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Journal of Chromatography A, 954 (2002) 33–40

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Initial study of using a laminar fluid diffusion interface for sample preparation in high-performance liquid chromatography

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Received 11 December 2001; received in revised form 11 February 2002; accepted 12 February 2002

Abstract

This report describes a new microfluidic device called the H Filter for sample preparation prior to HPLC. The H Filters make possible a diffusional transfer of an analyte from a sample stream into a stream of a ''receiver'' fluid. Existing mathematical models can be used for optimizing experimental conditions. The authors have selected the extraction of the antibiotic cephradine from blood to demonstrate the utility of the new device. The extracts of blood samples spiked with cephradine levels between 0.2 and 100 μg/ml were analyzed using a C_8 reversed-phase column and UV detection at 260 nm. The HPLC results were in good agreement with theory. The recovery of 32.2±2.8% was uniform over the entire range of cephradine concentrations. The new method completely avoids the use of centrifuges, that is otherwise typical for most current methodologies for the preparation of blood samples prior to HPLC analysis.

Keywords: Microfluidics; Laminar fluid diffusion interface; Instrumentation; Sample preparation; Cephradine; Antibiotics

1. Introduction

The laminar fluid diffusion interface (LFDI) is observed in fluidic microchannels whenever two or more streams are running parallel and under conditions characterized by a low value of Reynolds number ($Re \ll 1$).

The Reynolds number is defined as $Re = (\rho \nu d)/\mu$ where ρ is the density of the fluid, ν is the mean flow velocity, d is a characteristic dimension of the channel and μ is the viscosity. Microfluidic devices making possible LFDI were evaluated, for example, for diffusional transfer of low molecular mass compounds ($M_r < 300$) from a stream of blood [1,2], as continuous concentration monitoring devices [3] and as switching devices for microchannels [4].

The diffusional transfer of small molecules from blood under the conditions of LFDI holds promise for preparation of blood samples prior to HPLC.

Several other promising HPLC sample preparation methods have also been introduced for small molecules in complex biological matrices. These include, for example, restricted access media [5,6] and mi-

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00160-7

crodialysis [7]. However, our report focuses entirely on the evaluation of LFDI devices.

Currently, most HPLC-related sample preparation methods for blood can still be characterized as laborious and tedious. Most of the procedures use a centrifuge, at least once, to remove the red blood cells and to generate plasma or serum. Many of the sample preparation methods utilize a centrifuge in at least one additional step, for example, in the removal of proteins from the sample by precipitation. The necessity to centrifuge creates major obstacles for any efforts to automate the sample preparation. Even with expensive robotic equipment and on line centrifuges, centrifugation and the preceding agitation and vortexing remain the time-limiting steps of the entire protocol.

The high-throughput requirement typical of many pharmaceutical laboratories and the need for automation in that environment led us to select a drug analysis in blood for our evaluation of an LFDI device. Additionally, we have also searched for an example of a chemically stable drug compound being analyzed in blood using a sample preparation procedure involving at least two centrifugation steps. Applying the above criteria, we have selected a recently published procedure by Johnson et al. [8] describing the determination of cephalosporin cephradine in human plasma. Interestingly, Johnson et al. discuss and propose a solution for an additional problem related to the common deproteinization protocols. Johnson et al. assume the perchloric acid sample matrix (pH 1) stemming from the protein removal step not to be compatible with silica-based reversed-phase columns. They present new evaluation results with the help of a polymeric reversed-phase column. Their use of polymeric rather than silica-based columns should enable longer column lives.

In this report, we are describing an application of an LFDI device that eliminates not only the blood to plasma conversion and protein precipitation but also any use of HPLC-column damaging reagents.

2. Experimental

2.1. Chemicals

All chemicals were of reagent grade or better purity. The water we used in preparation of standards, for sample preparation solutions and chromatographic mobile phases was freshly drawn from a laboratory water purification unit (Milli Q, Millipore Corp., Bedford, MA, USA). The organic solvent for the chromatographic mobile phase ''Acetonitrile UV'' was obtained from Burdick and Jackson (Muskegon, MI, USA). Initially, we tried to utilize a ready-made phosphate-buffered saline solution (PBS) from a commercial supplier as a receiver fluid for the sample preparation step. However, all commercial compositions we tried produced strongly interfering chromatographic peaks with UV detection at 260 nm. There was no interference when we prepared our own PBS solution from the individual components and with our ultra-pure water (0.014 *M* NaCl, 0.0083 *M* NaH_2PO_4, 0.0014 *M* Na_2HPO_4). The cephradine solid (~90%) was purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation and handling of standards

The aqueous stock solution and aqueous standard dilutions of cephradine were prepared as described in Ref. [8]. Additionally, we have used the aqueous stock solution to prepare 0.2, 0.8, 4.0, 20, and 100 mg/l dilutions of cephradine in PBS freshly for each of the sample preparation experiments. The PBS-containing standard dilutions were submitted to exactly the same treatment as the PBS-containing H-Filter extracts of blood, i.e. storage on solid ice overnight, thawing and chromatographic processing within the next 24 h. The composition of PBS-containing standard dilutions was validated by comparison with purely aqueous standards analyzed on the same day. The results of PBS-based and aqueous standards were always identical within the experimental error. The observed chromatographic reproducibility (RSD) of cephradine peak areas from aqueous and PBS dilutions was between 0.5 and 1.6% for a set of six injections ($V = 15.0$ or 33.0 μl). The calibration curves for the above six concentration levels were of essentially the same quality as previously reported [8], i.e. correlation coefficients >0.9990.

2.3. Preparation of spiked blood and plasma samples

Blood used in the experiments was collected into

EDTA (purple) Vacutainer tubes on the same day we performed the experiments.

2.3.1. Spiking of whole blood samples

For each sample, 500.0 μl of blood were transferred from the Vacutainer tube into a 1.0-ml microcentrifuge tube. In the next step, we added 5.0 μl of a 100× concentrate of cephradine solution to each sample to achieve the final concentration. Each addition of the 100× concentrate was followed by gentle mixing.

2.3.2. Spiking of plasma samples

For each sample, 1.5 ml of whole blood from the Vacutainer tube were transferred into a microcentrifuge tube and spun down to separate the plasma from red blood cells. From each tube, 500 μl of plasma were transferred into a different microcentrifuge tube for an addition of 5.0 μl of a 100× concentrate (100× higher than the targeted spike level in plasma) of cephradine. Each concentrate addition was followed by gentle mixing.

2.4. The LFDI apparatus

All LFDI devices or ''H Filters'' used for our initial evaluation study were from Micronics (Redmond, WA, USA). A top view of a typical H Filter is shown in Fig. 1. The device is constructed of multiple layers of polyester sheets. Narrow segments of some of the inner layers of the polyester material were removed by cutting with UV laser light. When put together and laminated, these cutouts form a three-dimensional pattern of narrow fluidic channels depicted in Fig. 1 [2,9].

The three sets of two concentric circles denoted V1, V2, and V3 in Fig. 1 are valves. They are pneumatically actuated by means of the three narrow diagonal channels shown in Fig. 1. The three pump interfaces S1, S2, and S3, are connected to three syringe pumps SP1, SP2 and SP3 (not shown in Fig. 1). The syringe movement and switching of valves are synchronized and controlled by a software program developed at Micronics.

The LFDI portion of the H Filter is shown in the

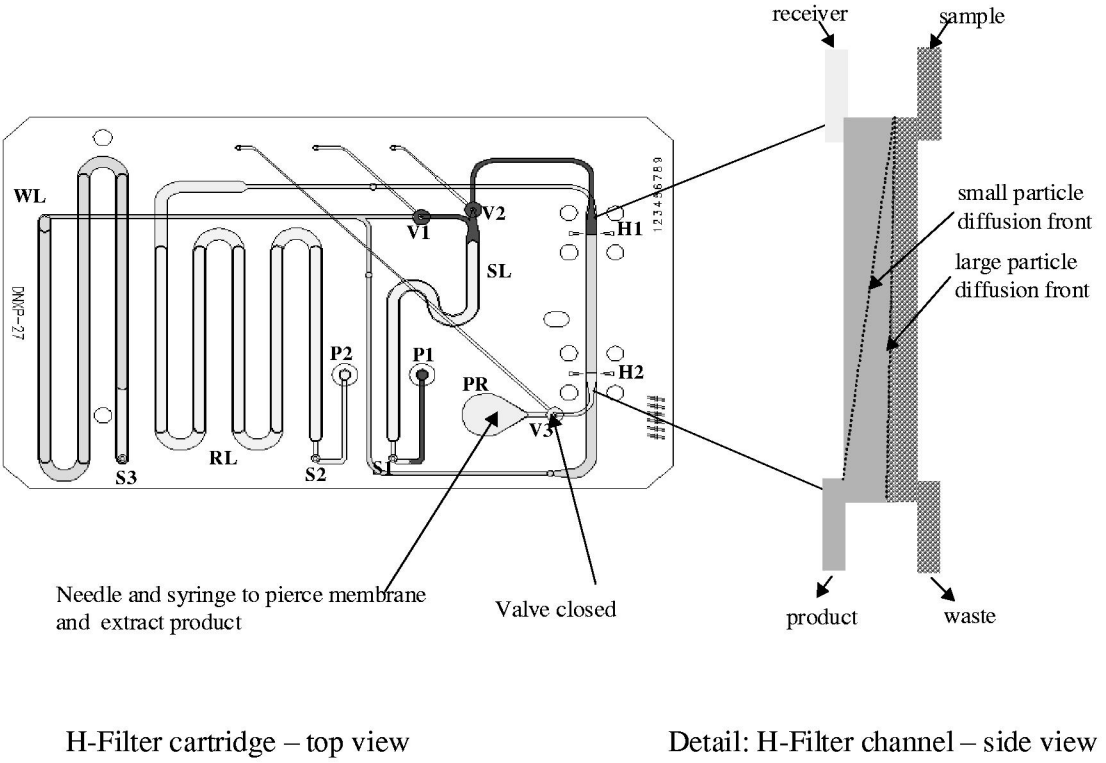

Fig. 1. H-Filter cartridge. See Experimental section for a detailed description.

inset enlargement. The stream of whole blood is pumped at the bottom overlaid by a stream of receiver solution. The dimensions of the H filter channel were ~0.1×1.2×20 mm (depth×width×length). While the geometry of the LFDI channel remained unchanged in all our experiments, the dimensions of the sample loop (SL) and receiver holding loop (RL) were changed several times as we progressed with the optimization of the sample preparation process. The drawing in Fig. 1 (scale 1.5:1) depicts the H Filter version deemed to be most suitable by the authors for the sample preparation of whole blood. Additional details of the H Filter process and description of optimization of the flow-rates are given in the Results and discussion section.

2.5. Determination of protein concentration in H Filter extracts

Protein concentrations in the extracted solutions were measured by the method of Lowry et al. [10]. The absorption of the Folin complex was measured in 10-mm cuvettes in a Turner SP870 Spectrophotometer set to 620 nm. A standard curve was established with BSA concentrations ranging from 0 to 50 μg/ml.

2.6. HPLC apparatus and separation conditions

A HPLC system consisted of a Gina50 autosampler, P580 low-pressure gradient pump, Model STH585 column thermostat, and Model UVD340S detector. All modules were from Dionex (Sunnyvale, CA, USA). The system control and data acquisition were carried out with the help of Dionex PeakNet 6.2 software.

The C_8 reversed-phase column (Dionex Acclaim 120) had the dimensions of 250×4 mm and was used with a 10×4 mm guard column filled with the same type of 5 μm reversed-phase particles as the main column.

The column and guard were placed in the column thermostat and kept at 35 °C.

The eluent composition described in Ref. [8] (20 m*M* NaH_2PO_4, 10% acetonitrile, pH 2.7 adjusted with phosphoric acid) was used for all separations discussed in this report. The flow-rate was always 1 ml/min and detection was carried out at 260 nm.

3. Results and discussion

3.1. Using the H Filter for extracting cephradine from blood samples

The main features of the H Filter are described in the Experimental section. Fig. 1 illustrates the position of liquids at the end of a sample preparation. The dark segment represents an unprocessed aliquot of the whole blood. At the beginning of a sample preparation, that segment (blood sample) extends from point P1 to point V2. The darker shade of gray is used for the processed blood aliquot. The lighter shade of gray depicts the receiver and ''pusher'' fluids. In all our experiments, receiver and pusher fluids have an identical composition.

The device operates as follows. The H Filter is placed into a holder. The pre-aligned liquid connections to syringe pumps SP1, SP2 and SP3 are established. In the beginning of the process, the valve V2 closes. The operator injects the blood sample (80 μl) into the inlet port P1. This is followed by the injection of the receiver solution (133 μl) into the inlet port P2. Valve V1 closes, and valve V2 opens. Syringe pump SP1 starts pumping blood up to the start of the H-Filter (position H1). Syringe pump SP2 then pumps receiver solution past the point H1 up to the point H2, touching the blood stream in the process. Syringe pump SP3, connected to inlet S3, now back fills the waste loop up to the position H2. The cartridge is fully primed now and ready for the extraction. Pumps SP1 and SP2 start pushing, and SP3 starts pulling. As represented in the enlarged portion of Fig. 1, a diffusive mass transfer of cephradine and other low molecular mass compounds is taking place. The red blood cells and other blood particles remain in the sample stream and are removed into the waste loop WL. Under optimized conditions, a large portion of high molecular mass compounds (i.e. proteins) also remains in the original sample and ends up in waste. However, a diffusion-based transfer of a small portion of proteins can never be entirely prevented.

The cephradine-enriched stream of the receiver is removed from the LFDI interface at the point H2 and collected in the product reservoir PR. At the end, V3 closes, and the product (cephradine-enriched receiver) is removed by the operator into a suitable

autosampler vial ($V = 300$ μl). The sample is ready for an injection into the HPLC system.

3.2. Optimization of laminar fluid interface

The optimization process begins with the choice of appropriate geometry of the LFDI microchannel of the H Filter (see Experimental). The correct chemistry of the receiver solution is also important. Multiple possibilities of disturbing effects (blood coagulation, clotting etc.) triggered by a contact of blood with incorrectly chosen receiver fluid have to be avoided. The phosphate buffered saline has been utilized successfully in many LFDI experiments involving blood [2,9].

The final optimization steps follow the mathematical model developed by Bardell [11].

One of the final decisions involves choosing a sample flow-rate to total flow-rate ratio. How this ratio influences the final distribution of blood components is visualized in the plot of Fig. 2. The approximate upper limit of optimal conditions is indicated by a vertical line in the plot. The flow ratios higher than the indicated optimum improve the transfer of cephradine only very little, while increasing the contamination by the proteins and eventually also by the red blood cells.

Another form of visualization of the mathematical

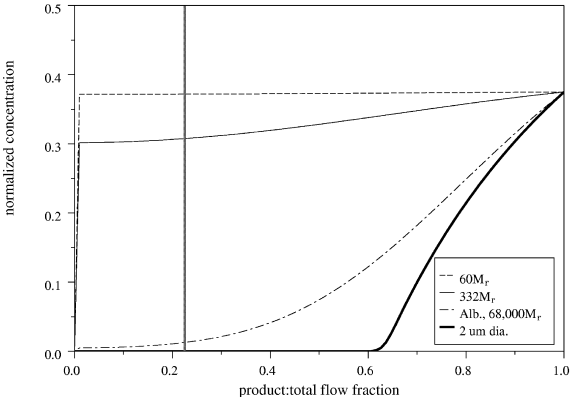

Fig. 2. Visual representation of mathematical model [7] for the whole blood and PBS streams. The M_r 332 represents fluorescein. This compound is frequently utilized because it can be seen easily. The cephradine has a very similar value of M_r of 349. The model is calculated for 25 °C. All LFDI experiments reported here were carried out at ambient temperature.

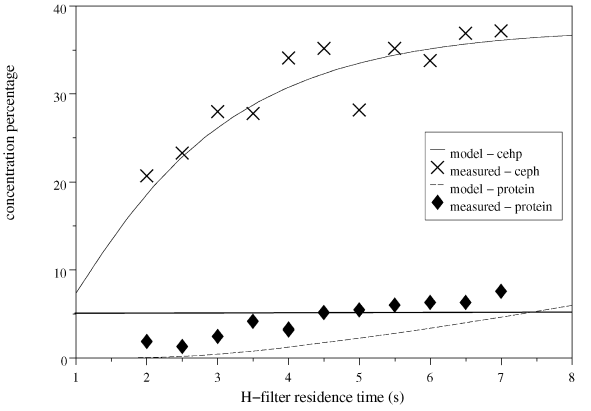

Fig. 3. Cephradine and protein concentration vs. H-Filter residence time. The curves represent the data predicted by mathematical modeling [7] assuming the conditions indicated by the vertical line in Fig. 2. See Results and discussion section for additional comments.

model is represented by the two curves of the plot in Fig. 3. The predicted sample concentration percentages (with the blood concentrations as 100%) of protein and cephradine are calculated assuming the flow-rate ratio marked in Fig. 2 (sample/total flow = 0.22 corresponding to blood/receiver = 0.6). The variable parameter is the sample residence time in the LFDI portion of the H Filter. The predicted optimum residence time is between ca. 4.0 and 5.5 s. At residence times shorter than 4 s, the concentration recovery of cephradine is far from its maximum possible value. At residence times that are higher than ca. 5.5 s, the concentration recovery increases only very little at the cost of much increased contamination by the blood proteins. Searching the published literature, we were unable to find any guidelines regarding the maximum recommended protein levels for samples injected onto silica-based reverse phase columns. The horizontal line in Fig. 3 is an arbitrary level of protein concentration that we decided not to exceed in our experiments.

3.3. Evaluation by HPLC

Prior to the work with H-Filter extracts, we validated our HPLC method by comparing reproducibility of cephradine standards prepared by dilution in PBS with that of purely aqueous standard dilutions. The standard deviation of both types of

standards (sets of six injections each, injection volume 30.0 μl, cephradine at 10 mg/l) was found identical within the error of measurement. In the first series of experiments combining LFDI and HPLC, we processed 10 blood samples using H filter residence between 2.0 and 7.0 s. In all experiments, all H Filters were used only once. The H Filters were pre-filled by 80 μl of whole blood (spiked to a level of 100 mg/l of cephradine, except for control blanks) and by 133 μl of PBS. We were able to recover a total volume of 48 μl of cephradine-enriched receiver from each experiment. The recovered volume was split into two parts. We transferred 25.0-μl aliquots into autosampler vials for HPLC analysis and utilized the rest for the photometric total protein determination. The HPLC injection volume was 15 μl. Other experimental conditions were as described in the Experimental section.

As documented in Fig. 3, the HPLC results for cephradine and photometric readings of total protein concentration follow the model-based predictions reasonably well considering the model calculations are based on a single protein (albumin, M_r 68 000) only.

On the negative side, we noted that the separation efficiency of the column set deteriorated at the end of the residence time study. Before the study, we calculated 14 000 theoretical plates for cephradine peak from an injection of aqueous standard (15 μl 100 mg/l). After the study the same peak showed an efficiency of only 9000 theoretical plates. However, we were able to restore the separation efficiency by replacing the guard column. The separation efficiency appears to have been affected by residual protein levels contained in samples having residence time over 4 s. Deciding not to exceed the residence time of 4 s in any of the future experiments, we proceeded with processing spiked blood samples in the entire range of concentration required in pharmacokinetics and bioequivalency studies (0.2 to 100 mg/l cephradine [8]). We continued checking the column status. The decrease of separation efficiency has not been observed again during the subsequent weeks of continuing work with the same column. Before starting the concentration range study, we also verified that the HPLC limit of detection was sufficiently lower than the expected lowest concentration of cephradine we needed to analyze. The HPLC limit of detection, determined as three multiples of noise and using PBS-diluted cephradine

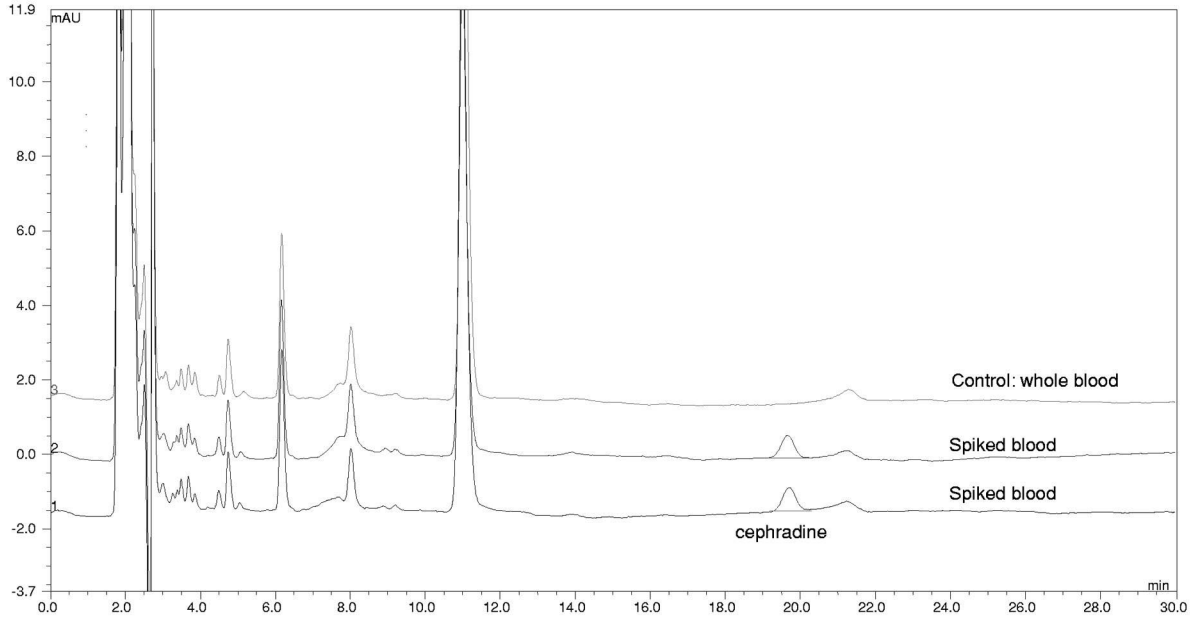

Fig. 4. Chromatograms of two whole blood samples spiked with 1.6 mg/l of cephradine. A separation of an unspiked aliquot of whole blood is included for comparison. See Experimental section for a complete description of chromatographic conditions.

Table 1
Concentration range study

Spiked level mg/l	Cephradine extracted in mg/l	Recovery[a] %	Standard deviation	Average	RSD %	Data for
0.2	0.059	29.6				
0.2	0.059	29.7				
0.2	0.054	27.0				
0.2	0.053	26.7	0.003	0.1	5.6	0.2 mg/l
0.8	0.262	32.7				
0.8	0.252	31.5				
0.8	0.296	37.0				
0.8	0.289	36.1	0.021	0.3	7.7	0.8 mg/l
1.6	0.555	34.7				
1.6	0.548	34.3				
1.6	0.531	33.2				
1.6	0.583	36.5	0.022	0.6	3.9	1.6 mg/l
4.0	1.293	32.3				
4.0	1.202	30.1				
4.0	1.292	32.3	0.052	1.3	4.1	4.0 mg/l
20.0	6.040	30.2				
20.0	5.821	29.1				
20.0	6.433	32.2	0.310	6.1	5.1	20 mg/l
100.0	34.352	34.4				
100.0	34.361	34.4				
100.0	33.489	33.5				
100.0	32.520	32.5				
100.0	30.470	30.5	1.672	32.7	5.1	100 mg/l

The H Filters were pre-filled by 80 μl of whole blood (spiked with indicated levels of cephradine, except for control blanks) and by 133 μl of PBS. We were able to recover a total volume of 48 μl of enriched receiver and transferred 38 μl to HPLC autosampler vials. The rest of the volume (10) μl was used for total protein determination. The residence time in the H Filter was 4 s.

[a] Mean recovery: 32.2±2.8%.

standards, was 0.036 mg/l and the expected lowest extracted concentration was ca. 0.060 mg/l.

The overlay of three chromatograms in Fig. 4 compares two chromatograms of LFDI extracts of whole blood spiked with 1.6 mg/l and a chromatogram of unspiked blood. Although there are some peaks of other blood constituents present in the chromatogram, the peak of cephradine is well resolved and free of interference. The entire sample processing, from the filling of the H Filter with blood to the transfer of the extract into the autosampler vials, takes only 287 s.

A summary of the quantitative data for the entire concentration range is presented in Table 1.

The mean value of concentration recovery agrees very well with that predicted by theory in Fig. 3 (32.2 vs. 32.0%). The fact that the value of recovery is similar for all spiking levels also indicates a good linearity of extraction over the entire range of investigated concentrations. The reproducibility of results as measured by RSD is comparable for all spiking levels. The RSD value at the lowest level confirms that the limit of quantitation was not exceeded even with the sample defining the lowest limit of the required concentration range. Injecting a cephradine standard before and after the concentration range study we obtained identical values of theoretical plates. Injections of blood extract samples listed in Table 1 (plus three blood controls) thus did not have any negative effects on the column set used in that experiment.

4. Conclusions

The data in this report (see Figs. 3, 4 and Table 1) represent a first documented instance of successful application of LDFI for sample preparation of blood

samples prior to HPLC. The new sample preparation procedure avoids any utilization of centrifuges and should be easily accessible to automation. Also notable are the reduced time requirements per sample: ca. 5 min vs. ca. 30 to 60 min by conventional methods.

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