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Development and validation of HPLC/UV-procedure for efavirenz quantitative determination

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Abstract

Efavirenz is a synthetic compound from the group of non-nucleoside reverse transcriptase inhibitors used for treatment of HIV infection. There are cases of acute poisoning due to administration of efavirenz, including cases of suicide attempts. The aim is to develop HPLC/UV-procedure of efavirenz quantification using the system of HPLC-analyzer MiLiChrome® A-02. HPLC microcolumn of Ø2×75 mm dimension and reversed phase ProntoSIL 120-5-C18 AQ, 5 µm were used as an analytical column. *Eluent A* (0.2 M $LiClO_4$ – 0.005 M $HClO_4$) and *Eluent B* (acetonitrile) were used as the mobile phase components. The mobile phase was run in gradient elution mode, namely from 5% to 100% *Eluent B* for 40 min, then 100% *Eluent B* for 3 min. All analysis was carried out at 40°C and flow rate of 100 µl/min. Detection was performed at 247 nm. The specificity of the used chromatographic conditions has been confirmed in relation to other antiretroviral medicine. Retention time for efavirenz is 11.95 min. Ethanol has been proposed for preparation of efavirenz solutions. To prove the possibility of the proposed procedure application in further analysis its step-by-step validation has been carried out in the variants of the method of calibration curve, method of standard and method of additions. Such validation parameters as in process stability, linearity/calibration model, accuracy and precision (repeatability) have been estimated by model solutions. New procedure of efavirenz quantitative determination by the method of HPLC/UV has been developed; its acceptability for application has been shown.

Keywords: efavirenz, high-performance liquid chromatography, validation, method of calibration curve, method of standard, method of additions

INTRODUCTION

Efavirenz is a synthetic compound attributed to the group of non-nucleoside reverse transcriptase inhibitors; it is used for treatment of HIV infection as a first-line antiretroviral medicine [1].

Efavirenz is active only to HIV-virus of type 1 because of its action mechanism – efavirenz noncompetitively suppresses reverse transcriptase (the enzyme of HIV-1 virus) and does not inhibit α-, β- and γ-DNA-polymerases [2 – 4].

Treatment with efavirenz accompanies with quite a number of side effects showed 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 – 12].

The studies of efavirenz showed that in 20 – 50% of cases the toxic concentrations of the medicine in blood were fixed [13 – 16]. There are cases of acute poisoning due to administration of efavirenz, including cases of suicide attempts [17 – 19].

Use of efavirenz can produce a false positive result in blood and urine tests for marijuana [20].

HPLC is used to analyse efavirenz in pharmaceuticals and biological liquids widely enough [21 – 25]. The main disadvantage of the present procedures is their application exclusively for efavirenz quantification; both chromatographic conditions and sample preparations are specially chosen to analyse efavirenz. It is usual situation for pharmacokinetic studies, but in forensic toxicology it is impossible to use individual procedures for each analyte, it is necessary to use unified technics of sample preparation and unified screening chromatographic conditions, so called HPLC-analyzer system.

So this research is conducted to develop HPLC/UV-procedure of efavirenz quantification using the system of HPLC-analyzer MiLiChrome® A-02, which is implemented in practice of forensic medical laboratories in Russia and Ukraine. Step-by-step validation of the developed procedure has been performed according to the offered approaches [26 – 32] in the variants of the method of calibration curve (MCC), method of standard (MS) and method of additions (MA) to choose the optimal variant for further application. Another purpose of our experiment is to accumulate experience of application of the offered standardized validation procedures for method development.

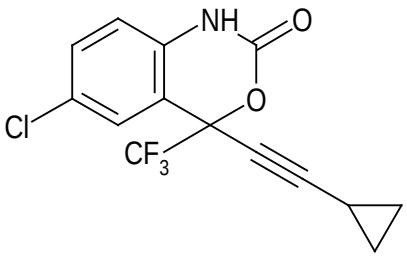


Figure 1. Chemical structure of efavirenz

MATERIALS AND METHODS

Instrumentation and chromatographic conditions

The HPLC/UV analyses were performed using high pressure liquid chromatograph MiLiChrome® A-02 (EcoNova, Russia) equipped with double syringe gradient pump, autosampler (sample volume is 0 – 99 µI), column oven (35 – 90°C) and double-beam multiwave UV-spectrophotometer as a detector. Analitika-Chrom® software (Analitika SPF, Ukraine) was used for integration and processing of chromatograms. HPLC microcolumn of Ø2×75 mm dimension and reversed phase ProntoSIL 120-5-C18 AQ, 5 µm (BISCHOFF Analysentechnik und -geräte GmbH, Germany) were used as an analytical column. All analysis was carried out at 40°C and flow rate of 100 µl/min. The mobile phase was run in gradient elution mode, namely from 5% to 100% *Eluent B* for 40 min, then 100% *Eluent B* for 3 min. Detection was performed at 247 nm. The volume of injection was 2 µL.

Weighing was carried out using digital analytical balance AN100 (AXIS, Ukraine) with d = 0.0001 g.

Glassware satisfied ISO 648:2008 «Laboratory glassware – Single-volume pipettes», ISO 1042:1998 «Laboratory glassware – One-mark volumetric flasks», ISO 4788:2005 «Laboratory glassware – Graduated measuring cylinders», ISO 385:2005 «Laboratory glassware – Burettes» and calibrated according to ISO 4787:2010 «Laboratory glassware – Volumetric instruments – Methods for testing of capacity and for use» and «Guidelines for calibration in analytical chemistry» [33] was used throughout this study.

Reagents and chemicals

Efavirenz was of pharmacopoeial purity. Acetonitrile CHROMASOLV®Plus for HPLC and perchloric acid (70%, puriss. p.a., ACS reagent) were purchased from Sigma-Aldrich Co. LLC (USA), lithium perchlorate trihydrate was purchased from Panreac Química S.L.U. (Spain). Ethanol was of analytical grade.

Mobile phase preparation

Eluent A (0.2 M $LiClO_4$ – 0.005 M $HClO_4$) and *Eluent B* (acetonitrile) were used as the mobile phase components. *Solution 1* and *Solution 2* were obtained for *Eluent A* preparation.

Solution 1 (4.1 M $LiClO_4$ aqueous solution): 330.00 g of $LiClO_4 \cdot 3H_2O$ were dissolved in 450 ml of bidistilled water while stirring and heating to 50°C, the solution obtained was cooled to ambient temperature and transferred to the measuring flask with the capacity of 500.0 ml, the solution was diluted to the volume with the same solvent and then filtered through the membrane filter Millex® HA Filter (0.45 μm pore size, mixed cellulose esters, PVC housing) purchased from Merck Millipore Corporation (USA).

Solution 2 (4 M $LiClO_4$ solution in 0.1 M $HClO_4$ solution): 2.2 ml of $HClO_4$ was measured by the pipette with the capacity of 5.0 ml into the measuring flask with the capacity of 250.0 ml, the solution was diluted to the volume with *Solution 1*.

Eluent A: 10.0 ml of *Solution 2* was measured by the pipette into the measuring flask with the capacity of 200.0 ml, the solution was diluted to the volume with bidistilled water.

Reference and model solutions

The method of calibration curve and the method of standard (Scheme 1)

The stock solutions 1 and 2 (100 μg/mL) were prepared by dissolving 50.0 mg of efavirenz in ethanol and the solutions were diluted to 500.0 mL with the same solvent. The reference solution (8 μg/mL) was prepared by diluting 4.00 mL of the stock solution 1 to 50.0 mL with ethanol. The stock solution 2 was diluted with ethanol to prepare the model solutions 1 – 7 having concentrations of 2; 4; 6; 8; 10; 12 and 14 μg/mL respectively.

The method of additions (Scheme 2)

The stock solution 3 (100 μg/mL) was prepared by dissolving 50.0 mg of efavirenz in ethanol and the solution was diluted to 500.0 mL with the same solvent. The addition solution 1 (300 μg/mL) was prepared by dissolving 60.0 mg of efavirenz in ethanol and the solution was diluted to 200.0 mL with the same solvent. The stock solution 3 was diluted with ethanol to prepare the model solutions 8 – 13 having concentrations of 10; 10; 20; 30; 40; 40 μg/ml respectively. The model solutions 8.1 – 13.1 were prepared by diluting 10.00 mL of the model solution 8 – 13 to 50.0 mL with ethanol. For preparing the model solutions 8.2 – 13.2 10.00 mL of the model solutions 8 – 13 were mixed with 1.00 mL of the addition solution 1 and diluted to 50.0 mL with ethanol.

When experiments carrying out each solution (excepting in process stability studying) was chromatographed 3 times or, as required, more following the requirements to repeatability of peaks areas *S* for replicate injections offered by us [26] – the relative standard deviation of the mean RSD_{nom} calculated towards the nominal value of peak area S_{nom} should not exceed:

$$RSD_{nom} = \frac{S}{S_{nom}} \cdot 100\% \le \max RSD_{nom} = \frac{0.1 \cdot \max\Delta_{As} \cdot \sqrt{n}}{t(95\%; n-1)} = \begin{Bmatrix} 1.21\%;\ n=3 \\ 1.74\%;\ n=4 \\ 2.15\%;\ n=5 \\ 2.49\%;\ n=6 \end{Bmatrix},$$

where S_{nom} – the mean peak area obtained when analysing the model solution 1. The mean values were used in further calculations.

RESULTS AND DISCUSSION

Chemically, efavirenz is (*S*)-6-chloro-4-cyclopropylethynyl-1,4-dihydro-4-trifluoromethyl-2*H*-3,1-benzoxazin-2-one and has the structural formula as shown on Figure 1.

We have previously [34] shown the possibility of application of direct UV-spectrophotometry for efavirenz quantitative determination using two solvents – 96% ethanol and 0.1 M sodium hydroxide solution (analytical wavelengths λ_{max} are 247 nm and 267 nm respectively). Both solvents provide sufficient stability of the medicines solutions [34].

Taking into account the chromatographic conditions used in experiment with application of the HPLC-analyzer «MiLiChrome® A-02» [35] (pH of mobile phase is more than 2.3) the most optimal solvent is ethanol for preparation of efavirenz solutions for development of quantification procedure, since it does not affect pH of eluent. In this case detection should be carried out at 247 nm, which corresponds to the absorption maximum of non-ionized form of efavirenz (Figure 2).

Previously the specificity of the proposed chromatographic conditions has been confirmed in relation to other antiretroviral medicine (lamivudine, zidovudine, tenofovir, abacavir, stavudine and didanosine). Retention time for efavirenz is 11.95 min, unlike for lamivudine (6.18 min), zidovudine (9.34 min), tenofovir (21.46 min), abacavir (11.18 min), stavudine (8.46 min) and didanosine (4.51 min).

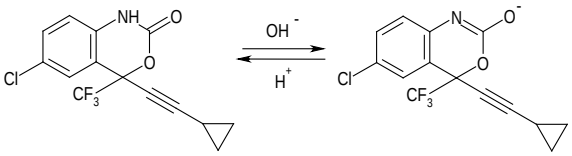


Figure 2. Transformations in the efavirenz solutions when changing the medium pH

Method validation *(Scheme 3)*

Validation of the developed procedure has been carried out in the variants of the method of calibration curve [26 – 30], method of standard [26, 31] and method of additions [26, 32].

Such validation parameters as in process stability, linearity/calibration model, accuracy and precision (repeatability) have been estimated by model solutions.

Method validation by model solutions according to Scheme 3 suggested previously [26] allows to assess the suitability of the actual analytical procedure for further work.

The validation provides application of the normalized coordinates:

$$X_i = \frac{C_i}{C_{st}} \cdot 100\%; \qquad Y_i = \frac{S_i}{S_{st}} \cdot 100\%$$

i. e. transition from the equation $S_i = b_1 \cdot C_i + a_1$ to the equation $Y_i = b_2 \cdot X_i + a_2$, that allows to calculate the validation characteristics, which do not depend on the analyte and features of the method of analysis.

The efavirenz concentration in the model solution for the point of 100% in the normalized coordinates $C_{100\%}^{model}$ has been chosen as the concentration provided the «signal/noise» ratio at the level of 40.

For normalization of the obtained experimental data the reference solution with the analyte concentration of $C_{reference}^{model} = C_{100\%}^{model}$ is used.

The analytical ranges *D* of the method application are 25 – 125%, 25 – 150% and 25 – 175%; the number of concentration levels *g* equals 5, 6 or 7 respectively in constant increments of

25%.

Acceptability criteria for validation parameters have been formed on the basis of systematic application of "insignificance concept" [36, 37] – the confidence interval Δ_2 is insignificant as compared with the confidence interval Δ_1 at the conventional level p = 95%, if the following inequality is correct:

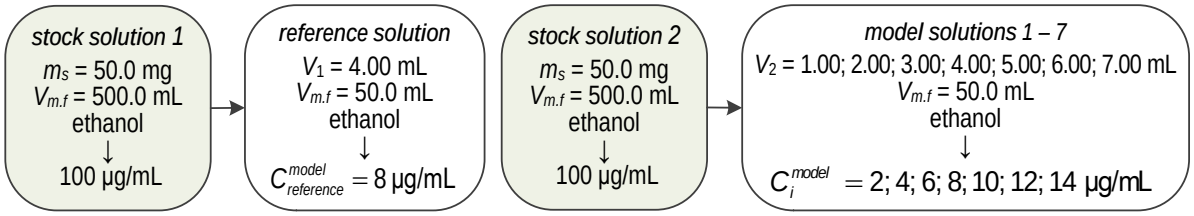
$$\Delta_2 \le 0.32 \cdot \Delta_1$$

and proceeding from the value of extreme uncertainty Δ_{As} for the methods in analytical toxicology, which equals 25% and 20% [38, 39] – for the lowest point of the analytical range of the method application and for the rest of range.

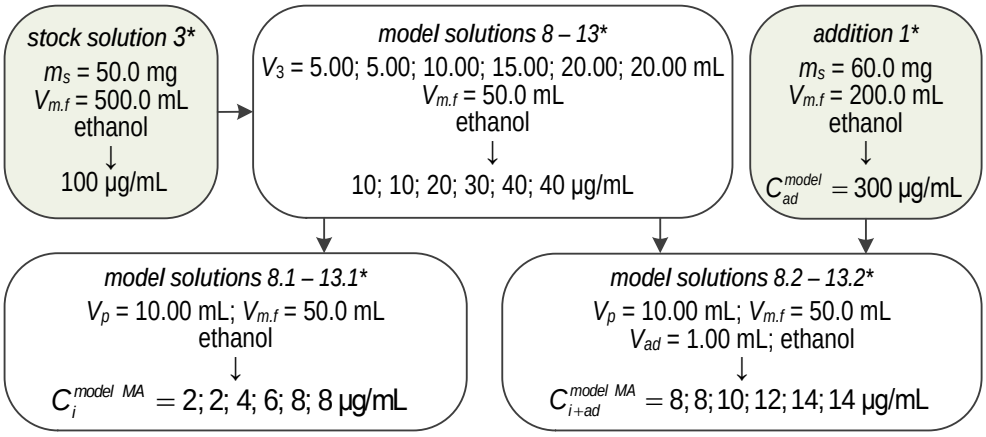
In the *MCC* acceptability criteria for linear dependence and precision have been found proceeding from the equality of uncertainty of plotting the calibration curve Δ_{cal} and uncertainty of analysis of the sample to be analysed Δ_{sample}.

Acceptability criteria for validation parameters have been calculated proceeding from the assumption that uncertainty of analyte quantification in model solutions Δ_{As}^{model} is insignificant as compared with total uncertainty Δ_{As}:

$$\begin{gathered}\max \Delta_{As}^{model} = 0.32 \cdot \max \Delta_{As} = 0.32 \cdot 20.00\% = 6.40\%; \\ \max \Delta_{cal}^{model} = \max \Delta_{sample}^{model} = \frac{\max \Delta_{As}^{model}}{\sqrt{2}} = 0.707 \cdot \max \Delta_{As}^{model} = 0.707 \cdot 6.40\% = 4.52\%; \\ \max \delta^{model} = 0.32 \cdot \max \Delta_{As}^{model} = 0.32 \cdot 6.40\% = 2.05\%.\end{gathered} \quad (5)$$



Scheme 1. The preparation procedure for reference and model solutions of efavirenz



Scheme 1 . The preparation procedure for model solutions of efavirenz for MA

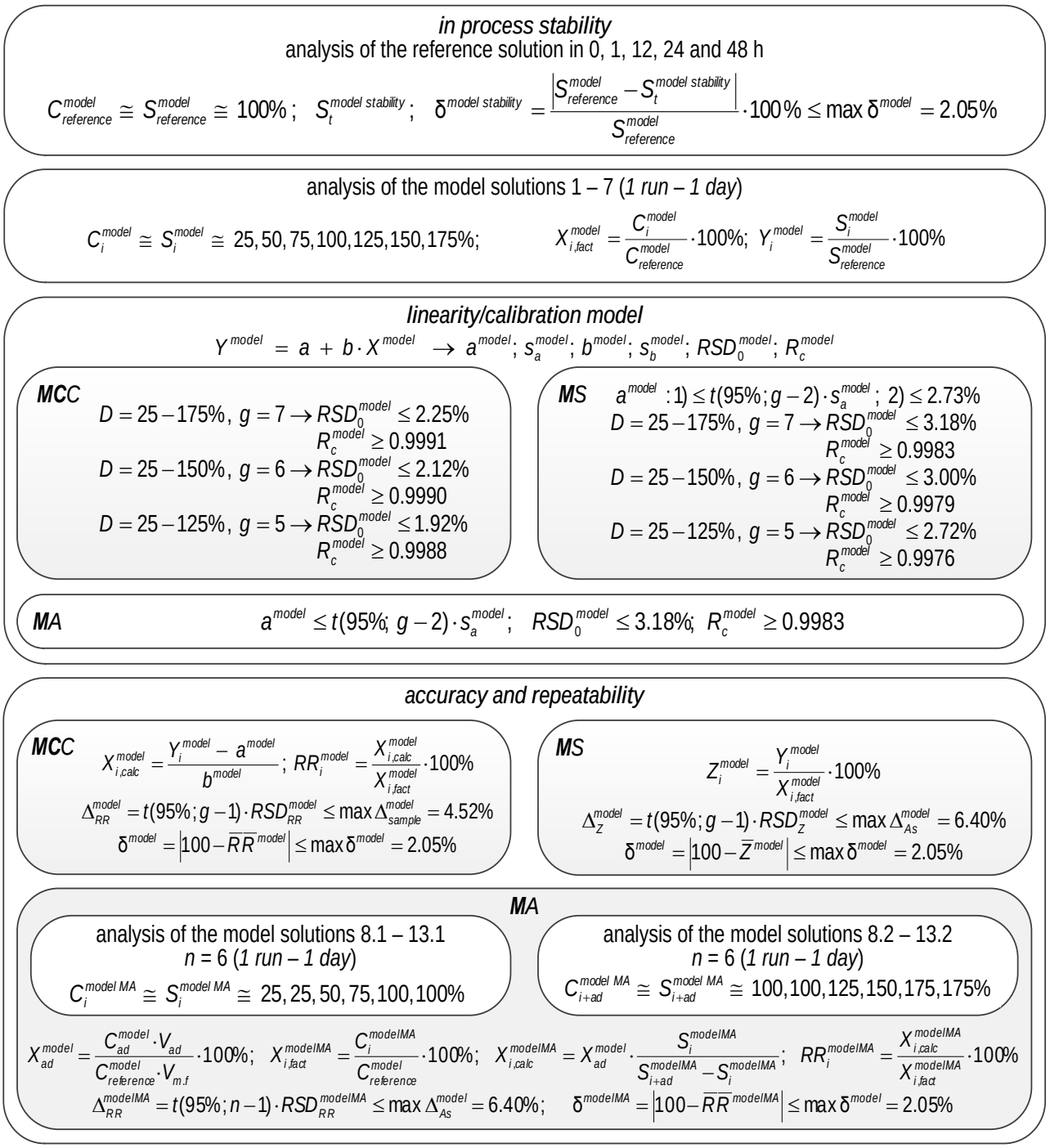
Validation results

In process stability of efavirenz in the model solution was verified by chromatographing the reference solution immediately and in 1, 12, 24 and 48 hours after its preparation, and the systematic error $\delta^{model\ stability}$ was calculated and assessed (Table 1). *In process stability* of efavirenz in model solutions is satisfied the acceptability criteria for all periods of time.

To determine *linearity/calibration model* the model solutions 1 – 7 were analysed within 1 run, correlation coefficient R_c^{model}, rest standard deviation RSD_0^{model} and also absolute term a^{model} (if it is necessary) were calculated and assessed (Table 2).

To estimate *precision (repeatability) and accuracy:*

- *MCC:* the model solutions 1 – 7 concentrations were calculated using the linear dependence obtained and the values «found/given» RR_i^{model} were used to determine the confidence interval Δ_{RR}^{model} and the systematic error δ^{model} respectively (Table 3);
- *MS:* the ratios Z_i^{model} for the model solutions 1 – 7 were calculated and used to determine the confidence interval Δ_Z^{model} and the systematic error δ^{model} respectively (Table 4);
- *MA:* the model solutions 8.1 – 13.1 and 8.2 – 13.2 were analysed within 1 run, the model solutions 8.1 – 13.1 concentrations were recalculated and the values «found/given» $RR_i^{model\ MA}$ were used to determine the confidence interval $\Delta_{RR}^{model\ MA}$ and the systematic error $\delta^{model\ MA}$ respectively.



***Scheme 2*. The validation stages of HPLC/UV-procedures for efavirenz determination**

Table 1

The results of in process stability verification for efavirenz in model solutions

Parameter	Values					
	0 h	1 h	12 h	24 h	36 h	48 h
$S^{model\ stability}$	0.007578	0.007512	0.007536	0.007547	0.007523	0.007542
$S_0^{model\ stability} - S_t^{model\ stability}$	–	0.000066	0.000042	0.000031	0.000055	0.000036
$\delta^{model\ stability}$,%	–	0.87	0.55	0.41	0.73	0.48
$\delta^{model\ stability} \le \max \delta^{model} = 2.05\%$	–	satisfied	satisfied	satisfied	satisfied	satisfied

Table 2

The results of linearity verification of efavirenz determination procedures by the method of HPLC/UV

Parameter	Values	Acceptability criterion		
		MCC	***MS***	***MA***
D = 25 – 175% (g = 7)				
b^{model}	1.019	–	–	
s_b^{model}	0.014	–	–	
a^{model}	–1.765	–	≤ 2.73%	
s_a^{model}	1.539	–	$a^{model} \le 2.015 \cdot s_a^{model}$	
RSD_0^{model}	1.821	≤ 2.25%	≤ 3.18%	
R_c^{model}	0.9995	≥ 0.9991	≥ 0.9983	
D = 25 – 150% (g = 6)				
b^{model}	0.996	–	–	–
s_b^{model}	0.004	–	–	–
a^{model}	–0.205	–	≤ 2.73%	–
s_a^{model}	0.363	–	$a^{model} \le 2.015 \cdot s_a^{model}$	–
RSD_0^{model}	0.389	≤ 2.12%	≤ 3.00%	–
R_c^{model}	1.0000	≥ 0.9990	≥ 0.9979	–
D = 25 – 125% (g = 5)				
b^{model}	0.994	–	–	–
s_b^{model}	0.006	–	–	–
a^{model}	–0.114	–	≤ 2.73%	–
s_a^{model}	0.457	–	$a^{model} \le 2.015 \cdot s_a^{model}$	–
RSD_0^{model}	0.436	≤ 1.92%	≤ 2.72%	–
R_c^{model}	1.0000	≥ 0.9988	≥ 0.9976	–

Table 3

The results of accuracy and precision verification (MCC) of efavirenz determination procedures by the method of HPLC/UV

Factual concentration of efavirenz in model solution ($C_{reference}^{model} = 8$ μg/mL)		Peak area S_i^{model}	Found in % to standard peak area Y_i^{model},%	Calculated concentration of efavirenz in model solution $X_{i,calc}^{model}$,%			RR_i^{model},%		
C_i^{model}, μg/mL	$X_{i,fact}^{model}$,%			25 – 175%	25 – 150%	25 – 125%	25 – 175%	25 – 150%	25 – 125%
2	25	0.001872	24.81	26.07	25.12	25.06	104.28	100.47	100.26
4	50	0.003708	49.15	49.94	49.55	49.54	99.88	99.10	99.07
6	75	0.005650	74.90	75.20	75.40	75.42	100.26	100.53	100.57
8	100	0.007516	99.62	99.45	100.22	100.29	99.45	100.22	100.29
10	125	0.009347	123.89	123.26	124.59	124.69	98.60	99.67	99.75
12	150	0.011266	149.34	148.22	150.13	–	98.81	100.09	–
14	175	0.013547	179.57	177.87	–	–	101.64	–	–
$S_{reference}^{model} = 0.013060$			$\overline{RR}^{model}$,%				100.42	100.01	99.99
$\delta^{model},\% = \lvert 100 - \overline{RR}^{model} \rvert$							0.42	0.01	0.01
$\delta^{model} \le \max \delta^{model} = 2.05\%$							satisfied	satisfied	satisfied
$RSD_{RR}^{model},\%$							1.98	0.54	0.59
$\Delta_{RR}^{model},\% = RSD_{RR}^{model} \cdot t(95\%; g-1)$							3.85	1.10	1.26
$\Delta_{RR}^{model} \le \max \Delta_{sample}^{model} = 4.52\%$							satisfied	satisfied	satisfied

Table 4

The results of accuracy and precision verification (MS) of efavirenz determination procedures by the method HPLC/UV

Factual concentration of efavirenz in model solution ($C_{reference}^{model} = 8$ μg/mL)		Peak area S_i^{model}	Found in % to standard peak area Y_i^{model},%	Z_i^{model},%		
C_i^{model}, μg/mL	$X_{i,fact}^{model}$,%			25 – 175%	25 – 150%	25 – 125%
2	25	0.001872	24.81	99.25	99.25	99.25
4	50	0.003708	49.15	98.30	98.30	98.30
6	75	0.005650	74.90	99.86	99.86	99.86
8	100	0.007516	99.62	99.62	99.62	99.62
10	125	0.009347	123.89	99.11	99.11	99.11
12	150	0.011266	149.34	99.56	99.56	–
14	175	0.013547	179.57	102.61	–	–
$S_{reference}^{model} = 0.013060$		$\overline{Z}^{model}$,%		99.76	99.28	99.23
$\delta^{model},\% = \left\|100 - \overline{Z}^{model}\right\|$				0.24	0.72	0.77
$\delta^{model} \le \max\delta^{model} = 2.05\%$				satisfied	satisfied	satisfied
$RSD_Z^{model},\%$				1.35	0.55	0.60
$\Delta_Z^{model},\% = RSD_Z^{model} \cdot t(95\%; g-1)$				2.63	1.11	1.27
$\Delta_Z^{model} \le \max\Delta_{As}^{model} = 6.40\%$				satisfied	satisfied	satisfied

Table 5

The results of accuracy and precision verification (MA) of efavirenz determination procedures by the method of HPLC/UV

Factual concentration of efavirenz in model solution ($C_{reference}^{model} = 16$ μg/mL)		Absorbance		Calculated concentration of efavirenz in model solution $X_{i,calc}^{model\ MA}$,%	$RR_i^{model\ MA}$,%
$C_i^{model\ MA}$, μg/mL	$X_{i,fact}^{model\ MA}$,%	$A_i^{model\ MA}$	$A_{i+ad}^{model\ MA}$		
4	25	0.001848	0.007575	24.20	96.79
4	25	0.001922	0.007629	25.26	101.03
8	50	0.003703	0.009337	49.30	98.61
12	75	0.005637	0.011265	75.12	100.16
16	100	0.007629	0.013265	101.52	101.52
16	100	0.007575	0.013465	96.47	96.47
$\overline{RR}^{model\ MAД},\%$					99.10
$\delta^{model\ MA},\% = \left\|100 - \overline{RR}^{model\ MA}\right\|$					0.90
$\delta^{model\ MA} \le \max\delta^{model} = 2.05\%$					satisfied
$RSD_{RR}^{model\ MA},\%$					2.15
$\Delta_{RR}^{model\ MA} = t(95\%; n-1) \cdot RSD_{RR}^{model\ MA}$					4.34
$\Delta_{RR}^{model\ MA} \le \max\Delta_{As}^{model} = 6.40\%$					satisfied

The values of confidence interval and systematic error were compared with the respective acceptability criteria.

Validation of the procedure has been carried out within 3 different analytical runs using different batches of reagents and different glassware; experiments have been performed by three different analysts. The results obtained within one analytical run are presented in Tables 1 – 5, but results of other analytical runs are at the same range of values.

The total results of validation allow to point to the conclusion about acceptable *linearity*, *accuracy* and *precision* of HPLC/UV-procedure of efavirenz quantitative determination in the variant of MCC, MS and MA for all ranges of the method application. It gives us the possibility to recommend this procedure for further application in forensic toxicology with the purpose of development of the methods of biological liquids analysis for efavirenz quantification.

The procedure in the variant of MA is characterized by the worst value of accuracy and the worst level of precision. In turn, the procedure in the variant of MS is characterized by the best values of precision and the middle values of accuracy. For the variant of MCC the best accuracy and the middle values of precision are observed. Thus application of the method of calibration curve is optimal for analysis.

CONCLUSIONS

New procedure of efavirenz quantitative determination by the method of HPLC/UV has been developed. Its validation by such parameters as stability, linearity, accuracy and precision in the variants of the method of calibration curve, method of standard and method of additions has been carried out and acceptability for application has been shown.

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