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Column switching in capillary liquid chromatography–tandem mass spectrometry for the quantitation of pg/ml concentrations of the free basic drug tolterodine and its active 5-hydroxymethyl metabolite in microliter volumes of plasma

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Abstract

A capillary column switching system was developed for the determination of low, unbound concentrations of the basic drug tolterodine and its active 5-hydroxymethyl (5-HM) metabolite in human plasma. Free concentrations of tolterodine and 5-HM at p*M* and n*M* (pg/ml and ng/ml) levels were obtained by ultrafiltration of 40–400 μl plasma at 37°C. The free fraction (%) was independent of the plasma concentrations of the analytes. Detection of the analytes was performed by sheathless electrospray tandem mass spectrometry in the multiple-reaction monitoring mode. The selectivity of the mass spectrometric detection and the additional clean-up on the pre-column allowed direct injection of the ultrafiltrated plasma samples. Tolterodine and 5-HM were pre-concentrated on a reversed-phase capillary pre-column (1 cm×200 μm) and subsequently backflushed onto the separation column (25 cm×200 μm). The stability of the chromatographic system was good; a large number of ultrafiltrated plasma samples could be injected and the relative standard deviation of the retention times was typically ≤1% (within-day). The accuracy was between 86 and 105% and the precision was between 1 and 7% without the use of an internal standard. Linear calibration curves were obtained between 100 p*M* and 100 n*M*.

Keywords: Column switching; Ultrafiltration; Tolterodine

1. Introduction

It is well known that most drug molecules bind to plasma proteins like albumin and/or α_1-acid glycoprotein (AGP) in blood [1]. Analytical methods for determination of the free, non-protein bound fraction have been described for several drugs in plasma [2–7].

The rationale for this type of analysis is that the free concentration is often more relevant than the total, since it correlates better with the measured pharmacological effect [1].

This was also found to be true for tolterodine, a new muscarinic receptor antagonist for the treatment of incontinence, which binds strongly to AGP [8]. In addition an active metabolite is formed in most individuals which also binds to AGP, but to a lesser extent [8]. For tolterodine, as is also seen for other drugs bound to AGP, variation in the free concentration between individuals result from differences in plasma protein concentration. For these reasons an analytical procedure for the determination of the free concentration of tolterodine and the active

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5-hydroxymethyl (5-HM) metabolite, in plasma is highly demanded. Several aspects make this analysis a challenge to analytical chemists. Firstly, as a consequence of the low dosage in which tolterodine is administered and its high protein binding, free concentrations are very low (p*M*–n*M* range). Hence, a high detection sensitivity of the analytical method is required. Secondly a separation needs to be accomplished between the free and the protein-bound fraction of the drug in blood under equilibrium conditions.

Equilibrium dialysis [2,3], ultrafiltration [4–6] and frontal analysis [7] have mainly been applied. Whereas equilibrium dialysis is a rather slow technique, the sample preparation time per sample is strongly reduced by using ultrafiltration. In frontal analysis separation of the free and protein-bound fraction is achieved on a liquid chromatography (LC) column. The technique has the advantage of giving the free and total concentration simultaneously, but the applicability in bioanalysis is not as straightforward as e.g., with ultrafiltration. Finally, for a high sample throughput and applicability for both in vitro and in vivo studies the analytical method must be fast with small sample consumption. LC–tandem mass spectrometry (MS–MS) with electrospray ionisation (ESI) is well suited for sensitive quantification of medium polar, non-volatile drug compounds and has become a widely applied technique in recent years [4,9–14].

In combination with MS detection, capillary LC columns (I.D.$<$0.5 mm) offer some advantages over conventional columns: (i) the flow-rate which is in the range between 1–10 μl/min is highly compatible with ESI and (ii) since ESI-MS behaves as a concentration sensitive detector [15], the detection limit in terms of absolute amount is improved due to less dilution in the column. Thus if the available sample volume is limited, the use of capillary LC columns is a good choice. However, the sample volumes injected onto capillary LC columns are typically very small (60–200 nl) and restrict the concentration sensitivity. Pre-concentration performed either on-column or on a pre-column can be applied to improve the sensitivity considerably, provided that the sample is dissolved in a weaker solvent than the mobile phase.

Pre-concentration on a pre-column in a coupled column system has the additional possibility of performing on-line sample clean-up, which can significantly simplify the analytical method, thereby increasing the sample throughput.

Column-switching techniques for LC of drugs in biological samples are widely applied and have been reviewed by Campins-Falco et al. [16]. However only a few papers describe the use of capillary columns in LC switching systems for quantitative [10,17,18] or qualitative [19,20] analysis.

In this article we describe a capillary column switching system coupled to tandem mass spectrometry. This LC–MS–MS system in combination with ultrafiltration is used for quantification of low free concentrations of tolterodine and 5-HM in small plasma volumes. The performance of various ultrafiltration devices is compared and the whole method is validated in terms of accuracy, precision and linearity.

2. Experimental

2.1. Materials

Tolterodine (PNU-200583) and the 5-hydroxymethyl metabolite (5-HM, PNU-200577) (Fig. 1) were supplied by Pharmacia & Upjohn (Uppsala, Sweden). Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA).

Methanol and formic acid (98%) were purchased from Merck (Darmstadt, Germany) and tris(hydroxymethyl)aminomethane (Tris) was obtained from Bio-Rad Labs. (Richmond, CA, USA).

All fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA).

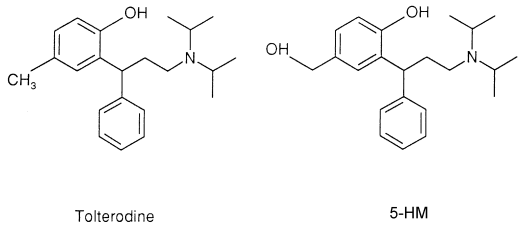

Fig. 1. Structural formulas of tolterodine and the active metabolite 5-HM.

2.2. Plasma samples

Human plasma was adjusted to pH 7.4 by purging CO_2 gas through and spiked with tolterodine and 5-HM to concentrations in the range between 100 p*M* and 100 n*M*. For each assay a new sample was prepared.

Prior to ultrafiltration the plasma samples were placed in a water bath at 37°C for at least 30 min to assure drug–protein binding. Portions of 40 to 400 μl of plasma were ultrafiltrated at 37°C in a pre-heated centrifuge for 10–20 min at 2000 *g*.

The concentrations of tolterodine and 5-HM in the plasma ultrafiltrate were quantified by comparing peak areas with calibration standards prepared in Tris buffer (pH 7.4, ionic strength, $I=0.15$ mol/l).

2.3. Ultrafiltration

A centrifuge (2K15, Sigma, Osterode am Harz, Germany) equipped with a 33° fixed angle rotor and a heating element was used for the ultrafiltration. Ultrafiltration devices were purchased from different suppliers. Centrifree MPS, Microcon 30 and Ultrafree 30.000 from Millipore, Nanosep 30K from Pall Gelman Sciences (Lund, Sweden) and Centrisart C4 from Sartorius (Goettingen, Germany).

Standard solutions for recovery measurements were prepared in Tris buffer, pH 7.4, $I=0.15$ mol/l. Ultrafiltration of the solutions was performed in two identical vials until the desired ultrafiltrate volume was obtained. The ultrafiltrates were pooled and analysed with the LC–MS–MS system. The recovery was calculated by comparing peak areas from the ultrafiltrate and standard solution.

2.4. Preparation of capillary columns

Fused-silica capillaries of approximately 20–25 cm×200 μm I.D. were packed with 5 μm Kromasil C_{18} particles (Eka Nobel, Bohus, Sweden) using a supercritical packing method as previously described [21]. An empty 50 μm I.D. capillary was connected to the column by a piece of PTFE tubing to keep the fiber glass frit at the end of the column.

Capillary pre-columns were packed in 200 μm I.D. fused-silica capillary with the same C_{18} particles. Before packing this 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. The 50 μm I.D. capillary retains the fiber glass frit and packing material and allows connection of both sides of the pre-column to the switching valve. The two pieces of capillary were fixated with epoxy glue and dried at 100°C for about 1 h. Then the 200 μm I.D. capillary was connected to a packing reservoir and slurry packed with acetonitrile as slurry and packing solvent. The pressure was maintained at 100 bar for 30 min. The pre-column was flushed with mobile phase for 30 min and then cut to the desired length. At this column end a very small section (≅0.2 mm) of the packing was removed by carefully heating the capillary. The empty part was filled with a fiber glass frit. A metal screen filter in the injector was sufficient to hold the packing inside the capillary. However, it was our experience that the column lifetime was improved by using a fiber glass frit. Capillary separation columns and pre-columns are commercially available.

2.5. Chromatography

Two LC pumps (PU-980, Jasco, Tokyo, Japan) were used to deliver the loading solvent for the pre-column and mobile phase for the separation column. A manual six-port injector with a 2.5- or 10-μl sample loop and an electrically actuated six-port switching valve were used, both from Valco (Schenkon, Switzerland). A schematic of the set-up is shown in Fig. 2.

The sample was loaded on the pre-column with formic acid buffer (pH 3.4)–methanol (90:10, v/v) at a flow-rate of 30 μl/min. The time between sample injection and backflushing was 2 min for a pre-column of 1.0 cm. This time was based on the breakthrough volume for 5-HM (the least retained compound) on the pre-column. These were determined either by changing the loading time and measuring the peak areas of the transferred analytes on the separation column, or by directly detecting the analytes in the effluent from the pre-column.

Separation of tolterodine and 5-HM was performed on a reversed-phase capillary column with a mobile phase consisting of 10 m*M* formic acid buffer (pH 3.4)–methanol (40:60, v/v).

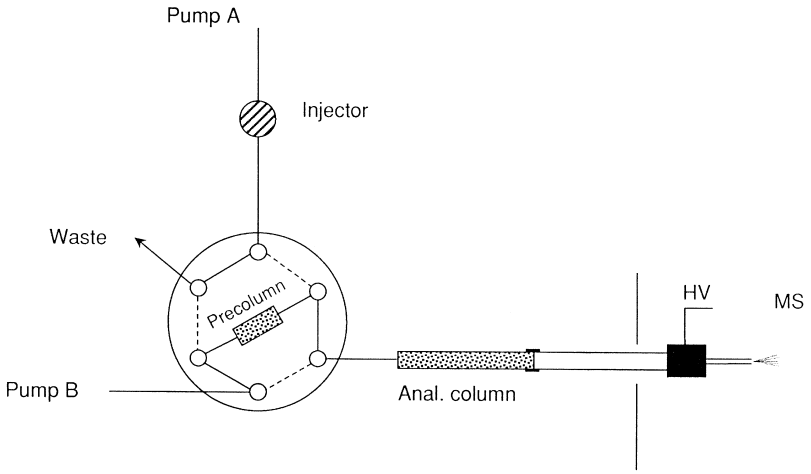

Fig. 2. Experimental set-up of the capillary switching LC–MS–MS system. Pre-column: 3–16 mm×200 μm I.D., packed with C_{18} particles. Separation column: 20 cm×200 μm. Capillary connecting column to interface: 20 cm×50 μm I.D. Other details as in Section 2.

2.6. Mass spectrometry

A PE-Sciex API 365 triple quadrupole mass-spectrometer (PE-Sciex, Concord, Canada) equipped with a sheathless MicroIon interface was used for detection of the analytes. This interface is composed of a low dead volume stainless steel union connecting the column and a spray capillary. A 50 μm I.D. capillary connected the packed capillary column to the union. To the other side of the union a 6 cm long fused-silica spray capillary with an I.D. of 15 μm was connected (see Fig. 2). The tip of this capillary was unmodified and positioned approximately 5 mm in front of the orifice.

The potential for the electrospray process was applied to the union, the conducting mobile phase supplied the electrical contact to the end of the spray capillary.

The MS signal was optimised during the continuous infusion of a solution of the analytes dissolved in the mobile phase. The signal stability was checked each day before chromatography was started. Adjustments of the electrospray potential or the position of the spray capillary were sometimes needed.

Once the signal was optimised, the infusion capillary was removed from the union and replaced by the transfer capillary (50 μm I.D.), which was butt connected to the separation column.

The mass spectrometer was operated in positive ion mode. The orifice potential was kept at 30 V and the electrospray voltage was around 3300 V. No nebulizer gas was used. The dwell time was set to 800 ms using multiple-reaction monitoring (MRM). The first quadrupole was set to transmit the molecular ions MH^+ at 342 m/z for 5-HM and at 326 m/z for tolterodine. These molecular ions were fragmented by collision-activated dissociation (CAD) with nitrogen as collision gas in the second quadrupole. The most abundant fragment ions, 147 m/z for tolterodine and 223 m/z for 5-HM, were selected for detection.

3. Results and discussion

3.1. Ultrafiltration

In ultrafiltration, adsorption of the analytes to the ultrafiltration vial can be a problem and is sometimes stated as a drawback of the technique [7]. Both the membrane and the polymeric materials used in these devices can cause adsorption of the analytes. Ultrafiltration devices are available in a wide variety of different sample sizes, types of membrane and applied materials. These characteristics are likely to have a strong influence on the solute adsorption, especially when analysing trace levels of hydrophobic compounds. Therefore five different ultrafiltration devices were studied for recovery of the compounds. All vials had membranes with a mass

cut-off value of M_r 30 000 except Centrisart C4 which had a cut-off value of M_r 20 000. To measure possible adsorption losses of the compounds during ultrafiltration, a standard solution containing 20 n*M* tolterodine and 5-HM in buffer (pH 7.4, I=0.15 mol/l) was ultrafiltrated. The recoveries for the devices are listed in Table 1.

Tolterodine and 5-HM are moderate hydrophobic compounds that bear a positive charge at pH 7.4. As a result, both hydrophobic and electrostatic interactions with the membrane and/or device could contribute to adsorption. As can be seen the recovery is consistently higher for 5-HM compared to tolterodine. This could be an indication that hydrophobic interactions contribute to adsorption, since tolterodine is more hydrophobic than 5-HM. The low recoveries obtained for the cellulose triacetate and polyethersulphone membranes are possibly the result of electrostatic interactions.

The Ultrafree 30.000 vial, which was selected for further measurements, was subjected to a more detailed study of the recovery. In addition to buffer, also ultrafiltrated plasma was used as matrix. Plasma was ultrafiltrated, the ultrafiltrate spiked and ultrafiltrated once again. These experiments, which were performed at two concentrations, are expected to approach the ultrafiltration of real plasma samples better. The results are listed in Table 2.

Very reasonable recoveries of respectively 90 and 104% for tolterodine and 5-HM were still obtained at a concentration of approximately 2 n*M*.

While adsorption losses are virtually absent for 5-HM, some adsorption of tolterodine to the ultrafiltration device occurs. These results were considered satisfactory for free concentration measurements.

3.2. On-line sample clean-up and chromatography

One objective when developing the method was to minimize manual sample work-up. Direct injection of the ultrafiltrate onto the chromatographic system was therefore highly desired. However, direct injection of the required volume (a few microliter) of ultrafiltrated plasma onto the capillary separation column changed the retention of the analytes and was therefore not feasible. Apparently sample constituents adsorbed onto the column packing. This is not surprising since the sample is still relatively dirty

Table 1
Recovery of 5-HM and tolterodine for various ultrafiltration vials

Device	Materials (sample reservoir/ support base/collection cup)	Membrane	Recovery (%)	
			5-HM	Tolterodine
Centrifree MPS	Styrene acrylonitrile/ polycarbonate/ polyethylene	Regenerated cellulose	75	54
Microcon 30	Polyethylene/ polycarbonate/ acetal	Regenerated cellulose	83	82
Ultrafree 30.000	Polyethylene/ polyethylene/ polyethylene	Regenerated cellulose	94	78
Nanosep 30	Polypropylene/ polypropylene/ polypropylene	Polyethersulphone	35	13
Centrisart C4	Polypropylene/ polypropylene/ polypropylene	Cellulose triacetate	52	28

Analyte concentrations were 20 n*M* for 5-HM and tolterodine. Measurements were performed in two vials.

Table 2
Recovery (%) of 5-HM and tolterodine for Ultrafree 30.000 ultrafiltration vials

Matrix	Concentration (n*M*)		Recovery (%)	
	5-HM	Tolterodine	5-HM	Tolterodine
Buffer	20	20	94	78
Ultrafiltrated plasma	20	20	102	86
Buffer	2	2	99	89
Ultrafiltrated plasma	2	2	104	90

Recoveries are measured for at least two ultrafiltration vials.

after ultrafiltration, containing plasma constituents up to a molecular mass of approximately 30 000. Only after flushing the column for about 1 h with mobile phase could the original performance be restored. In the light of the above a capillary column switching system was developed with the purpose of on-line sample clean-up on a pre-column. In such a system the analytes are retained on the pre-column while polar compounds and salts are washed off. After the on-line clean-up the retained analytes are back-flushed from the pre-column with a stronger eluting mobile phase to reduce peak broadening. An additional advantage of the coupled column system is that sample injection can be performed faster, since the flow-rate through the pre-column is much higher than through the separation column; in the present study respectively, around 30 and 1 μl/min.

In order to perform the on-line sample clean-up most effectively, i.e., washing off as much of the sample matrix compounds as possible, the breakthrough volume for 5-HM was measured with a 2.5-μl sample loop. 5-HM is the least retained compound and backflushing must be performed before 5-HM starts to elute from the pre-column. With 10% (v/v) methanol in the loading solvent the breakthrough volume was 70 μl per cm pre-column. With this mixture breakthrough volumes for 5-HM were measured on three pre-columns with lengths between 3.5 and 16 mm to study the reproducibility. The relative standard deviation of the breakthrough volume was 3.4%. This indicated that the preparation of the pre-columns was reproducible.

To study the suitability of the capillary pre-columns in the column switching system, retention of the analytes was examined by injecting standard solutions and spiked ultrafiltrated plasma. Retention of the analytes in the coupled capillary LC system was very stable and no difference was found in retention times measured for ultrafiltrate samples or standards. Another favourable aspect was the small variation in retention times; the relative standard deviation in retention times was typically $\leq$1% (within-day). Although this might be of less importance in this study since mass spectrometric detection was used, it clearly demonstrates the effectiveness of the on-line clean-up of the ultrafiltrated plasma sample on the pre-column.

An illustrative separation of the two drug compounds on the capillary LC switching system is shown in Fig. 3. Ten μl of spiked ultrafiltrated plasma was injected onto the pre-column. Plate numbers were typically around 15 000 for both compounds and capacity factors of 5-HM and tolterodine were around 0.3 and 1.3, respectively. The mobile phase was chosen such that 5-HM eluted well after the aqueous plug from the void volume of the

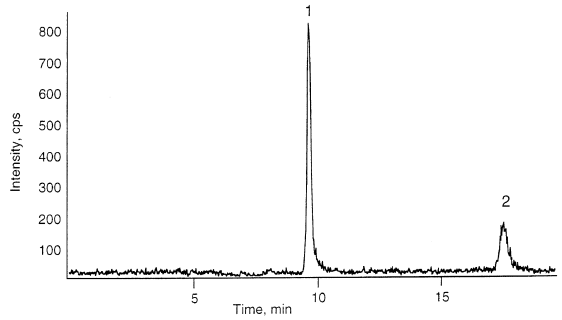

Fig. 3. Separation of tolterodine and 5-HM on the capillary column switching system. Sample concentration: 100 p*M* tolterodine and 5-HM in ultrafiltrated plasma. The injection volume was 10 μl. Mobile phase for the pre-column: 10 m*M* formic acid buffer (pH 3.4)–methanol (90:10, v/v), for the separation column: buffer–methanol (60:40, v/v). Detection: MRM 342→223 *m/z* for 5-HM and 326→147 *m/z* for tolterodine.

pre-column and the retention time of tolterodine was still reasonable.

Elution of 5-HM after the aqueous plug (buffer–methanol, 90:10, v/v) is important since the electrospray process and consequently the detection sensitivity can be affected by the solvent composition. A disturbance of the liquid electrospray spray cone at the end of the spray capillary could be observed with a stereoscope during the elution of this plug. This disturbance is the result of a temporarily changed effective electrospray voltage, as studied in more detail by Vanhoutte et al. [22]. Since the electrical contact at the tip of the spray capillary is established through the capillary, a change in liquid composition results in a change in electrical conductance and thus in the effective electrospray voltage. With the presently selected mobile phase the electrospray cone was re-established before elution of 5-HM and quantification could be performed with great precision as further discussed below.

3.3. Mass spectrometric detection

The required sensitivity to detect p*M* concentrations excluded UV detection as an option. Furthermore, UV detection at 210 nm, the absorption maximum for tolterodine, is not selective. Tandem mass spectrometry is the most selective detector available for LC and is very sensitive as well. This detector in combination with the on-line clean-up allowed for direct injection of the ultrafiltrated plasma samples.

The stability of the MS signal with the MicroIon interface was measured during infusion-MS and was satisfactory. However, occasionally the spray capillary got blocked during LC–MS–MS measurements, despite the use of several 0.2-μm filters in the injector and switching valve.

Detection was performed in MRM mode with CAD of the analytes in the second quadrupole with nitrogen gas. The most intense fragments, 147 m/z for tolterodine and 223 m/z for 5-HM, were selected for detection. These fragments are not obvious from the structures of the mother ions (see Fig. 1). To elucidate the structure of these fragments, deuterium exchange experiments and fragmentation of deuterated compounds were performed. Spectra from these experiments (not shown) showed that the 223 m/z fragment of 5-HM is most likely formed by cleavage of the amine group and a loss of water. For tolterodine the 147 m/z fragment is most probably formed by cleavage of the amine group, the unsubstituted phenyl ring and loss of the proton from the hydroxyl group.

The influence of the nebulizer gas on the stability and signal intensity was examined during continuous infusion-MS. Stable signals were obtained at no or a low nebulizer gas flow (450 ml/min). Although the absolute signal intensity was higher with the use of nebulizer gas, the signal-to-noise ratios were similar. Therefore all measurements were conducted without nebulizer gas.

The performance of the capillary LC switching system, combined with tandem mass spectrometry, was validated by analysing spiked ultrafiltrated plasma over the course of three consecutive days. Precision and accuracy were measured for tolterodine and 5-HM at two concentrations and are given in Table 3. The accuracy was between 94 and 98% and no significant differences were observed between the two analytes or between the two concentrations. Since the ultrafiltrated plasma samples were quantified by comparing peak areas with standard samples prepared in buffer, it can be concluded that ion suppression by matrix effects were virtually absent. Ion suppression caused by the sample matrix has been reported [10,11,14] and can result in significantly lower sensitivity. This problem can be severe when the capacity factor is small, Zell et al. [10] reported a reduction of the sensitivity by a factor 2 for plasma samples cleaned by liquid–liquid extraction. However, the present results show not only that the sensitivity is not impaired by the matrix, but also that the use of calibration standards prepared in buffer is justified.

3.4. Free concentration analysis

The analytical method of ultrafiltration and LC–MS–MS quantification was used to determine free concentrations of tolterodine and 5-HM in human plasma. Aliquots of plasma, spiked with 5-HM and tolterodine to total concentrations between 1 and 100 n*M*, were ultrafiltrated and the ultrafiltrates were injected onto the coupled capillary LC system.

Calibration curves, which were measured with

Table 3
Precision and accuracy of the capillary LC–MS–MS system

Day	Spiked concentration (n*M*)	Found concentration (±S.D.)	R.S.D. (%) (*n*=5)	Accuracy (%)
5-HM				
1	50	46.81±1.81	3.9	93.6
2	50	47.11±3.30	7.0	94.2
3	50	48.71±0.82	1.7	97.3
Tolterodine				
1	50	47.51±2.48	5.2	95.0
2	50	47.70±1.22	2.6	95.4
3	50	47.79±1.44	3.0	95.5
5-HM				
1	5	4.88±0.15	2.7	97.6
2	5	4.91±0.15	3.2	98.1
3	5	4.91±0.23	4.8	98.3
Tolterodine				
1	5	4.84±0.21	4.5	97.0
2	5	4.72±0.12	2.5	94.5
3	5	4.75±0.25	5.1	95.1

Spiked ultrafiltrated plasma samples were quantified by comparing peak areas with standard samples prepared in buffer.

standards prepared in buffer, showed good linearity over the concentration range 100 p*M*–100 n*M*; correlation coefficients were typically between 0.994 and 0.9997 and *y*-residual plots versus concentration showed no structure.

The results of the free concentration measurements are given in Table 4. The listed free concentrations are mean values of several assays, each for which a new sample was prepared. The inter-assay precision for the ultrafiltrated plasma samples was between 1 and 11%, which is comparable with data obtained in other ultrafiltration studies [5,6].

Despite the inter-assay variation in the free concentration, the free fractions of tolterodine and 5-HM were consistent over the concentration range: approximately 11 and 60%, respectively.

The accuracy and precision was measured with spiked ultrafiltrated plasma at 5 n*M* using a 2.5-μl loop and at 1 n*M* using a 10-μl loop. The precision for these samples was between 0.6 and 6.3%, which

Table 4
Free concentrations of tolterodine and 5-HM measured in human plasma and inter- and intra-assay precision data

Total concentration (n*M*)	Free concentration (n*M*)	Free fraction (%)	Inter-assay precision (R.S.D., %)	Mean intra-assay precision (R.S.D., %)
Tolterodine				
100	10	10.0	7.1 (*n*=5)	5.9
10	1.29	12.9	1.4 (*n*=3)	–
1	0.098	9.8	– (*n*=2)	7.3
5-HM				
100	59.5	59.5	11.0 (*n*=5)	5.9
10	6.14	61.4	2.5 (*n*=3)	–
1	0.602	60.2	– (*n*=2)	6.4

is similar as the data presented in Tables 3 and 4. As a result of the stability of the LC–MS system, all measurements could be performed without the use of an internal standard. The accuracy was between 86 and 106%.

For ex vivo animal free drug concentration measurements the volume of the plasma sample can be an important aspect. Preferably small volumes of plasma, <100 μl, are taken from the animal for analysis. For this reason it is interesting to compare free concentrations measured for different starting volumes of plasma. A spiked plasma sample containing 100 n*M* of 5-HM and tolterodine was ultrafiltrated in two 50- and two 400-μl portions. Ultrafiltration of 50 μl plasma for 10 min generated around 20 μl ultrafiltrate, for 400 μl around 100 μl was collected in 20 min. The ultrafiltrates were pooled before analysis. No difference in free concentration for 5-HM and tolterodine between 50- and 400-μl plasma samples was measured. A similar experiment at low concentration showed that a volume of 40 μl plasma was sufficient for measuring a free concentration of 0.6 n*M* for 5-HM. These results indicate that this method may well be applied in animal studies.

The equilibration time for the sample between spiking and ultrafiltration was typically 30 min. To check whether this was long enough for the establishment of the drug–protein binding a comparison was made with an equilibration time of 2 h. These measurements resulted in similar free concentrations for 5-HM and tolterodine. Thus it can be concluded that the protein binding is established within 30 min for these compounds.

4. Conclusions

The combination of ultrafiltration with the capillary LC switching system coupled to tandem mass spectrometric detection is a fast and sensitive method for the determination of free concentrations tolterodine and 5-HM in plasma. Only small volumes of plasma, around 40 μl, are needed to measure free concentrations. This indicates that the method can well be applied for both in vitro and in vivo animal studies. The required sensitivity, down to around 100 p*M*, is obtained by injection of 10 μl ultrafiltrate directly on a capillary pre-column. The pre-column provides effective on-line clean-up of the sample and reduces the sample preparation to a single step. This aspect is of great importance if large numbers of samples need to be analysed. Moreover the capillary pre-columns are very stable; more than 100 ultrafiltrate injections could be performed without any loss of performance.

The described method should also be applicable for other basic drugs after minor changes. Presently we are working on the on-line coupling of microdialysis and the capillary LC switching system as described in this paper.

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