Development and validation of HPLC/UV-procedures of efavirenz determination in biological fluids

Efavirenz is a non-nucleoside reverse transcriptase inhibitor with a number of side effects manifested by psychiatric symptoms. There are cases of acute poisoning due to administration of efavirenz, including cases of suicide attempts; therefore, efavirenz may be approved as a toxic compound in forensic toxicology.

Aim. To apply the MiLiChrome® A-02 HPLC-analyzer system for efavirenz quantitative determination in biological fluids and perform validation of the procedures developed.

Results and discussion. Three HPLC/UV-procedures of efavirenz determination in blood and urine have been proposed. Validation of all procedures developed has been performed by such parameters as specificity, recovery, linearity, accuracy and precision in the variants of the methods of calibration curve and standard. The results of analysis have shown the absence of peaks with the retention time, which is coincident with the efavirenz retention time, on the chromatograms of blank-samples for all variants of procedures of the analyte isolation. All procedures of sample preparation show the high efficiency of efavirenz isolation both for blood and urine (at the level of 90 %). All procedures studied are characterized by the acceptable parameters of linearity, within-run and between-run accuracy and precision.

Experimental part. Sample preparation of blood and urine was carried out in three ways – 1) liquid-liquid extraction with the mixture of chloroform and 2-propanol (80 : 20); 2) 2-propanol extraction and salting-out with ammonium sulfate; 3) acetonitrile extraction and salting-out with ammonium sulfate. The chromatographic conditions were as follows: the column – Ø2 × 75 mm, ProntoSIL 120-5-C18 AQ, 5 μm; temperature – 40 °С; the flow rate – 100 μl/min; Eluent A – 0.2 M LiClO₄ – 0.005 M HClO₄; Eluent B – acetonitrile; the elution mode – linear gradient; detection – UV, 247 nm; the volume of injection – 2 μl.

Conclusions. The set of HPLC-procedures of efavirenz quantitative determination in blood and urine has been developed. Validation of the procedures developed has been performed.

Key words: efavirenz; high-performance liquid chromatography; blood; urine; sample preparation; validation
Efavirenz is a non-nucleoside reverse transcriptase inhibitor [1-2]; it was approved by the Food and Drug Administration on September 18, 1999 [3]. Efavirenz was developed by DuPont Pharmaceuticals as DMP-266 and marketed under the brand name Sustiva [1-3]. Efavirenz was the first antiretroviral medicine used as a single daily dose [1-4]. Currently, it is used for the treatment of HIV infection as a first-choice antiretroviral agent [1-2].

The action mechanism of efavirenz is noncompetitive suppression of reverse transcriptase (the enzyme of HIV-1 virus), at the same time efavirenz does not inhibit α-, β- and γ-DNA-polymerases [1-2]; therefore, it is used for the treatment of HIV-1 infection in combination with other antiretroviral medicines [1-4].

Efavirenz therapy accompanies with quite a number of side effects manifested by psychiatric symptoms, including insomnia, nightmares, memory loss, depression, and anxiety. Efavirenz is characterized by certain neuropsychological symptoms in 50 % of cases; its neurotoxicity exceeds other antiretroviral medicines [5-7]. The studies of efavirenz showed that in 20-50 % of cases the toxic concentrations of the medicine in blood were fixed [8-9]. There are cases of acute poisoning due to administration of efavirenz, including cases of suicide attempts [10-11]. Therefore, in our opinion, efavirenz may be approved as a potential toxic compound in forensic toxicology.

High-performance liquid chromatography (HPLC) with different detection methods is the most popular method used in the analysis of efavirenz [12-16], but the procedures described do not use unified methods of sample preparation and unified screening chromatographic conditions, the so-called HPLC-analyzer system. The MiLiChrome® A-02 HPLC-analyzer system [17] is widely used in the Ukrainian laboratories of forensic toxicology. Previously [18] we developed the HPLC/UV-procedure for efavirenz quantitative determination using this chromatographic system, performed its validation by model solutions and showed the possibility for application in analytical toxicology.

The aim of the present work is to apply the HPLC/UV-procedure described for efavirenz quantitative determination in biological fluids (whole blood and urine) using different types of sample preparation procedures, perform its validation by matrix samples in the variants of the methods of calibration curve (MCC) and standard (MS) and prove the acceptability of application for forensic toxicology.

Results and discussion

The HPLC/UV-method for efavirenz determination was proposed by the authors before [18] and its specificity in relation to other antiretroviral medicines (lamivudine, zidovudine, tenofovir, abacavir, stavudine and didanosine) was shown.

Taking into account the results of the efavirenz extraction study [21] we proposed to carry out the sample preparation of the blood and urine for efavirenz determination in three ways according to Scheme 1:

1) liquid-liquid extraction with the mixture of chloroform and 2-propanol (80 : 20);
2) 2-propanol extraction and salting-out with ammonium sulfate;
3) acetonitrile extraction and salting-out with ammonium sulfate.

As a result three HPLC/UV-procedures of efavirenz determination in the blood and urine were developed.

Validation of all procedures developed was performed by such parameters as specificity, recovery, linearity, accuracy and precision according to the approaches offered before in the variants of the methods of calibration curve and standard [19].

The validation procedure foresees application of the normalized coordinates. For normalization of the experimental data obtained the reference solution with the concentration of the analyte corresponded to its concentration in the end solution to be analyzed under the condition of zero losses for the point of 100 % in the normalized coordinates was used. The peak area for reference solution was corrected taking into account the value of recovery $R$, which significance and value was shown at the preliminary stage of validation, and used for normalization of peak areas for the model samples.

The range of the methods application was $D = 25-175$ %; the number of concentration levels was $g = 7$ in constant increments of 25 %; as 25 % the mean therapeutic efavirenz concentration in the blood [8-9] – 5 $\mu$g/mL – was accepted.

The validation of the methods was performed using model samples. The determination procedure and acceptability criteria [19] are presented in Scheme 2.

The total results of validation are presented in Tab. 1-4.

The results of analysis have shown the absence of peaks with the retention time, which is coincident with (or near to) the efavirenz retention time (11.95 min), on the chromatograms of blank-samples for all variants of procedures of the analyte isolation from biological fluids. The conclusion can be made about acceptable specificity of the methods developed as for the components of the biological matrix.

All procedures of sample preparation show the high efficiency of efavirenz isolation both for the blood and urine (at the level of 90 % and higher).

All procedures examined are characterized by the acceptable parameters of linearity. In all cases the values of $a$, $RSD_{\mu}$, $R_c$ are satisfied the acceptability criteria within both variants – by the method of calibration curve and the method of standard.

For MS accuracy and precision have been determined as within-run and between-run parameters by calibration samples, and for MCC – by calibration samples, as well as by model samples.

The values of systematic and random errors are within the acceptability criteria both in the variants
Scheme 2. The stages of validation of HPLC-procedures of efavirenz determination in biological fluids using calibration and model samples.
### Table 1

The total results of recovery determination for HPLC/UV-procedures of efavirenz determination in blood and urine

| Parameter | Procedure | Acceptability criterion |
|-----------|-----------|-------------------------|
| Blood     |           |                         |
| $\bar{R}$ | 82.91     | 93.76                   | 94.21                   |
| $\Delta_{CR}$ | 4.67 | 5.84 | 5.73 | $\leq 20.00 \%$ |
| $b^u/s^u$ | $-0.01/0.01$ | $0.02/0.01$ | $0.01/0.01$ |
| $a^u/s^u$ | 83.53/1.07 | 92.24/1.45 | 93.12/1.48 | $a^u > 1.812 \cdot s^u$ |
| $|100 - \bar{R}|$ | 17.09 | 6.24 | 5.79 | $\leq 6.40 \%$ |
| Urine     |           |                         |
| $\bar{R}$ | 82.85     | 89.82                   | 90.09                   |
| $\Delta_{CR}$ | 4.99 | 13.64 | 13.57 | $\leq 20.00 \%$ |
| $b^u/s^u$ | $0.02/0.01$ | $0.07/0.03$ | $0.07/0.03$ |
| $a^u/s^u$ | 81.33/1.03 | 83.92/2.68 | 84.32/2.75 | $a^u > 1.812 \cdot s^u$ |
| $|100 - \bar{R}|$ | 17.15 | 10.18 | 9.91 | $\leq 6.40 \%$ |

### Table 2

The total results of linearity verification for HPLC/UV-procedures of efavirenz determination in blood and urine

| Parameter | Biological fluid | Acceptability criterion |
|-----------|------------------|-------------------------|
|           | run 1 | run 2 | run 3 | mean | run 1 | run 2 | run 3 | mean | MCC | MS |
| Procedure 1 | $a$ | 0.029 | 0.039 | 0.019 | 0.028 | 0.028 | 0.029 | 0.032 | 0.028 |
| $s_a$ | 3.228 | 4.339 | 2.171 | 3.095 | 3.178 | 3.198 | 3.567 | 3.129 | $a \leq 8.53 \%$ |
| $RSD_o$ | 3.819 | 5.134 | 2.569 | 3.662 | 3.761 | 3.784 | 4.220 | 3.703 | $\leq 9.93 \%$ |
| $R_c$ | 0.9982 | 0.9968 | 0.9992 | 0.9984 | 0.9983 | 0.9983 | 0.9979 | 0.9984 | $\geq 0.9830$ |
| Procedure 2 | $a$ | 0.024 | 0.033 | 0.029 | 0.027 | 0.029 | 0.032 | 0.038 | 0.032 |
| $s_a$ | 2.651 | 3.650 | 3.228 | 3.003 | 3.295 | 3.577 | 4.208 | 3.558 | $a \leq 8.53 \%$ |
| $RSD_o$ | 3.136 | 4.319 | 3.819 | 3.554 | 3.899 | 4.233 | 4.979 | 4.210 | $\leq 9.93 \%$ |
| $R_c$ | 0.9986 | 0.9975 | 0.9980 | 0.9983 | 0.9979 | 0.9976 | 0.9967 | 0.9976 | $\geq 0.9830$ |
| Procedure 3 | $a$ | 0.024 | 0.029 | 0.027 | 0.030 | 0.030 | 0.032 | 0.037 | 0.032 |
| $s_a$ | 2.656 | 3.655 | 3.192 | 2.993 | 3.323 | 3.633 | 4.180 | 3.578 | $a \leq 8.53 \%$ |
| $RSD_o$ | 3.142 | 4.325 | 3.776 | 3.541 | 3.932 | 4.299 | 4.946 | 4.234 | $\leq 9.93 \%$ |
| $R_c$ | 0.9986 | 0.9974 | 0.9980 | 0.9983 | 0.9979 | 0.9975 | 0.9967 | 0.9976 | $\geq 0.9830$ |
The total results of precision and accuracy determination for HPLC/UV-procedures of efavirenz determination in blood and urine in the variant of the method of calibration curve

| Parameter                        | Biological fluid | Acceptability criterion |
|----------------------------------|------------------|-------------------------|
|                                  | run 1 | run 2 | run 3 | run 1 | run 2 | run 3 |
|                                  | \(RR^k\) | \(\delta^k\) | \(RSD^k_{RR}\) | \(\Delta^k_{RR}\) | \(RR^k_{\text{intra}}\) | \(\delta^k_{\text{intra}}\) | \(RSD^k_{\text{intra}}\) | \(\Delta^k_{\text{intra}}\)
| within-run Procedure 1          |       |       |       |       |       |       |       |       |
| between-run                     |       |       |       |       |       |       |       |       |
|                                  | 100.87 | 101.03 | 100.47 | 100.90 | 100.79 | 100.66 | –    | –     |
|                                  | 0.87   | 1.03   | 0.47   | 0.90   | 0.79   | 0.66   | \(\leq 6.40\%\) | \(\leq 6.40\%\)
|                                  | 3.94   | 5.19   | 2.37   | 4.23   | 3.55   | 3.69   | –    | –     |
|                                  | 7.66   | 10.09  | 4.61   | 8.22   | 6.90   | 7.17   | \(\leq 14.14\%\) | \(\leq 14.14\%\)
| within-run Procedure 2          |       |       |       |       |       |       |       |       |
| between-run                     |       |       |       |       |       |       |       |       |
|                                  | 100.75 | 100.98 | 100.67 | 100.94 | 101.11 | 100.87 | –    | –     |
|                                  | 0.75   | 0.98   | 0.67   | 0.94   | 1.11   | 0.87   | \(\leq 6.40\%\) | \(\leq 6.40\%\)
|                                  | 3.71   | 4.82   | 3.21   | 4.38   | 4.90   | 4.20   | –    | –     |
|                                  | 7.21   | 9.37   | 6.24   | 8.51   | 9.52   | 8.16   | \(\leq 14.14\%\) | \(\leq 14.14\%\)
| within-run Procedure 3          |       |       |       |       |       |       |       |       |
| between-run                     |       |       |       |       |       |       |       |       |
|                                  | 100.80 | 100.98 | –     | –     | –     | –     | –    | –     |
|                                  | 0.80   | 0.98   | \(\leq 6.40\%\) | \(\leq 6.40\%\)
|                                  | 3.95   | 4.53   | –     | –     | –     | –     | –    | –     |
|                                  | 6.82   | 7.82   | \(\leq 14.14\%\) | \(\leq 14.14\%\)
|                                  | 105.72 | 102.08 | –     | –     | –     | –     | –    | –     |
|                                  | 5.72   | 2.08   | \(\leq 6.40\%\) | \(\leq 6.40\%\)
|                                  | 4.27   | 1.90   | –     | –     | –     | –     | –    | –     |
|                                  | 7.67   | 3.42   | \(\leq 20.00\%\) | \(\leq 20.00\%\)
| within-run Procedure 3          |       |       |       |       |       |       |       |       |
| between-run                     |       |       |       |       |       |       |       |       |
|                                  | 100.75 | 100.98 | 100.67 | 100.95 | 101.12 | 100.86 | –    | –     |
|                                  | 0.75   | 0.98   | 0.67   | 0.95   | 1.12   | 0.86   | \(\leq 6.40\%\) | \(\leq 6.40\%\)
|                                  | 3.72   | 4.81   | 3.19   | 4.42   | 4.94   | 4.17   | –    | –     |
|                                  | 7.23   | 9.35   | 6.20   | 8.59   | 9.60   | 8.10   | \(\leq 14.14\%\) | \(\leq 14.14\%\)
| within-run Procedure 3          |       |       |       |       |       |       |       |       |
| between-run                     |       |       |       |       |       |       |       |       |
|                                  | 100.80 | 100.98 | –     | –     | –     | –     | –    | –     |
|                                  | 0.80   | 0.98   | \(\leq 6.40\%\) | \(\leq 6.40\%\)
|                                  | 3.94   | 4.54   | –     | –     | –     | –     | –    | –     |
|                                  | 6.79   | 7.83   | \(\leq 14.14\%\) | \(\leq 14.14\%\)
|                                  | 106.32 | 101.95 | –     | –     | –     | –     | –    | –     |
|                                  | 6.32   | 1.95   | \(\leq 6.40\%\) | \(\leq 6.40\%\)
|                                  | 4.64   | 2.14   | –     | –     | –     | –     | –    | –     |
|                                  | 8.34   | 3.84   | \(\leq 20.00\%\) | \(\leq 20.00\%\)
The total results of precision and accuracy determination for HPLC/UV-procedures of efavirenz determination in blood and urine in the variant of the method of standard

| Procedure | Parameter | Biological fluid | Acceptability criterion |
|------------|-----------|------------------|-------------------------|
|            |           | run 1 | run 2 | run 3 | run 1 | run 2 | run 3 |
| within-run | \(Z_k\)   | 104.26 | 104.29 | 104.69 | 104.31 | 105.62 | 104.66 | – |
|            | \(\delta_k\) | 4.26 | 4.29 | 4.69 | 4.31 | 5.62 | 4.66 | \(\leq 6.40 \%\) |
|            | \(RSD_k^z\) | 2.86 | 3.78 | 2.91 | 3.07 | 3.25 | 4.79 | – |
|            | \(\Delta_k^z\) | 5.56 | 7.35 | 5.65 | 5.97 | 6.32 | 9.31 | \(\leq 20.00 \%\) |
| between-run | \(Z_{\text{intra}}\) | 104.41 | – | 104.86 | – | – | – | – |
|            | \(\delta_{\text{intra}}\) | 4.41 | 4.86 | – | – | – | – | \(\leq 6.40 \%\) |
|            | \(RSD_{\text{intra}}^z\) | 3.21 | 3.78 | – | – | – | – | – |
|            | \(\Delta_{\text{intra}}^z\) | 5.54 | 6.52 | – | – | – | – | \(\leq 20.00 \%\) |
| within-run | \(Z_k\) | 96.84 | 97.88 | 97.45 | 96.71 | 98.21 | 97.40 | – |
|            | \(\delta_k\) | 3.16 | 2.12 | 2.55 | 3.29 | 1.79 | 2.60 | \(\leq 6.40 \%\) |
|            | \(RSD_k^z\) | 2.88 | 3.20 | 2.70 | 3.60 | 3.07 | 4.12 | – |
|            | \(\Delta_k^z\) | 5.60 | 6.22 | 5.25 | 7.00 | 5.97 | 8.01 | \(\leq 20.00 \%\) |
| between-run | \(Z_{\text{intra}}\) | 97.39 | – | 97.44 | – | – | – | – |
|            | \(\delta_{\text{intra}}\) | 2.61 | 2.56 | – | – | – | – | – |
|            | \(RSD_{\text{intra}}^z\) | 2.93 | 3.62 | – | – | – | – | – |
|            | \(\Delta_{\text{intra}}^z\) | 5.05 | 6.24 | – | – | – | – | – |
| within-run | \(Z_k\) | 97.14 | 97.57 | 97.12 | 97.07 | 98.54 | 97.75 | – |
|            | \(\delta_k\) | 2.86 | 2.43 | 2.88 | 2.93 | 1.46 | 2.25 | \(\leq 6.40 \%\) |
|            | \(RSD_k^z\) | 2.77 | 3.24 | 2.80 | 3.46 | 3.05 | 3.91 | – |
|            | \(\Delta_k^z\) | 5.38 | 6.30 | 5.44 | 6.72 | 5.93 | 7.60 | \(\leq 20.00 \%\) |
| between-run | \(Z_{\text{intra}}\) | 97.28 | – | 97.78 | – | – | – | – |
|            | \(\delta_{\text{intra}}\) | 2.72 | 2.22 | – | – | – | – | \(\leq 6.40 \%\) |
|            | \(RSD_{\text{intra}}^z\) | 2.95 | 3.49 | – | – | – | – | – |
|            | \(\Delta_{\text{intra}}^z\) | 5.09 | 6.02 | – | – | – | – | \(\leq 20.00 \%\) |

of the method of calibration curve and the method of standard.

The values of systematic errors are lower when application of MCC; precision is at the same level for both type of the experiment design.

**Experimental part**

Efavirenz was of pharmacopoeial purity.

The procedure of preparation of the calibration and model samples of blood and urine is presented in Scheme 3.

The stock solutions 1 and 2 (1000 μg/mL) were prepared by dissolving 200.0 mg of efavirenz in 20.00 mL of 0.1 mole/L sodium hydroxide solution, and the solutions were diluted to 200.0 mL with distilled water. The stocksolutions 1 and 2 were diluted with 0.01 mole/L sodium hydroxide solution to prepare:
- the stock solutions 1.1-1.7 having concentrations of 50; 100; 150; 200; 250; 300; 350 μg/mL, respectively;
- the stock solutions 2.1-2.4 having concentrations of 50; 100; 200 and 350 μg/mL, respectively.
Scheme 3. The order of preparation of the samples for validation of efavirenz determination procedures in blood and urine

Three batches (in 11 samples each) of the corresponding matrix (blood or urine) obtained from three different sources were used to prepare the calibration samples 1-7 and model samples 1-4 by spiking 10.00 mL of the matrix with 1 mL of the stock solutions 1.1-1.7 and 2.1-2.4, respectively.

After spiking all samples were vortexed for 1 hour and stored for 24 hours at ambient temperature before the sample processing.

Blank-samples were prepared by spiking 5 samples (10.00 mL) of the corresponding matrix (blood or urine) obtained from different sources with 1.00 mL of 0.01 mole/L sodium hydroxide solution.

The stock solution 3 (100 μg/mL) was prepared by dissolving 50.0 mg of efavirenz in 50.00 mL of 0.1 mole/L sodium hydroxide solution, and the solution was diluted to 500.0 mL with distilled water. The reference solution (8 μg/mL) was prepared by diluting 4.00 mL of the stock solution 3 to 50.0 mL with 0.01 mole/L sodium hydroxide solution. The stock solution 3 was diluted with 0.01 mole/L sodium hydroxide solution to prepare the model solutions 1-4 having concentrations of 2; 4; 8 and 14 μg/mL, respectively.

The procedures of sample preparation of biological fluids for efavirenz determination are presented in Scheme 1.

Procedure 1: 10.00 mL of blood or urine was diluted with 20.00 mL of distilled water and then acidified with 10.00 mL of 10 % trichloroacetic acid solution (pH ≤ 2). The mixture was vortexed for 1 hour, then centrifuged for 5 min at 5000 rpm. The supernatant (pH should be less than 2) was extracted with 10.00 mL of the mixture of chloroform and 2-propanol (80 : 20) three times. The organic extracts obtained were separated, filtered through the paper filter with 1 g of anhydrous sodium sulfate into a 50.0 mL measuring flask, and diluted to the volume with chloroform.

Procedure 2: 10.00 mL of blood or urine was diluted with 20.00 mL of distilled water and then acidified with 10.00 mL of 10 % trichloroacetic acid solution (pH ≤ 2). The mixture was vortexed for 1 hour and then processed twice with 10.00 mL of acetonitrile and vortexed for 1 hour each time. After adding 2 g of ammonium sulfate the mixture was filtered through the paper filter (wetted with acetonitrile) into the separating funnel and salted-out by adding ammonium sulfate till its dissolution stops. The top organic layer was separated, filtered through the paper filter with 1 g of anhydrous sodium sulfate into a 50.0 mL measuring flask, and diluted to the volume with acetonitrile.

Procedure 3: 10.00 mL of blood or urine was diluted with 20.00 mL of distilled water and then acidified with 10.00 mL of 10 % trichloroacetic acid solution (pH ≤ 2). The mixture was vortexed for 1 hour and then processed twice with 10.00 mL of 2-propanol and vortexed for 1 hour each time. After adding 2 g of ammonium sulfate the mixture was filtered through the paper filter (wetted with 2-propanol) into the separating funnel and salted-out by adding ammonium sulfate till its dissolution stops. The top organic layer was separated, filtered through the paper filter with 1 g of anhydrous sodium sulfate into a 50.0 mL measuring flask, and diluted to the volume with 2-propanol.

TLC-purification: 20.00 mL of the organic extract obtained were evaporated at 80 °C to complete removal of the organic layer; the dry residue was dissolved in ≈ 0.5 mL of chloroform and applied quantitatively on the start line of the chromatographic...
plate in the form of stripe 2 cm wide. 10 μL of efavirenz standard ethanol solution (1 mg/mL) were applied in the point (“testifier”) near the stripe. The plate was eluted in chloroform twice and then using the mixture of chloroform and methanol (90 : 10) as a mobile phase then it was dried out, the “testifier” stripe was developed in UV-light, and the spot of a violet color in the area of \( R_f = 0.35-0.55 \) was observed. The sorbent was carefully removed from the plate part with the area of 3 cm × 1 cm at the level of “testifier” into the glass bottle with 10.00 mL of 0.01 mole/L sodium hydroxide solution, the bottle content was vortexed for 5 min and filtered through the paper filter wetted with the corresponding solvent (the solution to be analyzed).

The chromatographic plates Sorbfil® PTLC-IIH-UV (silica gel STC-1HP, PETP, luminophor, silica sol, 8 ÷ 12 μm fraction, 100 μm layer thickness) were purchased from MID LLC (Russia).

The model and calibration samples, and also blank-samples were analyzed for each procedure of sample preparation.

The chromatographic conditions were as follows:

- device – MiLiChrome® A-02 high pressure liquid chromatograph (EcoNova, Russia);
- software – Analytika-Chrom® (Analytika SPF, Ukraine);
- column – Ø2 × 75 mm, ProntoSIL 120-5-C18 AQ, 5 μm (BISCHOFF Analysentechnik und -geräte GmbH, Germany);
- temperature – 40 °C;
- flow rate – 100 μL/min;
- Eluent A – 0.2 M LiClO₄ – 0.005 M HClO₄;
- Eluent B – acetonitrile;
- elution mode – linear gradient (from 5 % to 100 % Eluent B for 40 min, then 100 % Eluent B for 3 min);
- detection – UV, 247 nm;
- volume of injection – 2 μL.

Each solution to be analyzed was chromatographed 3 times or, as required, more following the requirements to repeatability of peaks areas \( S \) for replicate injections [19] – the relative standard deviation of the mean \( \text{RSD}_{\text{nom}} \) calculated towards the nominal value of peak area \( S_{\text{nom}} \) should not exceed:

\[
\text{RSD}_{\text{nom}} = \frac{s}{S_{\text{nom}}} \cdot 100 \% \leq \frac{0.1 \cdot \max \Delta_{\text{rel}} \cdot \sqrt{n}}{t(95 \%; n - 1)} = \begin{cases} 
1.47 \%; & n = 3 \\
1.88 \%; & n = 4 \\
2.22 \%; & n = 5 \\
2.52 \%; & n = 6 
\end{cases}
\]

where: \( \max \Delta_{\text{rel}} \) – is the extreme relative uncertainty of the procedure of analysis, \( \max \Delta_{\text{rel}} = 20 \% \) [20]; \( S_{\text{nom}} \) – is the mean peak area obtained when analyzing the corresponding solutions with the analyte concentration corresponded to the point of 25 % in the normalized coordinates.

**Conclusions**

For HPLC/UV-determination of efavirenz in blood and urine three types of the sample preparation have been proposed – 1) liquid-liquid extraction with the mixture of chloroform and 2-propanol (80 : 20); 2) 2-propanol extraction and salting-out with ammonium sulfate; 3) acetonitrile extraction and salting-out with ammonium sulfate. All procedures are effective by the recovery parameter, and may be used in laboratories of forensic toxicology according to the presence of the corresponding reagents and methods.

Additional purification has been carried out by the TLC method. The quantitative determination of efavirenz in the organic extracts obtained has been performed by the method of HPLC/UV using the MiLiChrome® A-02 HPLC-analyzer system.

Three HPLC/UV-procedures of efavirenz determination in blood and urine have been developed; validation of the procedures developed has been performed in the variants of MCC and MS by such parameters as specificity, recovery, linearity, accuracy and precision. According to the results of validation all procedures may be recommended for application in laboratories of forensic toxicology.

**Conflict of interests:** authors have no conflict of interests to declare.

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