Analysis of beta-blocking drugs in biological fluids using on-line and off-line sample preparation methods and Agilent 1100 Series LC systems

Application

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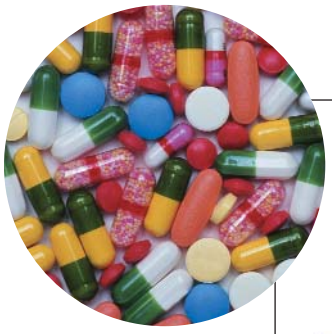

This application has been verified using an Agilent 1200 Series LC system, and showed comparable or even better performance.

Application Area
Sample preparation of drugs and their metabolites in biological fluids.

Introduction

Beta-blocking drugs ("beta-blockers") are used in the treatment of hypertension and glaucoma. Measuring the concentrations of these drugs in biological fluids is important during drug development and clinical tests as well as for monitoring purposes during medication of patients.[1,2,3] Sample preparation is still one of the bottlenecks in the analysis of drugs in physiological samples, as it is a labor-intense and time-consuming process, especially for plasma, urine and tissue samples. The goal of the following experiments was to find a sensitive and simple on-line or off-line sample preparation method for pindolol, timolol, metoprolol and propranolol as beta-blocking drugs. Different methods were tested, for example, using special cleaning columns (off-line and on-line), off-line fraction collection and standard procedures such as protein precipitation and liquid/liquid extraction. The focus was on evaluating the limits of detection in biological fluids rather than determining sample recoveries in detail.

Agilent Technologies

Equipment

The standard Agilent 1100 Series LC system was used in combination with UV, fluorescence and mass spectrometry detection. The modular system consisted of:

- Agilent 1100 Series binary pump for precise flow rates and micro degasser with an internal volume of 1 mL
- Agilent 1100 Series well-plate autosampler for precise injection in the µL range with cooling option for the samples
- Agilent 1100 Series column thermostat for precise retention times at room temperature, and above or below room temperature
- Agilent 1100 Series diode-array detector for highly precise quantitation and identification via UV
- Agilent LC/MSD quadrupole mass spectrometer with ESI source for identification and quantitation via MS
- Agilent 1100 Series fluorescence detector for multi signal and sensitive analysis of all compounds showing fluorescence activities.

The Agilent 1100 capillary LC system was used for experiments using capillary columns with an internal diameter of 0.3 mm. This modular system consisted of:

- Agilent 1100 Series capillary pump with electronic flow control for precise flow rates even with changing back-pressure, and a micro degasser with an internal volume of 1 mL
- Agilent 1100 Series micro well-plate autosampler for precise injection in the nL and µL range with cooling option for the samples
- Agilent 1100 Series column thermostat for precise retention times at room temperature, and above or below room temperature
- Agilent 1100 Series diode-array detector equipped with a 500-nL cell for sensitive analysis of small peak volumes
- Agilent XCT Ion Trap mass spectrometer with ESI source with micro-spray nebulizer for sensitive identification and structural analysis.

The Agilent 1100 Series fraction collection system was used for sample clean-up of biological fluids. This modular system consisted of:

- Agilent 1100 Series quaternary pump with seal wash option for precise flow rates and micro degasser with an internal volume of 1 mL
- Agilent 1100 Series well-plate sampler for precise injection in the µL range with cooling option for the samples
- Agilent 1100 Series diode-array detector for monitoring
- Agilent 1100 Series fraction collector for collecting fractions eluting from gel filtration or Multiple Affinity Removal System (MARS) column, performed in time-based mode.

Analytical columns

The following capillary and narrow-bore columns were used:

- 100 x 0.3-mm ZORBAX SB C-8, 3.5-µm capillary column
- 75 x 0.3-mm ZORBAX SB C-8, 3.5-µm capillary column
- 50 x 0.3-mm ZORBAX SB C-8, 3.5-µm capillary column
- 30 x 2.1-mm ZORBAX SB C-8, 3.5-µm narrow-bore column

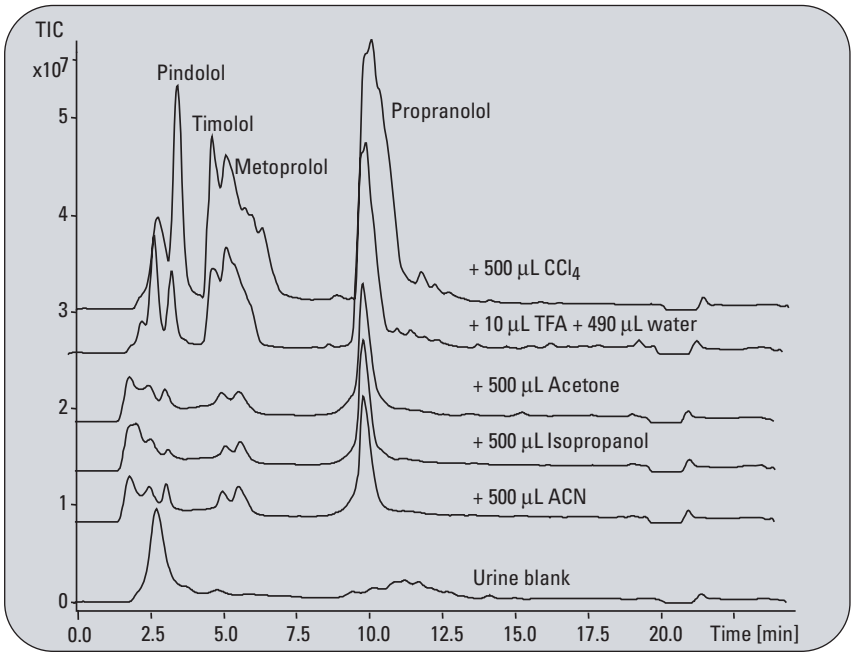

Figure 1
Evaluation of different protein precipitation methods using the Agilent 1100 Series MSD Ion Trap as detection system and capillary.

Sample preparation columns

The following columns were used:

- A standard bore 30 x 4.6 mm ZORBAX XDB C-18, 5-µm was used as cleaning column
- A gel filtration column 100 x 2.1 mm Poly LC INC Poly-hydro-xyethyl A, 3 µm, 500Å
- A Multiple Affinity Removal System (MARS) column, 4.6 x 50 mm, Hv-6

Samples of the beta-blocking drugs pindolol, timolol, metoprolol and propranolol were purchased from Sigma.

Results and discussion

Off-line sample preparation methods for LC/MS analysis

The combination of capillary LC and mass spectrometer detectors has proven to be an ideal solution for the sensitive analysis of beta-blocking drugs.[4] Using this combination the limit of detection for standards is in the low pg range. Experiments were carried out to find a sample preparation procedure which is compatible with MS detection. The following sample preparation procedures were tested.

- Protein precipitation for urine, plasma and tissue
- Liquid/liquid extractions
- Gel filtration offline with fraction collection for urine and plasma
- SPE extraction using a column for multiple affinity removal of high abundant proteins with fraction collection for human plasma

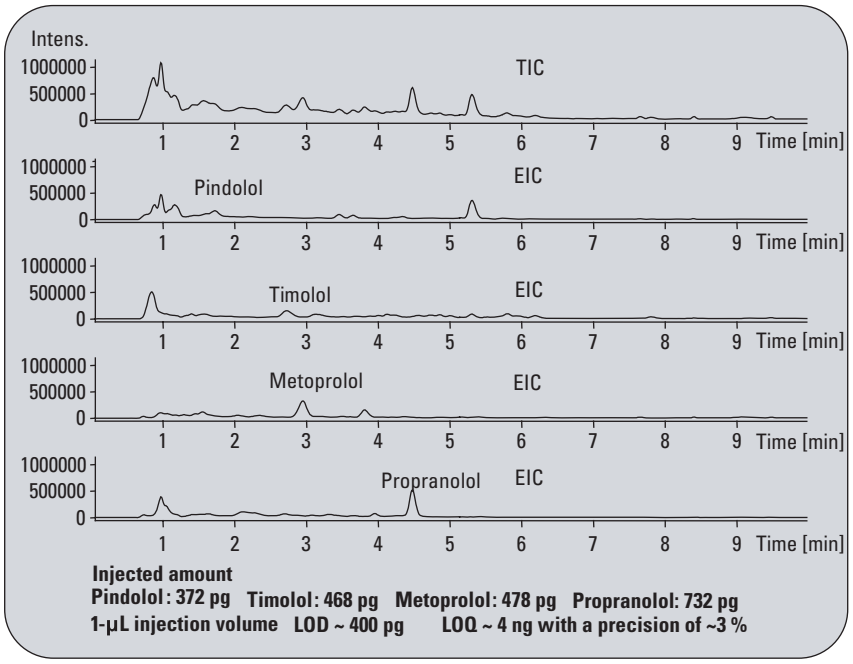

Figure 2
Chromatograms showing the LOD of beta-blockers in a spiked urine sample analyzed on a Agilent 1100 Series capillary LC/MSD system using TFA as a protein precipitation reagent. No sample concentration step was included.

Sample preparation
1mL spiked urine was mixed with 1-µL TFA, centrifuged at 12000 g for 15 min. and 4 °C. From the supernatant liquid 1-µL was injected without further concentration step.

Chromatographic conditions

Column:	50 x 0.3 mm ZORBAX SB C-8
Mobile phase:	water+ 0.05 % FA, acetonirile + 0.045 % FA
Flow rate:	0.01 mL/min Gradient: at 0 min 10 % ACN, at 10 min 90 % ACN with fast reconditioning
Injection volume:	1 µL with needle wash in flush port

MS conditions

Source:	ESI
Peak width:	0.1 min
Time filter:	On
SIM mode:	SIM ions 249, 260, 268,317, Fragmentor 100, Gain 10, Actual dwell 144
Gas temperature:	350 ºC
Drying gas:	5 L/min
Nebulizer pressure:	15 psig
Vcap:	4000 V positive
Scan:	120-450
Fragmentor:	60

Fast sample preparation for urine, plasma and tissue using protein precipitation and liquid/liquid extraction

The first series of experiments was mainly based on protein precipitation, which is the most popular sample clean-up procedure for biological samples prior to drug analysis. Different reagents for protein precipitation were tested (figure 1). Precipitation with either carbon tetrachloride or tri-

fluoroacetic acid produced the best results with respect to sample recovery. When using acetonitrile, the chromatogram had almost no co-eluting peaks, but sample recovery was lower. Because the MSD has the specificity to detect the beta-blockers in the presence of residual amounts of coeluting proteins, protein precipitation is typically sufficient for safe identification and acceptable quantitation in the low ng range. The limit of detection (LOD) for the spiked urine was about 400 pg on-column (figure 2). The method is well suited for fast identification of beta-blocking drugs in urine with acceptable sample recovery (see also table 3, page 10).

The LOD is in the low pg range for standards and between 200 and 400 pg for a spiked urine sample, when using the MSD.

- An advantage of this method is its speed and simplicity. It can be used for different matrices. Limits of detection (LOD) in the low to mid-picogram range can be achieved. Recovery rates for urine are between 70 and 90 %.
- Good quantitative date can be obtained in the low nanogram range with RSD for areas between 3 and 5 %.
- A disadvantage is that for tissue and plasma, sample recovery is below 50 %.

In a second series of experiments for urine and tissue samples liquid/liquid extraction steps in combination with protein precipitation were used. The biological matrix, 500 µL of either urine or S9 liver homogenizate, was spiked with beta-blockers in the low ng range. The spiked sample was

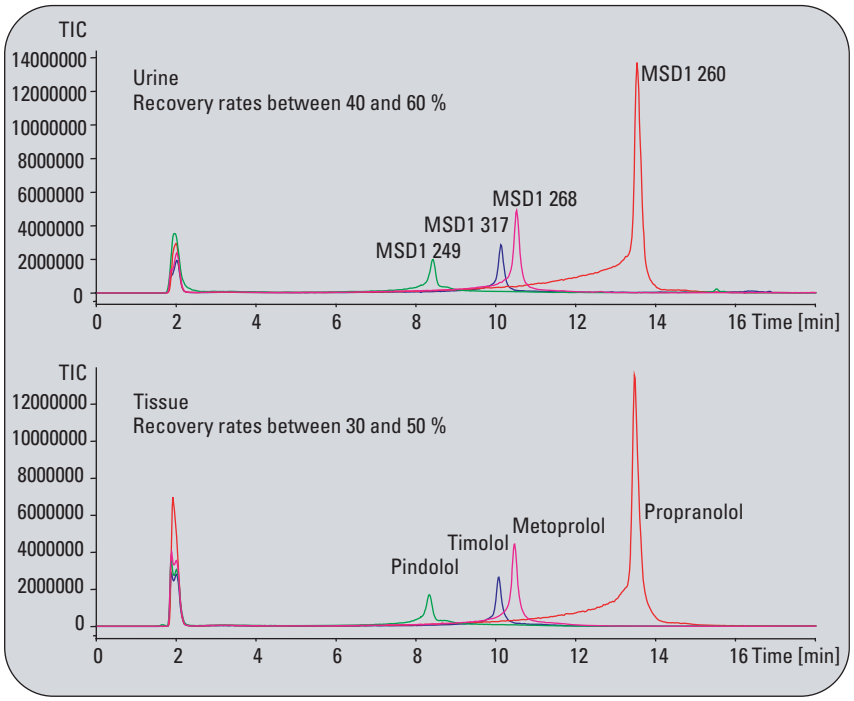

Figure 3
Acetonitrile fraction from ACN/sulfate sample preparation procedure.

Chromatographic conditions
1 µL was injected into the 1100 Series capillary LC/MS system.

Chromatographic conditions for the analytical system

Column:	0.3 x 100 mm ZORBAX SB C-8, 3.5 µm, 300 Å
Flow rate:	5 µL/min
Mobile phases:	Water + 0.1 %formic acid (A), acetonitrile + 1 % formic acid (B)
Gradient:	at 0 min 2 % B
	at 1 min 2 % B
	at 11 min 30 % B
	at 15 min 60 % B
	at 17 min 95 % B
	at 20 min 95 % B
Post time:	15 min

MSD conditions (figure 2)

Scan:	200-400
Peak width:	step size 0.25 Da

then mixed with the following reagents:

- 500 µL methanol
- 500 µL acetonitrile
- 500 µL acetonitrile in combination with ultrasonic bath extraction
- Addition of sodium sulfate (1 M solid) and 500 µL acetonitrile for extraction
- 500 µL ethanol/ethyl acetate (1:2),
- 500 µL 30 % HCl with citrate (1 M)
- Hexane/TFA (450:50 µL)
- Lyophilization using SpeedVac and extraction with 500 µL methanol in the ultra sonic bath, afterwards addition of 500 µL acetone for desalting

Samples were thoroughly mixed with vortex mixer. Incubation time was 15 minutes and then each phase, both organic and water, of

each sample was centrifuged for 15 minutes with 12000 g at 4 °C.

Acetonitrile/sodium sulfate was an efficient reagent for all 4 beta-blockers, yielding sample recoveries for urine between 40 and 60 % and recoveries for tissue between 30 and 50 % (figure 3). Using the hexane/TFA procedure, the recovery rates in the water phase (figure 4) were about 100 % except for pindolol, which cannot be found in acceptable recovery rates. The extracts from this sample preparation procedure are very clean and limits of detection in the low pg range are possible. In tables 1 and 2 results of the sample preparation procedures are summarized.

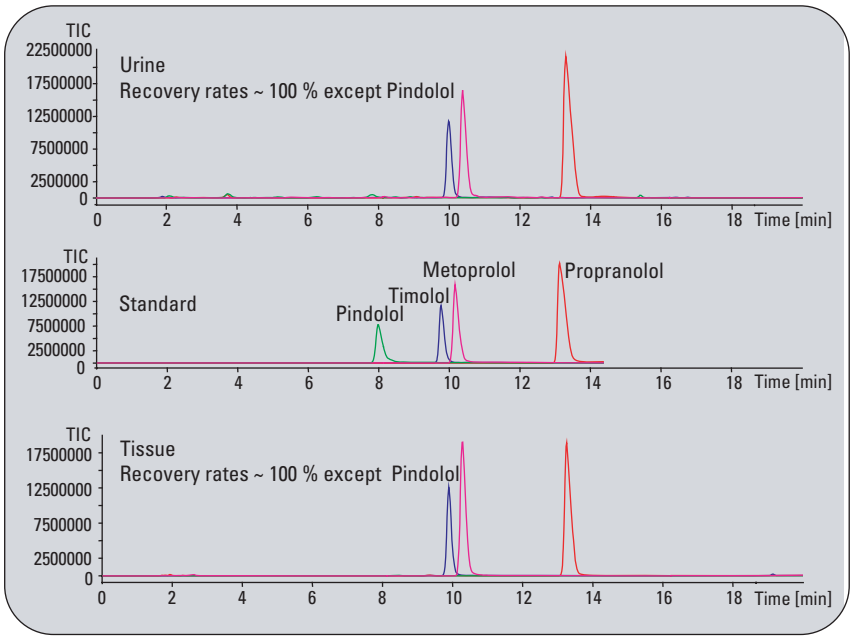

Figure 4
Water fraction from hexane/TFA sample preparation procedure

Compounds → Precipitation and extraction reagent	Pindolol 37.2 ng/µL Recovery rate (%)	Timolol 46.8 ng/µL Recovery rate (%)	Metoprolol 47.8 ng/µL Recovery rate (%)	Propranolol 73.2 ng/µL Recovery rate (%)
Acetonitrile	40	57	61	53
Acetonitrile/sulphate ACN Fraction	**28**	**36**	**48**	**73**
Acetonitrile/sulphate water fraction	18	49	25	7
ACN ultra sonic bath	34	50	54	56
Ethanol/ethyl acetate, ethyl acetate fraction	4	4	4	58
Ethanol/Ethyl acetate, water fraction	35	39	39	62
Hexane/TFA, hexane fraction	0.3	0.2	0.3	1.3
Hexane/TFA, water fraction	**1**	**100**	**116**	**77**
HCL, citrate	0.8	33	35	29
Lyophylisation/ methanol	5	8	7	17
Methanol	1.3	120	19	109

Table 1
Recovery rates for tissue sample preparation procedures

Compounds → Precipitation and extraction reagent	Pindolol 37.2 ng/µL Recovery rate (%)	Timolol 46.8 ng/µL Recovery rate (%)	Metoprolol 47.8 ng/µL Recovery rate (%)	Propranolol 73.2 ng/µL Recovery rate (%)
Acetonitrile	17	31	36	52
Acetonitrile/sulphate ACN fraction	**38**	**37**	**42**	**62**
Acetonitrile/sulphate water fraction	10	24	15	5
ACN ultra sonic bath	29	10	34	63
Ethanol/Ethyl acetate, ethyl acetate fraction	6	6	6	57
Ethanol/Ethyl acetate, water fraction	17	23	24	31
Hexane/TFA, hexane fraction	0.3	0.2	0.3	1
Hexane/TFA, water fraction	**7**	**102**	**102**	**104**
HCL, citrate	3	21	23	11
Lyophylisation/ methanol	19	30	32	60
Methanol	-	-	-	109

Table 2
Recovery rates for urine sample preparation

- The advantage of the two methods presented is speed and simplicity. They can be used for different matrices. Limits of detection (LOD) in the low to mid-picogram range can be achieved.
- A disadvantage is that for tissue, sample recovery is below 50 % for the ACN/sulfate procedure.
- A disadvantage is that with the hexane/TFA procedure pindolol shows very low recovery rates.

Sample preparation for urine and plasma using off-line gel filtration

The spiked matrix was injected onto a 200 x 2.1 mm polyhydroxyethyl gel filtration column. Based on time, one fraction containing the drugs of interest was collected using a fraction collector. The fractions were concentrated and injected onto an 1100 Series capillary LC/MS Ion Trap system. Plasma and urine matrices were tested (figure 5).

- An advantage of this sample preparation method is that it can be used for different matrices such as plasma, urine and tissue.
- A disadvantage of this method is that only small sample volumes can be injected into the gel filtration system. Fractions from several runs must be pooled, concentrated, and then analyzed by LC/MS to achieve the desired LOD.
- Another disadvantage is that salts and other small molecules might cause interference problems.

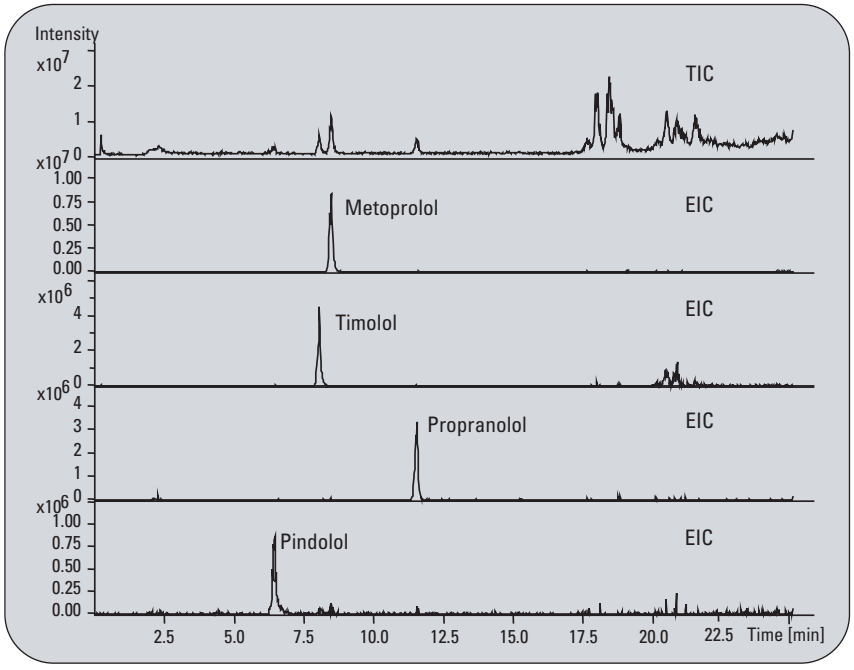

Figure 5
LC/MS analysis after gel filtration sample preparation procedure for plasma.

Sample preparation procedure
1 µL of sample, containing 37.3 ng/µL pindolol, 46.8 ng/µL timolol, 47.8 ng/µL metoprolol and 73.2 ng/µL propranolol, were injected on the Poly LC INC Polyhydroxyethyl A 200 x 2.1, 3 µm, 500 Å column. The compounds were eluted using water (70 %) and acetonitrile (30 %), with 0.1 % formic acid as mobile phase. The flow rate was set to 30 µL/min. A fraction was collected between 13 to 15 min. Fractions from five injections were pooled and resulted in a liquid volume of 300 µL. The pooled sample was concentrated to 25 µL using a speed vac. 1 µL was injected into the Agilent 1100 Series capillary LC/MS system.

Chromatographic conditions for the analytical system

Column:	0.3 x 75mm ZORBAX SB C-8, 3.5 µm, 300 Å
Flow rate:	5 µL/min
Mobile phases:	water +0.1 %formic acid (A), acetonitrile + 1 % formic acid (B)
Gradient:	at 0 min 5 % B
	at 20 min 60 % B
	at 22 min 95 % B
	at 25 min 95 % B
Post time :	15 min

MSD Ion Trap conditions

Scan:	200-400
Mass accumulation time:	50 ms
ICC:	50000
Standard mass resolution	
Width:	4Da

Sample preparation for human plasma using the MARS column

This column allows specific removal of the most abundant proteins from human serum: albumin, IgG, IgA, haptoglobin, transferrin and anti-trypsin.[6,8] Using this column and the Agilent 1100 Series fraction collection system, automated sample preparation of drugs in human serum is possible. A sample volume of 150 µL was injected and time-based fraction collection was performed. A portion of this sample was analyzed by LC/MS

without pre-concentration (figure 6) yielding excellent detection of the analytes at the level of 400-700 pg/µL of the original plasma. Although 300 µL plasma were cleaned with the multiple affinity removal system, only 1 µL was injected onto the LC/MS system. Here the LOD was in the low picogram range.

- The advantage of this method is that very low LODs may be achieved without extensive sample clean-up. If further concentration steps are used, even lower LODs may be achieved.
- A disadvantage is that this system is designed for one matrix only – human plasma.
- Another disadvantage is that the collected fractions contain highly concentrated buffers which might cause problems in the MS system, especially if further concentration steps are used.
- Low-abundance proteins are still present and might cause interference problems at very low analyte concentrations.

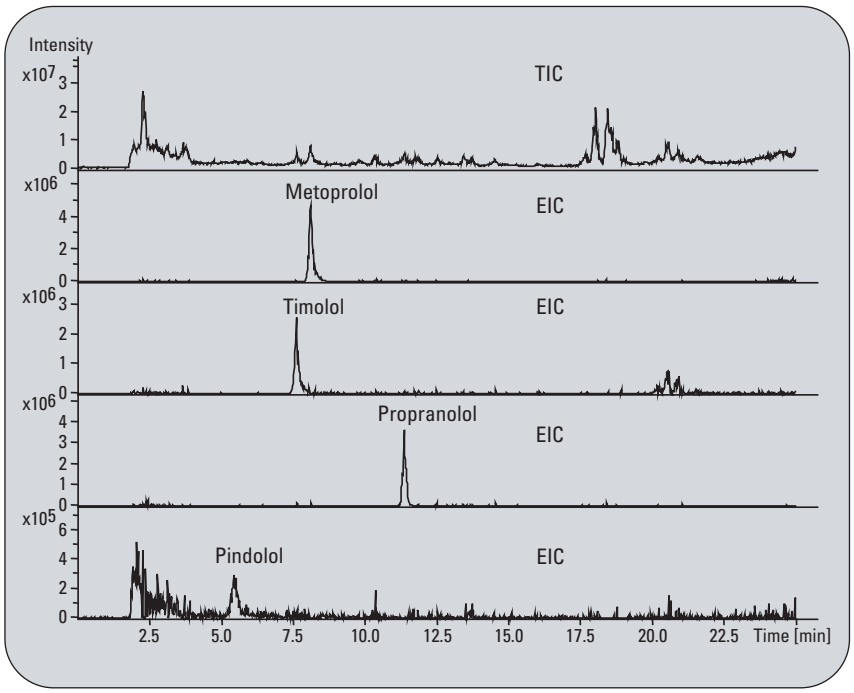

Figure 6
Analysis of beta-blockers in plasma by LC/MSD Ion Trap after the MARS sample clean-up procedure.

Sample preparation procedure
1 mL plasma was spiked with (start concentration):

Pindolol:	372 pg/µL
Timolol:	468 pg/µL
Metoprolol:	478 pg/µL
Propranolol:	732 pg/µL

Two injections of plasma (150 µL each) were performed, and a fraction was collected and pooled (1.5 mL total volume).
1 µL was injected into the 1100 capillary LC/MS Ion Trap system.

Chromatographic conditions for the analytical system

Column:	0.3 x 75mm ZORBAX SB C-8, 3.5 µm, 300 Å
Flow rate:	5 µL/min
Mobile phases:	water +0.1 %formic acid (A), acetonitrile + 1 % formic acid (B)
Gradient:	at 0 min 5 % B
	at 20 min 60 % B
	at 22 min 95 % B
	at 25 min 95 % B
Post time:	15min

MSD Ion Trap conditions

Scan:	200-400
Mass accumulation time:	50 ms
ICC:	50000

Tests for On-line sample preparation methods for urine

Our goal was to find a robust and easy-to-use on-line sample preparation method. The experiments have shown that on-line injection of biological fluids causes severe problems such as co-elution of matrix peaks and contamination of the system. Two examples are shown using the following hardware and samples:

- Valve system using a C-18 column as pre-column and direct injection of urine
- Valve system using a C-18 column

as pre-column and injection of urine after previous protein precipitation

On-line sample preparation for urine using C-18 material and a basic mobile phase for sample clean-up

For the experiments a 4.6 x 30 mm ZORBAX XDB C-18 column as clean-up column and a 2.1 x 30 mm ZORBAX SB C-18 analytical column were used. The mobile phase for the clean-up column was basic (pH 8.8), and the mobile phase for the analytical column was acidic (pH 2.2). A valve system as shown in figure 7 was used. Both UV and fluorescence detection were used. Figure 8 shows an example comparing the resulting chromatograms of a standard and a spiked urine sample with UV detection. It is quite obvious that this chromatogram shows many interfering peaks. Identification and quantitation is not possible. The analysis using fluorescence detection is more selective. Metoprolol and propranolol can be detected with less interference. Pindolol still shows an interference with another co-eluting peak. Timolol does not fluoresce and thus cannot be detected (figure 9).

- An advantage of this method is that no offline sample preparation step is needed.
- A disadvantage is that proper quantitation and identification may be difficult due to interference.

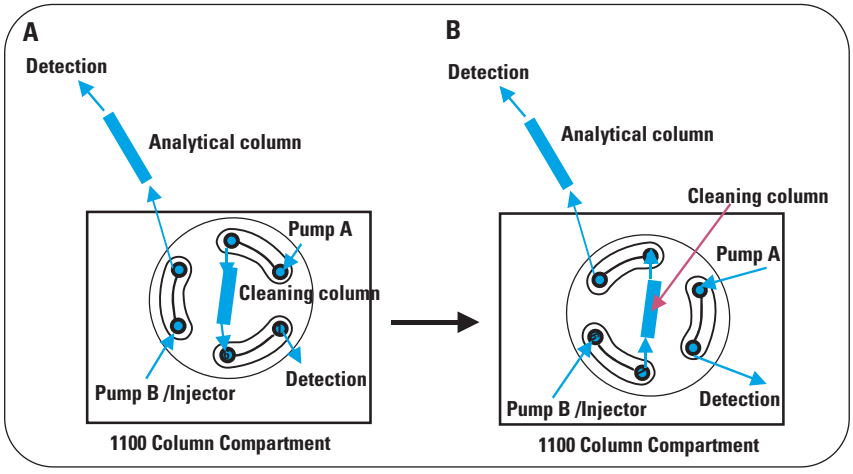

Figure 7
On-line sample clean-up flow diagrams. A: Sample loading and clean-up on an XDB C18 column, basic mobile phase. B: Backflush of the cleaned sample onto the analytical column, acidic mobile phase.

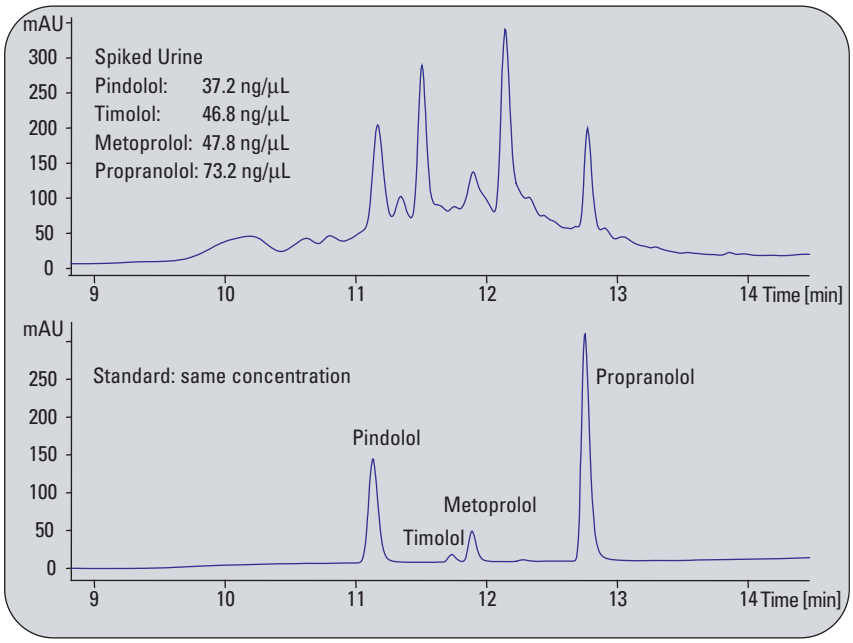

Figure 8
Chromatogram of standard and spiked urine sample using a column switching system, UV detection and different pH for cleaning and analytical column.

Chromatographic conditions

Column:	2.1 x 3.0 mm ZORBAX SB C-8 analytical column, 4.6 x 30 mm XDB C-18 ZORBAX cleaning column
Analytical mobile phases:	water + 0.05 % FA, acetonitrile + 0.045 % FA
Flow rate:	0.4 mL/min for analytical column
Gradient for analytical column:	7 min 10 % B, at 20 min 95 % B
Cleaning mobile phase:	4 % isopropanol, pH = 8.8 with NH4OH= A, acetonitrile in B for cleaning the injection system
Flow rate:	1 mL/min for cleaning
Gradient for cleaning pump:	100 % A 7 min, at 7.2 min 95 % B, at 21 min 100 % A
DAD signal:	220/10 nm, ref 500/100 nm, 290/40 nm, ref 550/100 nm
Fluorescence signal:	Ex265/Em320 and Ex220/Em320, PMT gain 12
Injection volume:	5 µL with needle wash in flush port
Column temperature:	20 ºC, at 7 min valve was switched

Combination of protein precipitation with a pre-column, UV and fluorescence detection

A combination of offline protein precipitation followed by further on-line clean-up with a pre-column provided good quantitative results in the low nanogram range using UV and fluorescence detection (figures 9 and 10). A UV and fluorescence detector in series were used to provide data for all injected beta-blockers.[5] The combination of protein precipitation and an additional pre-column sample clean-up can be used for the detection of beta-blocking drugs in the low nanogram range.

- The advantage of this method is that less interference is observed, and identification and quantitation becomes more reliable.
- Sample recovery is about 60 %
- The LODs for timolol, metoprolol and propranolol are in the low nanogram range for UV and fluorescence detection.
- A disadvantage is that pindolol is difficult to detect and quantitate. It elutes immediately after the valve switch.

Cleaning the system

On-line sample preparation means that biological fluids are injected directly into the LC system. All compounds present in the sample come into contact with the injector and the capillary connections. This can cause severe contamination in the needle, the seat, the seat capillary, the injection rotor, and the connecting capillaries. In extreme cases, blockage of parts with small internal dimensions, i.e., capillaries and frits, can occur.

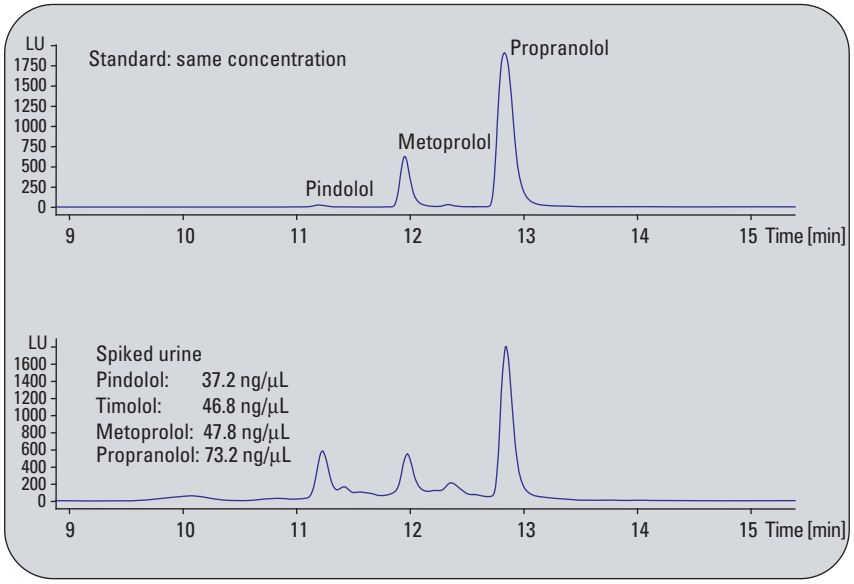

Figure 9
Chromatogram of standard and spiked urine sample using a column switching system, fluorescence detection and different pH for cleaning and analytical column.

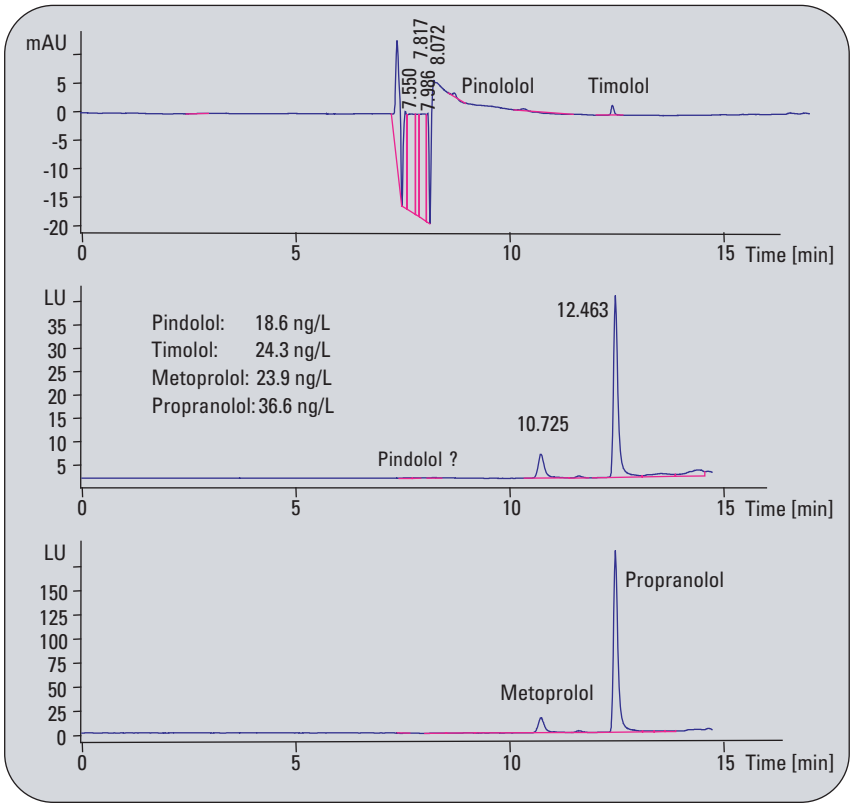

Figure 10
Analysis of spiked urine sample after off-line protein precipitation and with on-line pre-column.

The influence of contamination on the separation performance is shown in figure 11. This means that all parts that come into contact with the sample have to be cleaned on a regular basis. These cleaning procedures should include flushing with solvents of different polarities to purge the system of hydrophilic and hydrophobic materials. A good practice is to purge the system first with an acidic solution, followed by water, and then more non-polar solvents. It is also recommended to test the system daily for contamination with blank runs.

Conclusion

Several off-line and on-line sample preparation techniques were evaluated for the determination of beta-blockers in biological fluids. Off-line protein precipitation is a frequently used sample preparation method and is able to provide acceptable limits of detection for drugs in biological sample matrices. Off-line protein precipitation in combination with liquid/liquid extraction provides very clean extracts with excellent recovery rates and low limits of detection. A very simple and effective off-line method was based on the Agilent Multiple Affinity Removal system for removing six of the most abundant human plasma proteins, consisting of a special MARS column and special reagents. Originally this system was introduced to enable the analysis of low abundant proteins and has been adapted in this application to

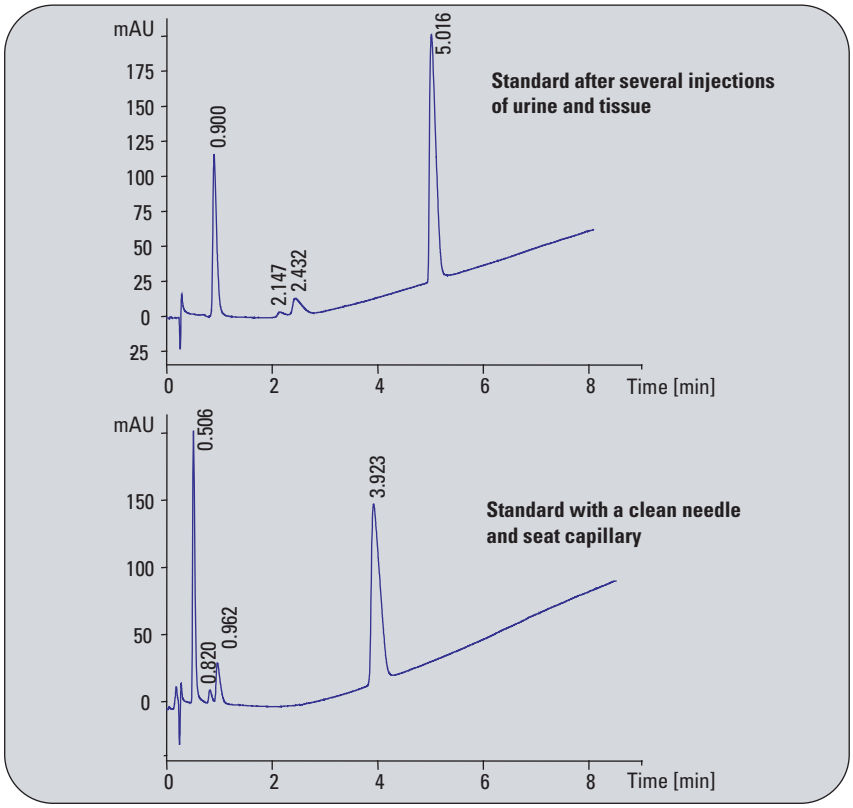

Figure 11
Influence of contamination on separation and peak shape after several injections of urine.

Offline sample preparation procedure	Protein precipitation	Precipitation/ Liquid/Liquid extraction using acetonitrile/sulfate	Precipitation/ Liquid/Liquid extraction using hexane/TFA	Gel filtration	Special extraction column for human plasma
LOD for biological samples	LOD = Low ng range Recovery rates 70 – 90 % for urine, < 50 % for plasma and tissue	LOD = Low to middle pg range Recovery rates between 40 to 60 % for urine and 30 to 50 % for tissue	LOD = Low pg range Recovery rates for urine and tissue close to 100 % except for Pindolol	LOD = Low to middle pg range with several poolings	LOD = Low to middle pg range with 2 poolings
LOD of standards no sample preparation	LOD ~2pg				
Instrument	Capillary LC/MS Ion Trap, 0.3-mm id column				

Table 3
Results from offline sample preparation experiments using capillary LC with MSD Ion Trap

extract small molecules from high-abundance proteins present in human plasma. Very good limits of detection are achieved (table 3) without further concentration steps. A disadvantage is that this system can be used for human plasma only. An alternative for other matrices is to use gel filtration. In combination with an LC fraction collection system, very good LODs are achieved. The disadvantage here is that only small sample amounts can be injected, and, therefore, pooling of different runs and/or concentration of the sample after clean-up may be necessary to achieve the desired detection limits. An Agilent 1100 Series LC system using valves in combination with UV and fluorescence detection was also tested as an on-line technique (table 4). This on-line technique was combined with off-line protein precipitation to achieve LODs in the low nano-gram range using UV and fluorescence detection. A disadvantage of the on-line method is that cleaning procedures have to be performed on a regular basis to remove matrix residues from all LC parts that come in direct contact with proteins, lipids, salts and other compounds that are present in biological material.

Offline sample preparation procedure	C-18 in line as cleaning column Recovery rates propranolol ~90 %	Combination of protein precipitation and cleaning column C-18 in line. Recovery rates ~60 % for urine
LOD for biological samples	LOD = Low ng range for propranolol only and fluorescence detection All other compounds showed co-elution with matrix peaks	LOD = Low ng range for UV/FLD detector combination
LOD of standards no sample preparation	LOD ~5 ng for UV and FLD in series	
Instrument	Agilent 1100 Series standard LC with UV and fluorescence and 2.1 mm id column	

Table 4
Results from online experiments.

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