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Quantitative determination of heparinoid mimetics in human and rat plasma by micellar electrokinetic chromatography

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Abstract

The investigated new class of heparinoid mimetics are spaced persulfated carbohydrates designed to increase the success rate of angioplasty and bypass surgery by preventing restenosis without increasing the risk of bleeding. Due to the presence of sulfate groups, these compounds are highly charged and, for preclinical kinetic investigations only small sample volumes of rat plasma were available. Therefore, capillary electrophoresis (CE) was applied. A bioanalytical method based on micellar elektrokinetic chromatography (MEKC) with UV-detection was developed for the selective quantitation of heparinoid mimetics (e.g., Ro 48-0843, Ro 48-3151, Ro 47-6199 and Ro 48-8722) in plasma. Using this method, only small volumes of plasma were required, which could be introduced directly into the separation capillary after 1:1 dilution with 100 m*M* aqueous sodium dodecyl sulfate (SDS). For increased sample throughput, an additional ultrafiltration step was performed after SDS-dilution of the plasma sample, improving both reproducibility and robustness of the method considerably. The sensitivity of the new method (3 μg/ml) was sufficient to follow plasma levels in pharmacokinetic studies. The practicability of this easy and rapid assay was demonstrated by the analysis of more than 350 plasma samples from pharmacokinetic studies performed in rats.

Keywords: Carbohydrate, persulfated; Heparinoid mimetics

1. Introduction

Atherosclerosis is still one of the most common causes of death in Europe, North America and Japan. A preferred method to restore blood flow in stenotic coronary arteries is angioplasty. The success rate is very high; however, 30–40% of the patients experience restenosis within a few months. The inhibition of neointimal thickening after vascular injury was first observed in a rat model [1]. As outlined in Fig. 1, the heparinoid mimetics used in this investigation are spaced persulfated carbohydrates [2] which were synthesized to prevent restenosis without substantially increasing the risk of bleeding [3,4]. For pharmacokinetic studies an accurate and precise bioanalytical assay for the determination of different heparinoid mimetics in human and rat plasma was required.

Capillary electrophoresis (CE) proved to be a very suitable method for the analysis of charged molecules and was already used for monitoring heparin or

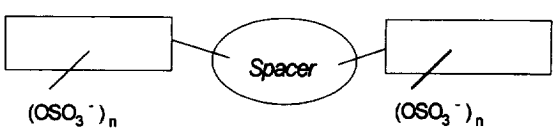

Fig. 1. Structure representation of the investigated heparinoid mimetics.

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heparin fragments by applying different separation modes. Capillary zone electrophoresis (CZE) [5] and micellar electrokinetic chromatography (MEKC) [6] were applied to evaluate the disaccharide composition of several proteoglycans by fractionating them as borate complexes at a high pH. The analysis of natural and synthetic low-molecular-mass heparin fragments at low pH was achieved by operating in the reversed polarity mode [7]. The separation of heparins based on charge-dependent complexation with ion-pairing reagents was developed by using laser-induced fluorescence (LIF) detection [8]. In addition, an MEKC method was described for monitoring sulfation reactions of disaccharides [9].

MEKC, as one of the most widely used CE modes, provides the possibility of separating neutral as well as charged compounds in one single run. The separation of neutral molecules is accomplished by the use of micelles formed by the addition of surfactants at a concentration above the critical micelle concentration. These micelles form a pseudo-stationary phase which is driven to the detector by the electro-osmotic flow [10,11]. When using sodium dodecyl sulfate (SDS) as a surfactant, direct injection of small volumes of biological fluids (e.g., plasma, urine) is possible [12,13].

A MEKC method for the determination of heparinoid mimetics in rat and human plasma samples (10–25 μl) using an easy and rapid pretreatment procedure is reported here. The new assay was validated for the compound Ro 48-8722 according to GLP-guidelines for bioanalytical method development.

2. Experimental

2.1. Reagents

Boric acid and disodium hydrogenphosphate dihydrate were purchased from Merck (Darmstadt, Germany). SDS was obtained from Bio-Rad (Hercules, CA, USA).

The running buffer was prepared by adjusting 10 m*M* disodium hydrogenphosphate with 10 m*M* boric acid to pH 8.0. Finally, 75 mmol/l SDS were added. The buffer was stored at 5°C, filtered before use (0.22 μm, Millipore, Bedford, MA, USA) and ultrasonicated.

A 100 m*M* SDS solution was prepared for sample pretreatment by dissolving 2.162 g SDS in 100 ml of water.

All buffers and solutions were prepared with deionized water (Milli-Q, Millipore).

2.2. Preparation of calibration and quality control standards

Stock solutions were prepared by dissolving 20 mg of the analyte in 1 ml of water and diluting with water, providing working solutions with concentrations of 10, 5, 2.5, 1.25, 0.625, and 0.31 mg/ml. Calibration standards were obtained by spiking human blank plasma (990 μl) with 10 μl of the corresponding working solution providing concentrations between 200 and 3 μg/ml.

Quality control samples were prepared by spiking rat and human blank plasma with the analyte at concentrations of 100 and 50 μg/ml.

All plasma standards were divided into aliquots of 25 μl and stored deep-frozen (−20°C) until required for analysis.

2.3. Sample pretreatment

Samples were thawed at room temperature and homogenized by vortex-mixing for about 10 s. Aliquots of plasma (25 μl) were diluted with the same volume of 100 m*M* aqueous SDS, which, after repeated vortexing, was ultrafiltrated by centrifugation through a membrane (Centrifree, Grace Amicon, M_r cut-off 16 000) for 15 min at 37°C and 5000 rpm. The resulting ultrafiltrate (about 35 μl) was then directly transferred to CE analysis.

2.4. Instrumentation

A Beckman P/ACE 2100 (Palo Alto, CA, USA) capillary system, equipped with a UV absorbance detector, was used. Untreated fused-silica capillaries (Beckman) with 75 μm I.D., 47 cm total length and 40 cm effective length were used for separation; detection wavelengths were set at 214 nm (Ro 48-8722 and Ro 48-0843), 254 nm (Ro 47-6199) or 280 nm (Ro 48-3151). The analysis temperature was

20°C. System operation, data acquisition and calculation of the corrected peak areas were performed with the Beckman GOLD V7.11 software package. The corrected peak area was calculated by multiplying the area with the velocity (length to the detector in cm divided by migration time in s). Further data evaluation was carried out by transferring data into the KINLIMS/UNICHROM software [14].

2.5. Electrophoretic conditions

Freshly installed capillaries were rinsed with 0.5 *M* sodium hydroxide for 30 min, followed by water and running buffer (15 min each). Afterwards, current and baseline stability were tested by applying a voltage of 30 kV for 15 min.

To ensure a complete run of a sample series (24 free vial positions at the maximum), two method files were created with identical conditions, but using different vials for the separation buffer, water and sodium hydroxide vials. These two methods were applied alternately and consisted of the following steps: (1) prerinse of the capillary with the run buffer for 2 min; (2) injection of the sample by applying pressure for 10 s (about 25 nl injection volume); (3) dipping the injection side of the capillary into an extra vial with run buffer to remove plasma traces from the outside of the capillary (0.02 min low pressure); (4) separation by applying 30 kV for 10 min at 20°C; (5) rinse with water for 2 min; (6) rinse with 0.5 *M* sodium hydroxide for 3 min; (7) additional rinse with water for 2 min. These steps were necessary to remove plasma contents from the capillary and to recondition the capillary surface. The run buffer was renewed every morning and evening. The total time needed for an electrophoretic run was 18 min.

2.6. Calibration and calculations

For calibration, seven spiked human plasma standards with concentrations between 3 and 200 μg/ml were processed together with the unknown biological samples. The calibration curves were established by weighted linear least-squares regression (weighting factor $=1/y^2$) of the corrected peak areas. The regression equation was then used to extrapolate analyte concentrations in unknown plasma samples.

3. Results and discussion

3.1. Sample pretreatment

Different pretreatment procedures, including protein precipitation with acetonitrile or methanol, ultrafiltration, and SDS addition, were tested. Direct injection of plasma was feasible (Fig. 2A); however, with increasing sample number the migration time of the analyte increased and the peak shape varied from injection to injection, possibly because of adsorption of plasma constituents to the inner capillary wall. In order to circumvent adsorption problems, plasma samples were ultrafiltrated through a membrane (M_r cut-off 16 000) before injection for removal of disturbing matrix components (Fig. 2B). Using this procedure, fast-migrating matrix peaks nearly disappeared, but recovery of the analyte was reduced due to plasma protein binding of the heparinoid mimetics. Diluting plasma samples with a 100 m*M* aqueous solution of SDS before injection effectively displaced the analyte from the plasma proteins (Fig. 2C). To minimize the risk of contamination of the capillary surface occurring at increased sample throughput, the sample was first diluted with aqueous SDS and then ultrafiltrated by centrifugation at 37°C, as described above (Fig. 2D).

3.2. Capillary electrophoresis analysis

Micellar electrokinetic chromatography (MEKC) was used for the selective separation of the analyte from plasma components and the anticoagulant (citrate, the huge peak behind the analyte in Fig. 1). All detectable plasma components were found within the migration window of the micelles (markers: methanol for EOF, $t_m=1.5$ min and Sudan III for the micelles, $t_m=4.85$ min). Neither the anionic analyte nor the citrate interacted with the micelles and both migrated behind the pseudostationary phase.

Capillaries with 75 μm I.D. were used under the conditions described in the experimental section, as a compromise between sensitivity, peak resolution and practicability.

The method showed good versatility for the separation of various heparinoid mimetics differing in the aromatic groups or the amounts of sulfate groups. Fig. 3 shows electropherograms of three

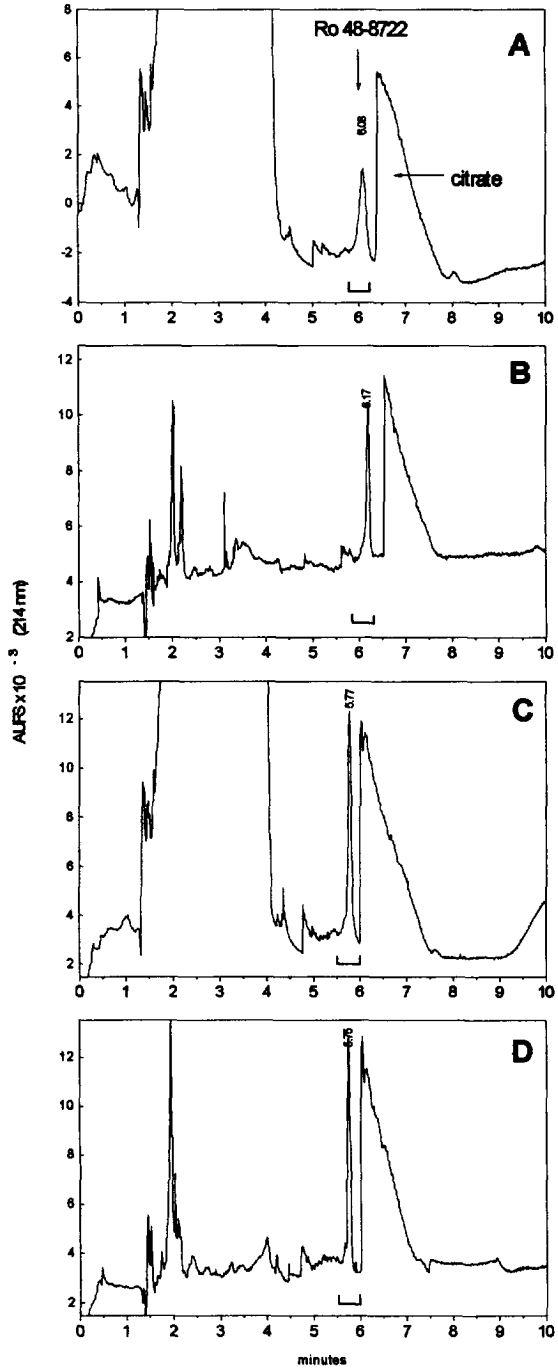

Fig. 2. Different sample work-up procedures for a rat plasma sample. Detection wavelength, 214 nm; for other conditions see Section 2. (A) Direct injection (5 s) without pretreatment. (B) Injection (5 s) after ultrafiltration. (C) Injection (10 s) after 1:1 dilution with 100 m*M* SDS; (D) injection (10 s) after 1:1 dilution with 100 m*M* SDS and ultrafiltration.

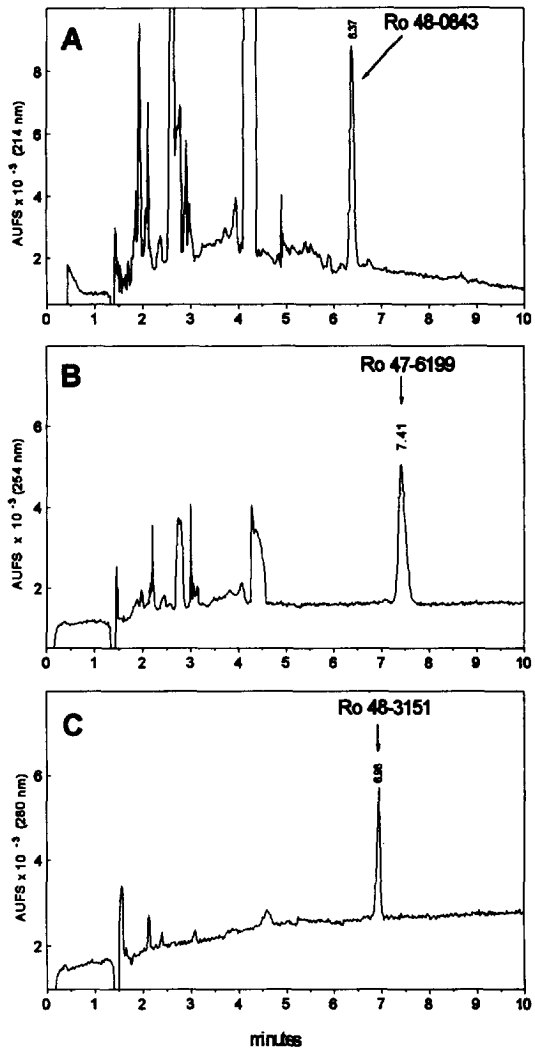

Fig. 3. Electropherograms of: (A) human plasma (EDTA) spiked with Ro 48-0843 at a concentration of 100 μg/ml; detection wavelength, 214 nm; (B) human plasma (EDTA) spiked with Ro 47-6199 at a concentration of 100 μg/ml; detection wavelength, 254 nm; (C) rat plasma sample (citrate) with Ro 48-3151 (pharmacokinetic study, concentration found, 108 μg/ml); detection wavelength, 280 nm. For other conditions see Section 2.

different compounds: Ro 48-0843 and Ro 47-6199 (both spiked in human plasma at 100 μg/ml) and Ro 48-3151 (rat plasma sample from a pharmacokinetic study). The presence of matrix components is increasingly masked with a higher detection wavelength (Fig. 3A–C) and the anticoagulant citrate

Table 1
Recovery of Ro 48-8722 from plasma

Species	Concentration added (μg/ml)	Number of replicates	R.S.D. (%)	Recovery (%)
Human	50.0	5	7.0	88.5
	12.5	5	9.0	82.1
Rat	50.0	5	8.2	87.8
	12.5	5	9.4	79.4

does no longer interfere with the analyte at a wavelength of 280 nm (Fig. 3C).

3.3. Recovery

The recovery was >80%, determined by spiking human and rat blank plasma with Ro 48-8722 at concentrations of 50 and 12.5 μg/ml. Control samples were obtained by spiking blank plasma with the same solution after 1:1 dilution with SDS and ultrafiltration. All samples were processed and analyzed by MEKC as described above. Recovery was calculated by comparing the corrected peak areas of the spiked samples with the corrected peak areas of the control samples, providing the 100% values (Table 1).

3.4. Precision, accuracy and linearity

The inter-assay precision was determined at different concentration levels in human and rat plasma. Three spiked plasma samples were prepared at concentrations of 100 and 50 μg/ml in rat plasma and 12.5 μg/ml in human plasma and analyzed on different days (using a separate calibration curve on each day). The data in Table 2 demonstrate a good precision and accuracy between 3 and 100 μg/ml.

The correlation between corrected peak area and concentration of the analyte was linear in this range. The standard deviations were not constant over the concentration range. Therefore, the calibration curve had to be calculated by means of a weighted linear least-squares regression procedure, using $1/y^2$ as weighting factor. A typical calibration curve showed a coefficient of determination of 0.9984, a mean deviation of 2.74%, and an x-intercept of +0.03 μg/ml.

3.5. Selectivity

The MEKC method fulfilled the selectivity criteria for the quantitation of all tested heparinoid mimetics (Ro 47-6199, Ro 48-0843, Ro 48-3151 and Ro 48-8722) in human and rat plasma. No interferences

Table 2
Inter-assay precision and accuracy

Concentration added (μg/ml)	Concentration found (mean±S.D.) (μg/ml)	Number of replicates	R.S.D. (%)	Inaccuracy (%)
100	100.1±6.7	5	6.7	0.1
50	50.3±3.1	5	6.1	0.6
25	24.9±2.3	5	9.3	−0.5
12.5	12.9±0.4	5	2.9	3.2
6.25	6.2±0.2	5	3.6	−0.4
100[a]	100.6±4.9	7	4.9	0.6
50[a]	49.8±3.0	10	6.0	−0.4
12.5	13.1±1.5	10	11.3	4.9

[a] Rat plasma.

with endogenous components or added anticoagulants were observed in different batches of human blank plasma and in rat blank plasma samples from placebo applications. Fig. 4 shows representative electropherograms of a rat placebo sample (Fig. 4A) and a rat plasma sample from an infusion application of Ro 48-8722 (Fig. 4B).

3.6. Limit of quantitation (LOQ)

The LOQ defined here as the minimum concentration that could be measured routinely with acceptable precision (<15%) and accuracy (>85%) was 3 μg/ml for Ro 48-8722.

3.7. Application to biological samples

The method has been applied successfully to more than 350 rat plasma samples from pharmacokinetic studies. The concentration range of Ro 48-8722 in these samples varied between 4 and 200 μg/ml plasma.

The required sample volume could easily be reduced to 10 μl plasma without losing precision. At smaller sample volumes precision was mainly determined by the feasibility of physically handling microliter volumes of liquids.

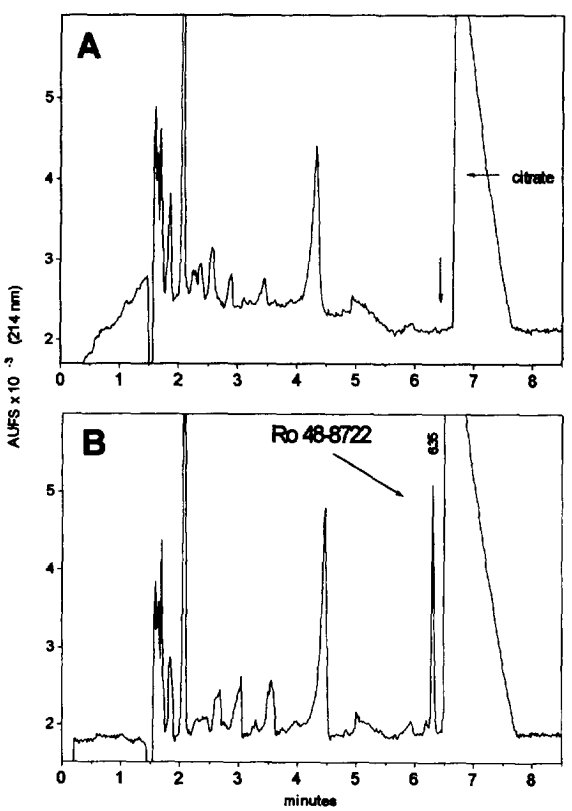

Fig. 4. Specificity of the method: (A) rat blank plasma (placebo); (B) rat plasma sample of a bolus experiment with Ro 48-8722; concentration found, 58 μg/ml; detection wavelength, 214 nm. For other conditions see Section 2.

4. Conclusion

MEKC, together with SDS pretreatment of samples, proved to be a suitable method for the quantitation of highly charged heparinoid mimetics in plasma samples. The sensitivity of the method (3 μg/ml) was sufficient to determine the pharmacokinetics of relevant plasma levels. Over 350 rat plasma samples were analyzed for Ro 48-8722 in the concentration range between 4 and 200 μg/ml. Only small sample volumes between 10 and 25 μl plasma were required. Being able to use small blood sample volumes allows the analysis of more time points in a pharmacokinetic study, and results in a better precision of the data.

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