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Capillary solid-phase extraction–tandem mass spectrometry for fast quantification of free concentrations of tolterodine and two metabolites in ultrafiltered plasma samples

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Abstract

A capillary solid-phase extraction (SPE) system has been coupled directly to electrospray tandem mass spectrometry for quantification of free tolterodine and metabolite concentrations in plasma. The unbound fraction of these compounds was obtained by ultrafiltration of plasma. The ultrafiltrate was directly injected onto the SPE capillary (4 mm×200 μm, 5 μm C_{18}). After desalting and clean-up of the sample, the analytes were eluted in backflush mode with methanol–1 m*M* triethylamine (70:30, v/v), providing considerable solute focusing. Elution from the SPE capillary was improved by inserting a short trapping capillary between the SPE capillary and the MS interface, by which analyte focusing was increased. The unresolved compounds eluted simultaneously with the remaining matrix compounds and were detected in a multiple-reaction monitoring (MRM) mode. No interference of the sample matrix on detection was observed, allowing aqueous standards to be used for calibration. Linear calibration curves were obtained between 0.05 and 1000 ng/ml (corresponding to 150 p*M*–3 μ*M*) and the limit of detection was 50 pg/ml injecting 10 μl. Equilibration of the SPE capillary, sample loading, elution and detection took less then 6 min per sample.

Keywords: Tolterodine

1. Introduction

The development of liquid chromatography–mass spectrometry (LC–MS) interfacing techniques, in particular atmospheric pressure ionization, has led to a widespread use of the mass spectrometer as detector in liquid chromatography. Robust quadrupole MS instruments are increasingly used for quantitative analysis, e.g., in bioanalysis [1–4]. With a costly detector like a (tandem) MS instrument the analysis time becomes an important aspect to consider. Reduction of the analysis time is generally achieved by simplifying the sample clean-up, keeping analyte retention small and/or avoiding gradient elution. However, serious matrix interference on detection can occur as a result of poor separation. Ionization suppression of analytes caused by co-eluting matrix constituents has been observed [1,2] and can impede detection sensitivity and accuracy of the method.

Recently, we developed a capillary column LC switching system coupled to electrospray ionization-tandem mass spectrometry (ESI-MS–MS) for quantification of free drug concentrations in plasma

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[4]. In that work no ionization suppression effect by the sample matrix was found, despite the small capacity factors of the analytes, which were in the range of 0.3 to 1.3. The favorable results stimulated us to investigate the possibility of omitting the separation of the analytes to reduce analysis time. The tremendous selectivity, provided by tandem mass filtering in a triple quadrupole instrument, should be sufficient to quantify the compounds of interest without interference from the matrix or the co-eluting analytes. Although, in principal, a single quadrupole instrument could be used, the probability of matrix interference is considerably higher. This solid-phase extraction (SPE)–MS–MS, technique has been used with narrow-bore size columns (2 mm I.D.) for the determination of clenbuterol in urine [5] and several drugs in plasma [6].

In this article we report on our experiences with capillary SPE directly coupled to MS–MS. This approach is used for fast quantitative determination of free concentrations of tolterodine and two metabolites in plasma, which have been obtained by ultrafiltration. Special attention is paid to the elution of the analytes from the SPE capillary.

2. Experimental

2.1. Materials

Tolterodine (PNU-200583), tolterodine acid, the 5-hydroxymethyl metabolite (5-HM, PNU-200577) and the deuterated 5-HM metabolite were supplied by Pharmacia&Upjohn (Uppsala, Sweden). The structural formulas can be seen in Fig. 1. Triethylamine (TEA) was obtained from Pierce (Rockford, IL, USA) and methanol and formic acid were purchased from Merck (Darmstadt, Germany). Tris was obtained from Bio-Rad Labs. (Richmond, CA, USA). Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). All aqueous solutions were prepared with water purified with a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Samples and ultrafiltration

Human plasma was ultrafiltered in Ultrafree 30.000 vials (Millipore) and spiked with analytes for assessment of matrix interference on detection. Four different sources of plasma were used for accuracy and precision measurements.

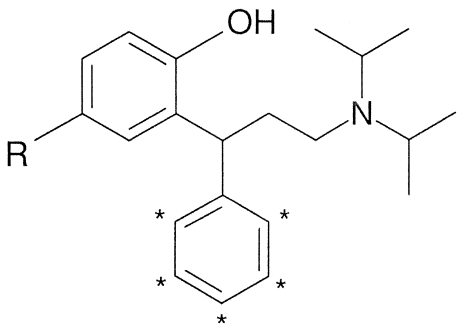

Fig. 1. Structures of the drug compounds. R-groups: tolterodine=CH_3, 5-HM=CH_2OH, and tolterodine acid=COOH. The internal standard 5-HM metabolite is deuterated at the asterisk.

For free drug concentration measurements, human plasma was adjusted to pH 7.4 by purging through CO_2 gas. Next the plasma was spiked with tolterodine, tolterodine acid and 5-HM to a total concentration of 5 or 50 ng/ml from a stock solution of 1.0 μg/ml of the analytes in water. Before ultrafiltration, plasma samples were placed in a water bath at 37°C for at least 30 min to assure drug–protein binding. A 400-μl volume of plasma was ultrafiltered at 37°C in a preheated centrifuge (2K15, Sigma, Osterode am Harz, Germany) for 20 min at 2000 *g*. Quantification was performed with calibration standards prepared in Tris buffer (pH 7.4, I=0.15 mol/l). Calibration standards of 0.2, 0.5, 1.0, 2.5 and 5.0 ng/ml were used for the low level determination and standards of 2.0, 5.0, 10.0, 25.0 and 50.0 ng/ml for the high level determination.

2.3. Preparation of the SPE capillary

SPE capillaries were slurry packed in a 200 μm I.D. fused-silica capillary with 5 μm Kromasil C_{18} particles. To retain the packing inside the capillary, a fiber glass frit followed by a second piece of fused-silica capillary of 50 μm I.D.×190 μm O.D. was inserted approximately 1 cm into the capillary and fixated with glue. A more detailed description of the procedure of the preparation of the SPE capillary can be found elsewhere [4].

Part of the experiments was performed with an extra trapping capillary to increase focusing of the

analytes. During these experiments a short (1 cm) 200 μm I.D. capillary packed with cyanopropyl silica particles was incorporated between the SPE capillary and the MS interface. The trapping capillary was held between two pieces of 50 μm I.D. capillary with PTFE tubing. A glass fiber frit retained the packing.

2.4. SPE

Two HPLC pumps (PU-980, Jasco, Tokyo, Japan) were used to deliver the loading and elution solvent, respectively. The loading solvent consisted of methanol–10 m*M* formic acid buffer, pH 3.4 (10:90, v/v). The elution solvent was a mixture of methanol–10 m*M* formic acid and 1 m*M* TEA, pH 4.8 (70:30, v/v). With the additional focusing capillary in the system, TEA was not added to the elution solvent.

Flow-rates for sample loading and elution were 30 and 2–3 μl/min, respectively. An injector with a 2.5- or 10.0-μl sample loop and an electrically actuated switching valve from Valco (Schenkon, Switzerland) were used. After sample injection, the SPE capillary was flushed with loading solvent to wash off salts and other polar compounds. The flush volume, 35 μl for a 6 mm column, was chosen to correspond to the breakthrough volume for 5-HM (the least retained compound on the reversed-phase SPE capillary) reduced by approximately 15%. The SPE capillary was equilibrated for 2 min before the next injection was performed.

2.5. Mass spectrometry

A PE-Sciex API 365 triple quadrupole mass spectrometer (PE-Sciex, Concord, Canada) was used for detection. A sheathless MicroIon interface was used for electrospraying the elution solvent. This interface is a low dead volume stainless steel union connecting the spray capillary (6 cm×15 μm I.D.) with a capillary from the switching valve (20 cm×50 μm I.D.).

The tip of the spray capillary was unmodified and positioned approximately 5 mm in front of the orifice. The potential for the electrospray process was applied to the union and the conducting elution solvent provided for the electrical contact with the end of the spray capillary.

The MS signal was optimized during continuous infusion of a solution of the analytes dissolved in the elution solvent. The mass spectrometer was operated in positive ion mode with an electrospray potential of 4200 V and an orifice potential of 30 V. The nebulizer gas flow-rate was set to 225 ml/min and the dwell time was set to 200 or 400 ms, depending on the peak width. The analytes were detected in multiple reaction monitoring (MRM) mode. Molecular transitions 326→147, 342→223, 347→228 and 356→237 m/z of tolterodine, 5-HM, the deuterated 5-HM metabolite and tolterodine acid, respectively, were selected for detection.

3. Results and discussion

3.1. Optimization of the elution profile

In the previously described capillary LC column switching system [4] a precolumn was used to desalt and clean-up plasma ultrafiltrate samples and to retain the analytes. In that system, elution of the analytes to the separation column was performed in backflush mode with a mobile phase optimized for the separation. Analyte focusing occurred both on the SPE capillary and at the head of the separation column resulting in normal peak shapes.

In the present system, with the separation column omitted, the analyte focusing did, however, only occur on the SPE capillary. It was found that special attention had to be paid to the elution of the analytes from the SPE capillary. Significantly tailing peaks were observed when the analytes were eluted with mixtures containing less than 50% (v/v) methanol. Increasing the methanol content to 70% (v/v) resulted in reasonable elution profiles. The peak tailing could be further reduced by adding TEA to the mobile phase at a concentration of 1 m*M*. The large excess of this tertiary amine is assumed to reduce the undesirable interaction of tolterodine and the metabolites with residual silanol groups. The effect of the modifier concentration and the addition of TEA on the peak shape of 5-HM are illustrated in Fig. 2. The disadvantage of the addition of TEA to the elution solvent was that detection sensitivity was slightly impeded.

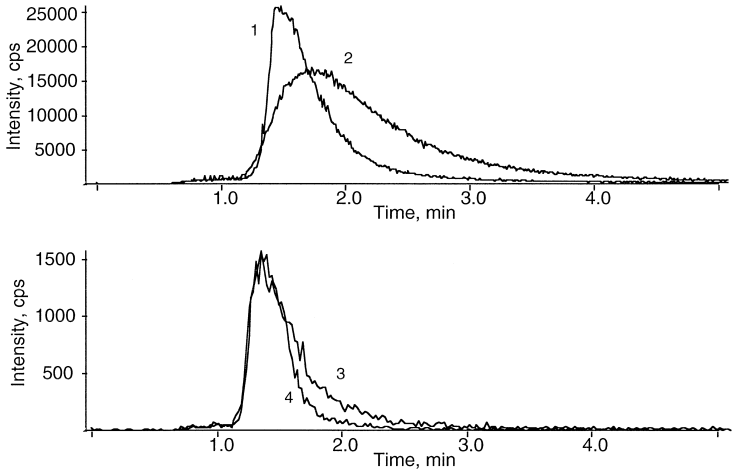

Fig. 2. Elution profiles of 5-HM from the C_{18} SPE capillary with various solvent compositions. Sample: (1) methanol–10 m*M* formic acid, pH 3.4 (70:30, v/v), (2) methanol–10 m*M* formic acid, pH 3.4 (30:70, v/v), (3) as 1, and (4) methanol–10 m*M* formic acid and 1 m*M* TEA, pH 4.8 (70:30, v/v).

3.2. Increased solute focusing with a trapping column

Although the analytes are focused during elution from the SPE capillary by the use of a stronger solvent mixture as compared with the loading solvent mixture, the peaks are still relatively broad and tailing. To improve the peak shape by increasing the analyte focusing, a 1 cm short trapping capillary packed with 5 μm cyanopropyl silica particles was placed between the SPE capillary and the MS interface. Since the purpose of this packed bed was focusing and not separation of the analytes, a cyanopropyl stationary phase was chosen that is less selectively retentive for tolterodine and its metabolites compared to a reversed-phase packing.

The effect of the trapping capillary on the peak shape is illustrated in Fig. 3, showing elution profiles for tolterodine and 5-HM. The peak shapes are significantly improved as a result of analyte focusing on the cyanopropyl capillary. Another advantage is that addition of TEA is no longer necessary for

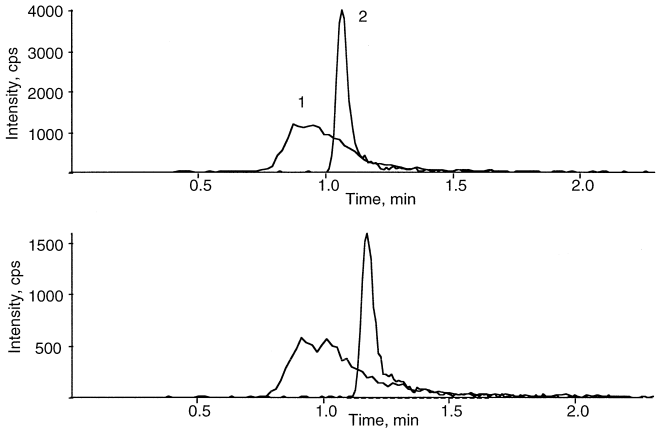

Fig. 3. Comparison of the elution profile of 5-HM (top ion trace) and tolterodine (bottom ion traces) without (1) and with (2) a cyanopropyl trapping capillary. Conditions: 1.0 ng/ml analyte concentration, 2.5 μl injection volume. For details on trapping column, see text.

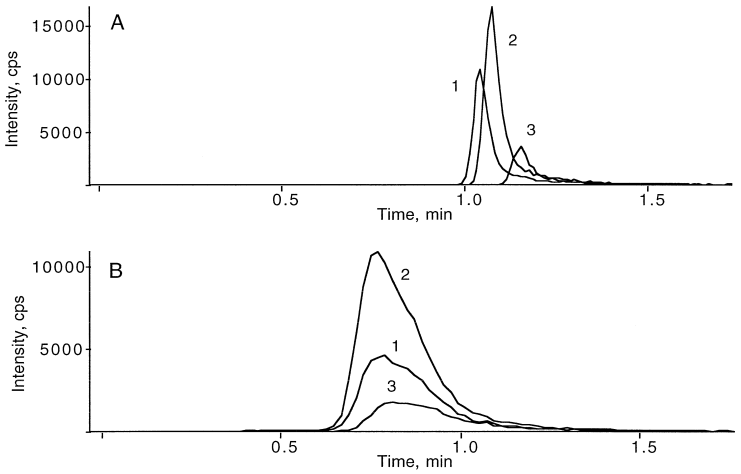

Fig. 4. Elution of the drug compounds with (A) and without (B) a focusing capillary. Sample: (1) 5-HM, (2) tolterodine acid, (3) tolterodine.

obtaining good peak shapes. In addition, the analytes are now slightly separated from each other as shown in Fig. 4.

3.3. SPE–MS–MS

Matrix interference, leading to suppressed ionization of the analytes, has been observed in LC–MS–MS studies with electrospray ionization [1–3]. Not only the detectability of the analytes is hampered if ion suppression is present, but it can also lead to poor accuracy and precision. Matuszewski et al. [2] has, among others, reported that separation (i.e., increased retention) and/or selective extraction of the analytes was needed in order to eliminate ionization suppression caused by the matrix. Ion suppression is, however, strongly dependent of the compounds ability to ionize in the matrix composition present at the time of ionization. For this reason, the influence of the matrix on the detection of the analytes may strongly vary between analytical methods, and this aspect of the present set-up was studied in more detail. Human plasma from four different individuals was thus ultrafiltered, spiked with the analytes to a concentration of 5 ng/ml and quantified with standards prepared in the buffer. These experiments were performed without the cyanopropyl focusing capillary on-line with the SPE column, i.e., without any separation. The accuracy and precision data obtained for these samples are listed in Table 1. The accuracy, calculated without internal standard, is within $100\pm10\%$ for all three analytes and different sources of plasma. This agrees well with the maximum bias in accuracy of 10%, that generally is accepted in bioanalytical studies. This also indicates that the matrix has no influence on analyte detection

Table 1
Accuracy[a] and precision[b] (in parentheses) data of the capillary SPE–MS–MS system for various plasma sources

Plasma	Without internal standard			With internal standard		
	5-HM	Tolterodine	Tolterodine acid	5-HM	Tolterodine	Tolterodine acid
I	98.8 (8.6)	105.3 (8.8)	94.9 (8.1)	101.6 (2.2)	108.1 (3.2)	97.6 (2.2)
II	100.1 (9.8)	101.6 (7.0)	97.6 (8.6)	101.3 (1.1)	102.9 (5.1)	99.0 (3.9)
III	106.4 (13.4)	99.3 (9.7)	101.3 (15.4)	97.8 (6.0)	91.1 (6.8)	93.7 (3.2)
IV	106.9 (7.9)	102.1 (7.7)	97.3 (6.9)	98.6 (2.1)	93.0 (5.1)	91.8 (4.2)

[a] Accuracy data is the percentage of measured concentration compared with the spiked concentration, 5 ng/ml plasma ultrafiltrate.
[b] Precision data is listed as relative standard deviation (%) in the measured concentration for at least four measurements.

for these analytes, by e.g., ionization suppression, under the present conditions. Hence, there appears to be no need for any separation of the analyte molecules from the matrix or for a more selective extraction of the compounds. It should be noted, however, that for quantitative studies the entire concentration range of interest should be tested for lack of suppression before an analytical step is removed that separates the analytes from the matrix before electrospray ionization and MS detection.

The use of deuterated 5-HM as internal standard substantially improved the precision of the measurements in this study. The precision of the measurements that were carried out with the focusing capillary was, however, also acceptable even without internal standard.

3.4. Free drug concentration analysis

The capillary SPE–MS–MS method was used to determine the free concentration of tolterodine and metabolites in human plasma. The results obtained with spiked plasma at two concentrations are listed in Table 2. The free fractions for tolterodine and 5-HM differ only slightly from the results obtained in a previous study [4]. In that study, free fractions of 5-HM and tolterodine of 59.5 and 10.0% respectively were found for plasma spiked with 33 ng/ml [4]. It should be noted that in both studies plasma from the same source had been used, the only difference being the time (i.e., several months) that it had been stored at −20°C. The inter- as well as the intra-assay variation of the free fraction determinations was acceptable and not different from previous results [4]. Both systems, i.e., with and without a focusing capillary, could be used for free drug determination in plasma and yielded similar results. However, the use of a focusing capillary is desirable, since both the detection sensitivity and the precision of the measurements were improved. Calibration curves were linear over the concentration range 0.05 to 1000 ng/ml with correlation coefficients typically between 0.995 and 0.9997. Limit of detection ($S/N=3$) was 50 pg/ml for tolterodine, which is similar as found previously [4] and is 10-times lower than the concentration that is required for clinical determination.

4. Conclusions

Fast elution is not only desired to reduce the analysis time, it also increases detectability of the analytes and facilitates quantification. Miniaturized capillary SPE–MS–MS proved to be a fast and quantitative method for bioanalytical analysis. Ultrafiltered plasma samples could be injected directly onto the SPE capillary for determination of free concentrations of tolterodine and two metabolites.

After elution from the packed SPE capillary, the analytes could be quantified without any separation due to the high selectivity of tandem MS detection. In addition, no interference of the matrix on detection of the drug compounds was observed, which resulted in the possibility to use aqueous calibration standards. The elution, optimized with respect to peak shape and analysis time, was performed with a solvent mixture containing methanol–10 m*M* formic acid and 1 m*M* TEA, pH 4.8 (70:30, v/v). With this solvent mixture reasonable analyte focusing was achieved on the SPE capillary. Additional analyte focusing resulting in sharper peaks and consequently

Table 2
Free concentrations of tolterodine, 5-HM and tolterodine acid measured in human plasma and inter- and intra-assay precision data

	Total concentration (ng/ml)	Free concentration (ng/ml)	Free fraction (%)	Inter-assay precision[a] (RSD, %)	Mean intra-assay precision (RSD)
Tolterodine	50	5.6	11.3	10.7	4.5
5-HM	50	26.3	52.6	3.0	5.9
Tolterodine acid	50	32.7	65.4	8.7	3.5
Tolterodine	5	0.62	12.4	7.5	3.6
5-HM	5	2.78	55.6	3.6	2.5
Tolterodine acid	5	3.43	68.6	4.5	2.9

[a] $n=3$.

increased detection sensitivity was attained by incorporating a 1 cm short cyanopropyl packed bed between the SPE capillary and the MS interface. With this system the limit of detection was 50 pg/ml for tolterodine injecting 10 μl ultrafiltrate.

The overall analytical system is simple and the analysis time is less then 6 min. Since equilibration and sample injection take an equal amount of time as elution and detection, the sample throughput could in principal easily be doubled by connecting a second SPE system to the mass spectrometer. Sample preparation, which is a single ultrafiltration step, can be performed simultaneously with multiple samples and does therefore not increase the analysis time.

Acknowledgements

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