproducibility are made on the SPE column materials. A disadvantage of off-line SPE is the need to evaporate elution solvents prior to injection into the LC–MS system, which can lead to analyte loss or artefact formation. More important is the rather long sample handling time associated with off-line SPE when evaporation steps are required.

In recent years, special SPE supports possessing restricted-access properties have been developed to allow the direct injection of biological matrices into on-line SPE–LC systems [14–23]. Restricted-access supports combine size-exclusion of proteins and other high-molecular-mass matrix components with the simultaneous enrichment of low-molecular-mass analytes at the often hydrophobic inner pore surface. A larger number of different restricted-access supports have been designed and commercialised in recent years [24,25].

In the majority of applications described in the literature, restricted-access supports are used in on-line, coupled-column SPE–LC systems, in combination with UV and other detectors. In this paper, we describe the on-line coupling of SPE based on restricted-access supports with LC–MS. Particular attention has been paid to the development of an efficient sample pre-treatment method. Using an on-line heart-cut technique, only the analyte-containing fraction eluting from the LC column is directed towards the MS, to protect the LC–MS interface from contamination. The whole system is operated in a parallel mode, that is, sample pre-treatment and LC–MS analysis are performed simultaneously to provide the shortest possible analysis time. Applications are shown for both the determination of arachidonic acid in urine and of cortisol and related compounds in plasma.

2. Experimental

2.1. Chemicals

Acetonitrile, methanol and acetic acid (Baker, Deventer, Netherlands) were of HPLC grade. Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Cortisol, cortisone, prednisolone and fludrocortisone were purchased from Sigma (St. Louis, MO, USA). Arachidonic acid and [$^2H_8$]arachidonic acid were obtained from Cayman Chemicals (Ann Arbor, MI, USA). All stock solutions for spiking biological samples were prepared in methanol (1 mg/ml) and stored in a refrigerator.

2.2. Apparatus

The instrumental set-up is shown in Fig. 1. The injection valves are depicted in the "load" position. The LC part consists of a Gilson (Villiers-le-Bel, France) ASPEC XL autosampler equipped with a Gilson 401C dilutor, two Rheodyne (Berkeley, CA, USA) six-port injection valves and an LKB (Brom-

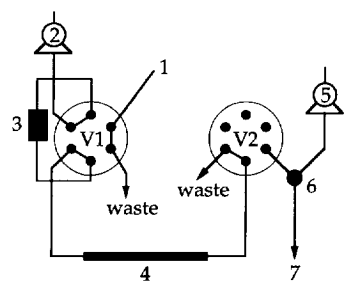

Fig. 1. Set-up of the analytical system. 1, syringe pump connected to autosampler; 2, HPLC pump; 3, precolumn packed with restricted-access support; 4, analytical column; 5, pump for make-up flow; 6, mixing union; 7, to MS interface; V1, V2, six-port injection valves shown in LOAD position. The pump for the make-up flow (5) and the mixing union (6) were only used in the determination of arachidonic acid.

ma, Sweden) Model 2150 LC pump. On-line preconcentration was performed on a precolumn (15×4 mm I.D.) packed with LiChrospher RP-18 ADS, 25 μm (Merck, Darmstadt, Germany). The flow-rate during the loading of the sample onto the restricted-access precolumn was 0.5 ml/min. Conditioning and washing steps were performed at 1.0 ml/min. Elution of compounds from the precolumn to the analytical column is performed by switching valve 1 to the "inject" position.

All experiments were performed on a Finnigan MAT TSQ-70 triple quadrupole mass spectrometer (San Jose, CA, USA). For the determination of arachidonic acid, a Finnigan MAT thermospray (TSP) interface was used with discharge on at 1 kV in the negative ionisation mode. The vaporiser and ion-source temperature were optimised and kept at 90 and 250°C, respectively. The repeller voltage was optimised daily to check for possible ion-source contamination and kept at the optimum voltage, which was between −100 and −140 V. Experiments were performed in selective reaction monitoring (SRM) mode, measuring the loss of CO_2 from the deprotonated molecule of arachidonic acid, using a collision pressure of 0.5 Pa and a collision energy of 15 eV.

For the determination of cortisol and related compounds, a custom-made atmospheric pressure chemical ionization (APCI) interface, which fits in the TSP ion-source, was used [26,27]. The stainless steel sampling capillary and ion-source temperature were kept at 250 and 200°C, respectively, whereas the nebulisation heater was kept at 500°C. Nebulisation at 1 bar was performed with a modified Finnigan MAT TSP II vaporiser at a temperature of 80°C. The TSP ion-source was pumped by a 28 m^3/h rotary pump and additional pumping was performed with a 12 m^3/h rotary pump at the atmospheric pressure region. To maintain 1 bar, this is performed with a leaking system, only removing the superfluous solvents, to prevent electrical breakdown. For optimal sensitivity, the ion-source pressure was regulated with a ball-valve and set at 500 Pa. To register this pressure, a Pirani, normally installed on the rotary pump, was installed between the ion-source and the ball-valve. The analysis was performed in negative ionisation mode using selective ion monitoring (SIM) of $[M+CH_3COO]^-$ of cortisol and fludrocortisone. Optimisation of the repeller voltage, which is ion-source pressure-dependent [26], was done at 500 Pa, the optimal voltage being −80 V.

Automation of the analytical system was performed using dedicated software written in Gilson 719 Pascal. The Gilson Samplib Pascal library was modified to allow for simultaneous sample processing and LC–MS analysis using heart-cut technology.

2.3. Procedure for the determination of arachidonic acid in urine

Urine samples were taken from volunteers and spiked with 100 ng/ml of $^2H_8$-labelled arachidonic acid as the internal standard, and 1 to 10 ng/ml of arachidonic acid, respectively. The internal standard was prepared freshly from 10 mg/ml [$^2H_8$]arachidonic acid in ethanol, by subsequent dilutions by factors of 100 and 10, to concentrations of 10 μg/ml in methanol. Aliquots containing 100 μl of the internal standard solution were added to 9.8 ml of urine. The urine samples were spiked to concentrations of 1 and 10 ng/ml of arachidonic acid by adding 100 μl of solutions containing 1 and 10 μg/ml arachidonic acid in methanol, respectively, to a total volume of 10 ml. It was assumed that endogenous arachidonic acid in the urine is not bound to other compounds.

The analysis of arachidonic acid was performed with an LC system consisting of a 200×3.0 mm I.D. stainless-steel column packed with Hypersil ODS (5 μm), which was operated at a flow-rate of 0.7 ml/min using acetonitrile–water containing 1 g/l acetic acid (75:25, v/v) as the mobile phase. Prior to the MS interface, a make-up flow (0.5 ml/min, see Fig. 1), consisting of acetic acid (1 g/l), was introduced, resulting in a total flow-rate of 1.2 ml/min and an acetonitrile content of 44%.

The ADS C_{18} precolumn was conditioned with 2 ml of methanol and 1 ml of acetic acid (1 g/l) at a flow-rate of 1 ml/min. After injection of 200 μl of the sample at a flow-rate of 0.5 ml/min, the precolumn was washed with 200 μl of acetic acid (1 g/l) at a flow-rate of 1 ml/min. The precolumn was desorbed in the backflush mode by switching valve 1 to the "inject" position. After 2 min, the precolumn was switched off-line ("load" position) and the next analysis was started. The first 5 min of the chromato-

graphic run were directed to waste to avoid ion-source contamination; the next 4 min of the chromatographic run were directed to the MS.

2.4. Procedure for the determination of cortisol and related compounds in plasma

Human plasma is spiked with concentrations of 10 to 400 ng/ml of cortisol, covering the relevant concentration range, and stored in the refrigerator in portions of 1 ml. No anticoagulant was added to the plasma samples. The calibration curve in water is made freshly each day. Before every run, the internal standard (prepared from a stock solution of 1 mg/ml in methanol) was added to the thawed plasma and the sample was centrifuged for 10 min at 5200 *g*. Together with the internal standard, methanol was added to the plasma sample to achieve a final methanol concentration of 10%. Samples containing 250 μl of plasma were used for analysis. The analytical method was tested by a three-fold assay on three consecutive days by recording calibration curves for both plasma and water samples. Three blank plasma samples, three calibration curves of plasma and two calibration curves of water samples are determined on one day.

The analysis of cortisol was performed with an LC system consisting of a 125×4 mm I.D. stainless-steel column packed with LiChrospher (Merck) 100 RP-18 (5 μm particles), which was operated at 0.5 ml/min using acetonitrile–water containing 1 g/l acetic acid (75:25, v/v) as the mobile phase. Before every injection, the ADS C_{18} precolumn was conditioned with 2 ml of acetonitrile and 2 ml of methanol–water (10:90, v/v) at a flow-rate of 2 ml/min. After injection of 200 μl of the sample, the precolumn was washed with 3 ml of methanol–water (10:90, v/v) at a flow-rate of 1 ml/min. The time course of the preconcentration procedure is shown in Fig. 2. The precolumn was desorbed in the forward flush mode by switching valve 1 to the "inject" position.

The time programme for the heart-cut sequence is shown in Fig. 2. At point A (start of the LC–MS analysis) the precolumn is switched on-line to the analytical column. At this point, valve 2 is in the "inject" position and the LC eluate is directed to waste. At point B (1 min after injection), the analytes

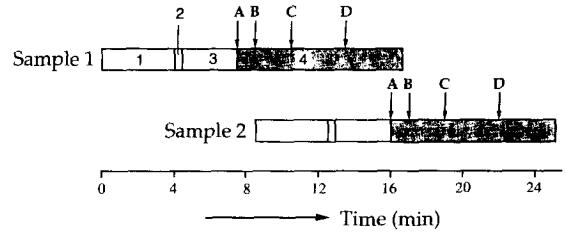

Fig. 2. Time scheme of the on-line SPE–LC–MS system including heart-cut. 1, Conditioning; 2, sample loading; 3, washing; 4, LC–MS analysis; A, precolumn is switched on-line to analytical column, LC eluate is directed to waste; B, precolumn is switched off-line; C, LC eluate is directed to MS; D, LC eluate is directed to waste.

are completely desorbed from the precolumn, which is switched off-line, and analysis of the next sample is started. At point C (3 min after injection), valve 2 is switched to the "load" position and the LC eluate is directed to the MS. At point C (6 min after injection), the LC eluate is directed to waste by switching valve 2 into the "inject" position.

3. Results and discussion

3.1. Design of the analytical system

The present system uses on-line column switching techniques for combining SPE on a restricted-access support with LC–MS. Moreover, heart-cut switching is used to direct only the analyte-containing part of the eluate to the MS. To obtain short analysis times, pre-treatment of a plasma sample is performed simultaneously with the chromatographic analysis of the previous sample. On-line sample handling involves four different steps, i.e., regeneration and conditioning of the precolumn, loading of the sample, washing to remove proteins and other interfering sample constituents and elution, which is simply performed by switching the precolumn on-line to the analytical column for 1 min. Due to the large particle size of the restricted-access material (25 μm), it was possible to use the syringe pump that is connected to the autosampler for all precolumn flushing procedures. We essentially used the same pre-treatment method, with different volumes for different analytes, in both plasma and urine analysis, although

with the latter the need for protein removal is less pronounced.

The time required to execute sample pre-treatment and heart-cut for the analysis of cortisol in plasma is shown in Fig. 2. During the first 3 min of the chromatogram, the HPLC eluate containing polar matrix components is directed to waste. After 1.0 min, the precolumn is switched off-line with the analytical column (point B in Fig. 2) and pre-treatment of the next sample starts. At exactly 3 min, valve 2 is switched to the "load" position, directing the HPLC eluate to the MS. After exactly 6.0 min, valve 2 is switched back to the "inject" position, sending the eluate to waste. The LC–MS run is finished after 8.0 min. The heart-cut technique effectively eliminates matrix components which were retained by the restricted-access precolumn and might interfere with the LC–MS analysis.

3.2. Determinations of arachidonic acid in urine

3.2.1. LC–MS conditions

Arachidonic acid is an endogenous compound present in urine at a low ng/ml level. The sensitivity using TSP ionisation was compared in both negative and positive ionisation mode, observing the deprotonated and protonated species, respectively. A two-fold higher sensitivity was obtained in the negative ionisation mode. The gain in the signal-to-noise ratio is even better due to fewer interferences by the background ions that are present. Moreover, in MS–MS, the negative mode is preferable since only two pronounced fragments are formed, while in the positive mode, the aliphatic backbone is cleaved subsequently. All further experiments were therefore carried out in the negative ionisation mode.

Optimisation of the TSP conditions was performed using flow injection analysis (FIA), with repetitive injections of 200 pg in SIM mode for arachidonic acid and D_8-arachidonic acid. Both compounds exhibited an equal response and a detection limit of 50 pg (signal-to-noise ratio, 3:1) was obtained. During development of the LC–MS system, a compromise had to be made with respect to the content of organic modifier in the mobile phase. Although the highest sensitivity in MS was obtained at an acetonitrile content of 50%, unfortunately, the retention time of arachidonic acid under these conditions was unacceptably long ($k'>15$). At an acetonitrile content of 90%, acceptable retention times for arachidonic acid were obtained but MS sensitivity decreased drastically. We finally chose a system with 75% acetonitrile at a flow-rate of 0.7 ml/min and a make-up flow of aqueous acetic acid at 0.5 ml/min (see Fig. 1). This resulted in a final acetonitrile content of the eluate entering the MS of 44% and a k' of 3 for arachidonic acid.

Using air as the collision gas, with an optimum collision energy of 15 eV, arachidonic acid loses CO_2 and C_7H_{14}. The loss of CO_2 is predominant and can be used in an SRM procedure, scanning the loss of CO_2 from arachidonic acid and [$^2H_8$]arachidonic acid, respectively. All further experiments were performed in the SRM mode.

3.2.2. Determination of arachidonic acid in urine

The recovery of arachidonic acid using an ADS C_{18} precolumn was tested by comparing these results with direct loop injections at 100 ng/ml levels. All experiments were repeated eight times, and the recovery of arachidonic acid was 77±5%. The automated LC–TSP–MS–MS system was tested with urine samples spiked with arachidonic acid at levels of 1 and 10 ng/ml, and with 100 ng/ml of [$^2H_8$]arachidonic acid. Several cycles were performed by injecting water, blank urine, 1 ng/ml spiked urine and 10 ng/ml spiked urine. The overall reproducibility at 10 ng/ml, calculated as the standard deviation of the internal standard, was 7% ($n=15$). At 1 ng/ml, the standard deviation for spiked urine was 10% ($n=5$). Arachidonic acid is an endogenous compound and can be detected in the blank urine. In Fig. 3, mass chromatograms for both blank urine and urine spiked with 1 ng/ml arachidonic acid are shown. Interpolating the results, a level of approximately 1 ng/ml arachidonic acid could be estimated in blank urine.

3.3. Determination of cortisol in plasma

3.3.1. LC–MS conditions

For the analysis of cortisol in plasma using MS detection, soft ionisation techniques can be used, whereas TSP is less favourable, due to thermal degradation in the vaporiser and the lack of sensitivity [28]. Ways to overcome these problems have been

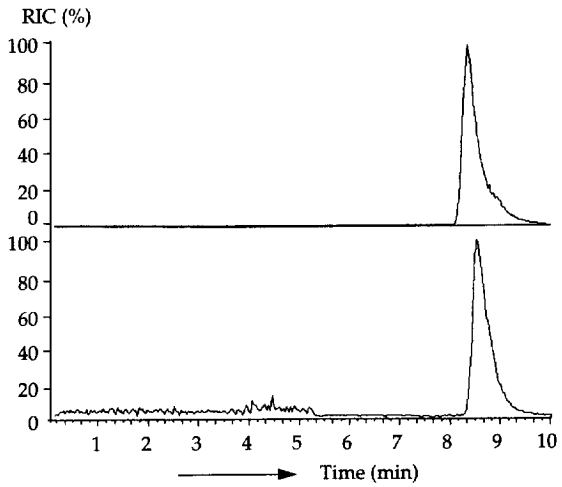

Fig. 3. Determination of arachidonic acid (10 ng/ml) (bottom) in urine using on-line SPE–LC–MS. Internal standard, [$^2H_8$]arachidonic acid (100 ng/l) (top). Conditions: see Section 2.

described previously by Paulson and Lindberg [29] using acetylation of cortisol to cortisol 21-acetate. We used APCI in negative ionisation mode and a strong signal can be observed, normally divided between several peaks, $[M-H]^-$, $[M+Cl]^-$ and $[M+CH_3COO]^-$. Addition of 1% glacial acetic acid to the eluent totally moves the mass spectrometric information to $[M+CH_3COO]^-$. The signals observed now in positive and negative ionisation mode are equal. Due to the fact that less interfering background ions are present in the negative mode, we employed this mode using TSP nebulisation for further experiments. Although in TSP severe fragmentation is observed, only minor loss of water is observed using TSP nebulisation at 1 bar. Under optimal conditions in FIA mode, 10 pg of cortisol could be detected, based on 20 μl injections.

A SIM procedure has been used, which alternatively scans *m/z* 419, 421 and 439. These masses reflect the $[M+CH_3COO]^-$ ion of cortisone and prednisolone (*m/z* 419), cortisol (*m/z* 421) and fludrocortisone (*m/z* 439), respectively. The signal of cortisone and prednisolone is involved in the procedure to check the separation and isotope influence as discussed below.

Normally plasma contains cortisone and endogenous cortisol at levels of 10 to 100 ng/ml. With the system described here, cortisone and cortisol can be separated by LC. Prednisolone, the active metabolite of prednisone, can be a bigger problem if patients are treated with this precursor. The separation of prednisolone and cortisol is difficult when working with reversed-phase chromatography, but using MS detection, it is not necessary, if *m/z* 419, the $[M+CH_3COO]^-$ of prednisolone and cortisone, is involved in the SIM procedure. The influence of the prednisolone signal, if present, on the signal of cortisol is based on the M+2 isotope (*m/z* 421) of prednisolone, which is 4.8%. By correcting the total *m/z* 421 signal, cortisol+prednisolone M+2, for the influence of the isotope, a correct concentration of cortisol can be obtained. To check this assumption, eight injections of 10 ng/ml cortisol in water together with several concentrations of prednisolone were made and data were evaluated using the following correction formula:

$$\text{Signal Cortisol} = [\text{signal } m/z\ 421] - 0.048[\text{signal } m/z\ 419]$$

In Table 1, the uncorrected and corrected ratios of internal standard, fludrocortisone, to cortisol are given. At a level of 100 ng/ml prednisolone, the signal of cortisol is already influenced by about 50% by the prednisolone M+2 isotope. We have used this

Table 1
Ratios of the internal standard, fludrocortisone, to cortisol, corrected and not corrected for the influence of prednisolone M+2

Concentration of prednisolone (ng/ml)	Uncorrected ratio			Corrected ratio		
	Area	S.D.	%	Area	S.D.	%
0	1.079	0.033	3.1	1.079	0.033	3.1
100	0.981	0.030	3.1	1.028	0.033	3.2
1000	0.715	0.013	1.8	1.096	0.044	4.0

correction equation for all those samples where prednisolone was detected at m/z 419.

3.3.2. Determination of cortisol in plasma

The overall recovery for cortisol using fludrocortisone as the internal standard was 105±12% (n= 15). When validating bioanalytical methods for cortisol in plasma it is common to produce a "blank" plasma by treatment with activated charcoal to remove endogenous cortisol. In this study, we have chosen to perform a validation with untreated plasma since we were primarily interested in the effect of plasma samples on the long term operational stability of the analytical system. Since the endogenous cortisol level of the plasma used was approximately 100 ng/ml, we tested our method by spiking untreated plasma with 100, 200 and 400 ng/ml cortisol. It should be emphasised that the detection limit for compounds such as prednisolone or fludrocortisone in plasma is 2 ng/ml (signal-to-noise ratio=3, 200 μl injections). In Fig. 4, the mass chromatogram of spiked plasma with 100 ng/ml of cortisol, cortisone, prednisolone and fludrocortisone is shown. The only sample pre-treatment performed was addition of methanol to a final concentration of 10% to disrupt cortisol–protein binding and subsequent centrifugation. The with-in day reproducibility of this method for cortisol at 100, 200 and 400 ng/ml was 14.4, 8.2 and 7.3%, respectively (n=3). The day-to-day (n= 3) reproducibility at the same levels was 11.2, 6.5 and 6.0%, respectively. The relatively high relative standard deviation for plasma analysis can be explained by the high level of endogenous cortisol, which exhibited slight variations over time of storage. The effective analysis time for plasma samples was approximately 8 min. Apart from the addition of a fixed amount of methanol to a final concentration of 10% and centrifugation, the complete analysis was performed automatically.

When injecting clear supernatants, no hardware problems (increased backpressure, contamination of LC–MS interface) occurred due to residual proteins or other matrix components. It should be noted, however, that upon prolonged storage at room temperature, particularly of plasma samples containing 10% methanol, plasma samples became turbid, probably due to proteins which had started to precipitate. Injection of these plasma samples leads to serious backpressure problems and, finally, clogging of the system. Storage of samples at 4°C may help to overcome this problem. The best solution, however, is to add methanol in appropriate amounts shortly before analysis using the liquid handling system of the autosampler. In this way, protein precipitation can be effectively reduced without requiring extensive, non-standard tray cooling equipment. In addition, an inlet-filter should be used prior to the restricted-access column. By freshly preparing plasma samples we are able to measure three calibration curves in plasma and two in water on three consecutive days without operational problems.

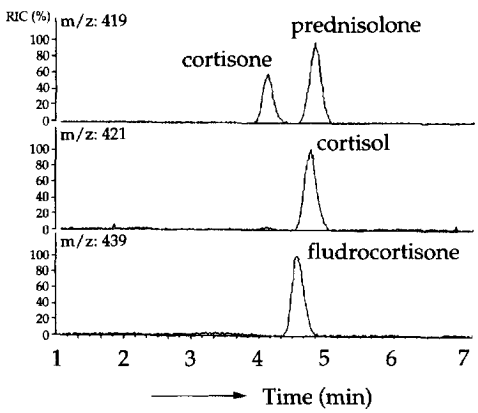

Fig. 4. Determination of cortisol, cortisone, prednisolone and fludrocortisone (100 ng/ml each) in untreated plasma using on-line SPE–LC–MS. Conditions: see Section 2.

4. Conclusions

The use of a restricted-access support for the direct injection of biological matrices without prior sample pre-treatment (except for centrifugation) significantly enhances the performance of LC–MS in bioanalysis. In both plasma and urine analysis, the time required for sample preconcentration and removal of interfering matrix components approximately equals the LC–MS run time. To ensure error-free unattended operation, it is crucial to remove particulate matter from the sample. This is particularly problematic when methanol or other organic solvents are added to the sample to decrease protein

binding of the analytes. Proper cooling of the sample tray or addition of the solvent shortly before the analysis using the liquid handling system of the autosampler are possibilities to circumvent this problem. Moreover, the use of an inlet-filter helps to prevent clogging of the precolumn due to the presence of particulate matter.

Sample throughput can be increased by simultaneously performing sample handling and LC–MS analysis of two samples. Implementing the on-line heart-cutting technique improves the robustness of the LC–MS system by eliminating remaining matrix components which might negatively effect the LC–MS interface. The heart-cut technique requires an additional electronically controlled switching valve; on the other hand, the reduction of down-times of the MS, for example, due to cleaning of the interface, is an important gain.

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