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O. I. Slabiak[1], I. M. Ivanchuk[1], L. Yu. Klimenko[2], Iu. S. Kolisnyk[2]

[1] Ivano-Frankivsk National Medical University
[2] National University of Pharmacy
53, Pushkinska str., Kharkiv, 61002, Ukraine. E-mail: lina_klimenko@nuph.edu.ua

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 °C; the flow rate – 100 μl/min; Eluent A – 0.2 M $LiClO_4$ – 0.005 M $HClO_4$; 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

О. І. Слаб'як, І. М. Іванчук, Л. Ю. Клименко, Ю. С. Колісник

Розробка та валідація ВЕРХ/УФ-методик визначення ефавіренцу в біологічних рідинах

Ефавіренц – ненуклеозидний інгібітор зворотної транскриптази з рядом побічних ефектів, що проявляються психіатричними симптомами. Описані випадки гострих отруєнь при застосуванні ефавіренцу, включаючи спроби самогубств, тому ефавіренц може бути сполукою, що має токсикологічне значення для судової токсикології.

Мета. Застосувати систему ВЕРХ-аналізатора MiLiChrome® A-02 для кількісного визначення ефавіренцу в біологічних рідинах і провести валідацію розроблених методик.

Результати та їх обговорення. Запропоновано три ВЕРХ/УФ-методики визначення ефавіренцу в крові і сечі. Валідацію всіх розроблених методик проведено за такими параметрами, як специфічність, ступінь ізолювання, лінійність, правильність і прецизійність у варіантах методів калібрувального графіка та стандарту. Результати аналізу показали відсутність піків з часом утримування, який співпадає з часом утримування ефавіренцу, на хроматограмах blank-зразків для всіх варіантів способів пробопідготовки. Всі процедури пробопідготовки показали високу ефективність ізолювання ефавіренцу як для крові, так і для сечі (на рівні 90 %). Всі розглянуті методики характеризуються прийнятними параметрами лінійності, within-run і between-run правильності та прецизійності.

Експериментальна частина. Пробопідготовку крові і сечі здійснювали трьома способами: 1) рідинно-рідинна екстракція сумішшю хлороформу та ізопропанолу (80 : 20); 2) екстракція ізопропанолом і висолювання амонію сульфатом; 3) екстракція ацетонітрилом і висолювання амонію сульфатом. Умови хроматографування: колонка – Ø2 × 75 мм, ProntoSIL 120-5-C18 AQ, 5 мкм; температура – 40 °С; швидкість потоку – 100 мкл/хв; елюент А – 0,2 М $LiClO_4$ – 0,005 М $HClO_4$; елюент Б – ацетонітрил; режим елюювання – лінійний градієнт; детектування – УФ, 247 нм; об'єм проби – 2 мкл.

Висновки. Розроблено комплекс ВЕРХ-методик кількісного визначення ефавіренцу в крові і сечі. Проведено валідацію розроблених методик.

***Ключові слова*:** ефавіренц; високоефективна рідинна хроматографія; кров; сеча; пробопідготовка; валідація

О. И. Слабяк, И. М. Иванчук, Л. Ю. Клименко, Ю. С. Колесник

Разработка и валидация ВЭЖХ/УФ-методик определения эфавиренца в биологических жидкостях

Эфавиренц – ненуклеозидный ингибитор обратной транскриптазы с рядом побочных эффектов, проявляющихся психиатрическими симптомами. Описаны случаи острых отравлений при применении эфавиренца, включая попытки самоубийств, поэтому эфавиренц может быть токсикологически значимым соединением для судебной токсикологии.

Цель. Применить систему ВЭЖХ-анализатора MiLiChrome® A-02 для количественного определения эфавиренца в биологических жидкостях и провести валидацию разработанных методик.

Результаты и их обсуждение. Предложены три ВЭЖХ/УФ-методики определения эфавиренца в крови и моче. Валидация всех разработанных методик проведена по таким параметрам, как специфичность, степень извлечения, линейность, правильность и прецизионность в вариантах методов калибровочного графика и стандарта. Результаты анализа показали отсутствие пиков с временем удерживания, которое совпадает со временем удерживания эфавиренца, на хроматограммах blank-образцов для всех вариантов способов пробоподготовки. Все процедуры пробоподготовки показали высокую эффективность извлечения эфавиренца как для крови, так и для мочи (на уровне 90 %). Все рассмотренные методики характеризуются приемлемыми параметрами линейности, within-run и between-run правильности и прецизионности.

Экспериментальная часть. Пробоподготовку крови и мочи осуществляли тремя способами: 1) жидко-жидкостная экстракция смесью хлороформа и изопропанола (80 : 20); 2) экстракция изопропанолом и высаливание аммония сульфатом; 3) экстракция ацетонитрилом и высаливание аммония сульфатом. Условия хроматографирования: колонка – Ø2 × 75 мм, ProntoSIL 120-5-C18 AQ, 5 мкм; температура – 40 °С; скорость потока – 100 мкл/мин.; элюент А – 0,2 М $LiClO_4$ – 0,005 М $HClO_4$; элюент Б – ацетонитрил; режим элюирования – линейный градиент; детектирование – УФ, 247 нм; объем пробы – 2 мкл.

Выводы. Разработан комплекс ВЭЖХ-методик количественного определения эфавиренца в крови и моче. Проведена валидация разработанных методик.

***Ключевые слова*:** эфавиренц; высокоэффективная жидкостная хроматография; кровь; моча; пробоподготовка; валидация

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.

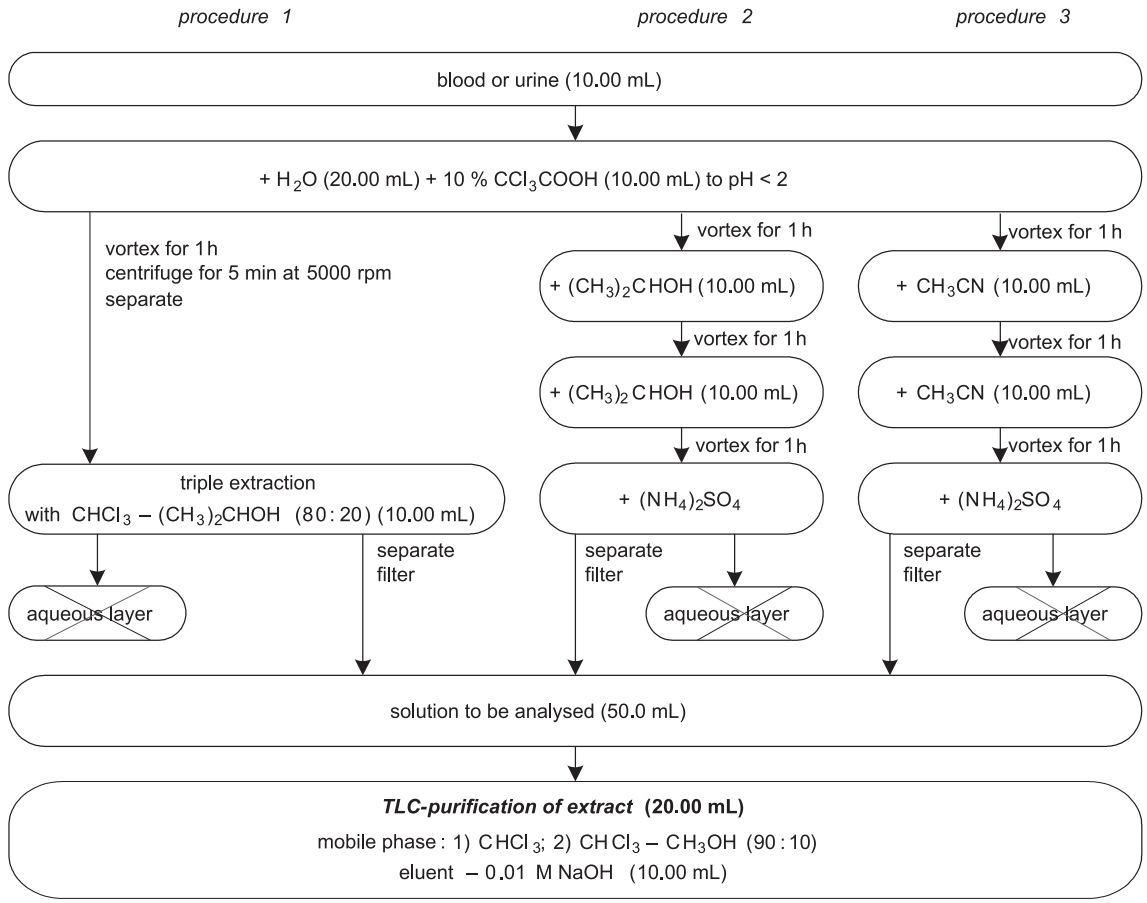
The aim of the present work is to apply the HPLC/UV-procedure described for efavirenz quantitative determination in biological fluids (whole blood and urine) using different types of sample preparation procedures, perform its validation by matrix samples in the variants of the methods of calibration 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 Sheme 1:

1) liquid-liquid extraction with the mixture of chloroform and 2-propanol (80 : 20);



Scheme 1. The main stages of the procedures of sample preparation of blood and urine for efavirenz determination by the method of HPLC/UV

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 μg/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 peakswith 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_0, 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

Analysis of the model solutions1–4 and reference solution (*1 run – 1 day*)

$C_i^{model} \cong S_i^{model} \cong 25, 50, 100, 175\ \%$

$C_{reference}^{model} \cong S_{reference}^{model} \cong 100\ \%$

Analysis of the calibration samples ; $g = 7$, $k = 3$ (*3 runs – 3 days*)

$C_i^{calibrator} \cong S_i^{calibrator} \cong 25, 50, 75, 100, 125, 150, 175\ \%$

Analysis of the model samples ; $n = 4$, $k = 3$ (*3 runs – 3 days*)

$C_i^{sample} \cong S_i^{sample} \cong 25, 50, 100, 175\%$

Specificity

Analysis of the blank-samples; $n = 5 \rightarrow \overline{S}_{blank}$ absence of peaks with analyte t_R or

$$RSD_{nom}\,(blank) = \frac{s}{S_{nom}} \cdot 100\ \% \le \frac{0.32 \cdot \max \Delta_{As} \cdot \sqrt{n}}{t(95\,\%, n-1)} = 6.71\ \%^1$$

$$S_{nom} = \overline{S}_{\min} = \overline{S}_{25\,\%}^{sample}$$

[1] if unsatisfied – n is increased

$$\delta_{blank\,(25\,\%)} = \frac{\overline{S}_{blank}}{\overline{S}_{25\,\%}^{sample}} \cdot 100\ \% \le \max \delta_{25\,\%} = 8.00\ \%^2$$

[2] if unsatisfied – it is necessary to modify the sample preparation procedure or chromatographing conditions

Recovery

Recovery determination (by model samples)

$$R_i = \frac{S_i^{sample}}{S_i^{model}} \cdot 100\ \%;\ \ \overline{R} = \frac{\sum R_i}{n \cdot k}$$

1) $\Delta_{R,r} = t(95\,\%,\ k \cdot n - 1) \cdot \sqrt{\frac{\sum (RSD_R^k)^2}{k}} \le \max \Delta_{As} = 20.00\ \%$

2) $R = a^R + b^R \cdot X \rightarrow a^R;\ s_a^R;\ b^R;\ s_b^R;$

$a^R > t(95\,\%;\ k \cdot n - 2) \cdot s_a^R;\ b^R \le t(95\,\%;\ k \cdot n - 2) \cdot s_b^R$

3) $\left|100 - \overline{R}\right| \le \max \delta = 6.40\ \%^3$

[3] if unsatisfied – the value of R is considered when calculating

Linearity

Normalization of the data obtained

$$X_{i,fact} = \frac{C_i^{calibrator}}{C_{reference}} \cdot 100\ \%;\ C_{reference} = C_{reference}^{model} \cdot K^4$$

$$Y_i = \frac{S_i^{calibrator}}{S_{reference}} \cdot 100\ \%;\ S_{reference} = \frac{S_{reference}^{model} \cdot \overline{R}}{100}$$

[4] dilution coefficient

Linearity/calibration model

within-run: $Y^k = a^k + b^k \cdot X^k \rightarrow a^k;\ s_a^k;\ b^k;\ s_b^k;\ RSD_0^k;\ R_c^k$

between-run: $Y = a + b \cdot X \rightarrow a;\ s_a;\ b;\ s_b;\ RSD_0;\ R_c$

MCC

$RSD_0 \le 7.02\ \%$

$R_c \ge 0.9915$

MS

a: 1) $\le t(95\,\%;\ g - 2) \cdot s_a$; 2) $\le 8.53\ \%$

$RSD_0 \le 9.93\ \%;\ \ R_c \ge 0.9830$

Accuracy and precision

Within-run

MCC

$$X_{i,calc}^k = \frac{Y_i^k - a^k}{b^k};\ RR_i^k = \frac{X_{i,calc}^k}{X_{i,fact}} \cdot 100\ \%$$

$$\Delta_{RR}^k = t(95\,\%;\ g - 1) \cdot RSD_{RR}^k \le \max \Delta_{sample} = 14.14\ \%$$

$$\delta^k = \left|100 - \overline{RR}^k\right| \le \max \delta = 6.40\ \%$$

MS

$$Z_i^k = \frac{Y_i^k}{X_{i,fact}} \cdot 100\ \%$$

$$\Delta_Z^k = t(95\,\%;\ g - 1) \cdot RSD_Z^k \le \max \Delta_{As} = 20.00\ \%$$

$$\delta^k = \left|100 - \overline{Z}^k\right| \le \max \delta = 6.40\ \%$$

Between-run *(by calibration samples)*

MCC

$$X_{i,calc} = \frac{Y_i^k - a}{b};\ RR_i = \frac{X_{i,calc}}{X_{i,fact}} \cdot 100\ \%$$

$$\overline{RR}^{intra} = \frac{\sum RR_i}{k \cdot g};\ RSD_{RR}^{intra} = \sqrt{\frac{\sum RSD_{RR,k}^2}{k}}$$

$$\Delta_{RR}^{intra} = t(95\,\%;\ k \cdot g - 1) \cdot RSD_{RR}^{intra} \le \max \Delta_{sample} = 14.14\ \%$$

$$\delta^{intra} = \left|100 - \overline{RR}^{intra}\right| \le \max \delta = 6.40\ \%$$

MS

$$\overline{Z}^{intra} = \frac{\sum Z_i^k}{k \cdot g} \cdot 100\ \%$$

$$RSD_Z^{intra} = \sqrt{\frac{\sum (RSD_Z^k)^2}{k}}$$

$$\Delta_Z^{intra} = t(95\,\%;\ k \cdot g - 1) \cdot RSD_Z^{intra} \le \max \Delta_{As} = 20.00\ \%$$

$$\delta^{intra} = \left|100 - \overline{Z}^{intra}\right| \le \max \delta = 6.40\ \%$$

Between-run *(by model samples)*

MCC

$$X_{i,fact}^{sample} = \frac{C_i^{sample}}{C_{reference}} \cdot 100\ \%;\quad Y_i^{sample} = \frac{A_i^{sample}}{A_{reference}} \cdot 100\ \%;\quad X_{i,calc}^{sample} = \frac{Y_i^{sample} - a}{b};\quad RR_i^{sample} = \frac{X_{i,calc}^{sample}}{X_{i,fact}^{sample}} \cdot 100\ \%$$

$$\overline{RR}^{sample} = \frac{\sum RR_i^{sample}}{k \cdot n};\quad RSD_{RR}^{sample} = \sqrt{\frac{\sum (RSD_{RR,k}^{sample})^2}{k}}$$

$$\Delta_{RR}^{sample} = t(95\ \%;\ k \cdot n - 1) \cdot RSD_{RR}^{sample} \le \max \Delta_{As} = 20.00\ \%$$

$$\delta^{sample} = \left|100 - \overline{RR}^{sample}\right| \le \max \delta = 6.40\ \%$$

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
		1	2	3	
Blood	$\overline{R}$	82.91	93.76	94.21	–
	$\Delta_{R,r}$	4.67	5.84	5.73	≤ 20.00 %
	b^R/s_b^R	–0.01 / 0.01	0.02/0.01	0.01/0.01	$b^R \le 1.812 \cdot s_b^R$
	a^R/s_a^R	83.53/1.07	92.24/1.45	93.12/1.48	$a^R > 1.812 \cdot s_a^R$
	$\lvert 100 - \overline{R} \rvert$	17.09	6.24	5.79	≤ 6.40 %
Urine	$\overline{R}$	82.85	89.82	90.09	–
	$\Delta_{R,r}$	4.99	13.64	13.57	≤ 20.00 %
	b^R/s_b^R	0.02/0.01	0.07/0.03	0.07/0.03	$b^R \le 1.812 \cdot s_b^R$
	a^R/s_a^R	81.33/1.03	83.92/2.68	84.32/2.75	$a^R > 1.812 \cdot s_a^R$
	$\lvert 100 - \overline{R} \rvert$	17.15	10.18	9.91	≤ 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	
		blood				*urine*					
		run 1	run 2	run 3	mean	run 1	run 2	run 3	mean	MCC	MS
Procedure 1	b	1.086	1.090	1.090	1.089	1.096	1.105	1.110	1.104		
	s_b	0.029	0.039	0.019	0.028	0.028	0.029	0.032	0.028		
	a	–3.558	–3.948	–3.263	–3.590	–4.240	–3.872	–4.774	–4.295	$a \le 2.015 \cdot s_a$ $a \le 8.53$ %	
	s_a	3.228	4.339	2.171	3.095	3.178	3.198	3.567	3.129		
	RSD_0	3.819	5.134	2.569	3.662	3.761	3.784	4.220	3.703	≤ 9.93 %	≤ 7.02 %
	R_c	0.9982	0.9968	0.9992	0.9984	0.9983	0.9983	0.9979	0.9984	≥ 0.9830	≥ 0.9915
Procedure 2	b	1.014	1.021	1.012	1.015	1.027	1.031	1.030	1.029		
	s_b	0.024	0.033	0.029	0.027	0.029	0.032	0.038	0.032		
	a	–3.593	–3.488	–2.961	–3.347	–4.689	–4.046	–4.400	–4.378	$a \le 2.015 \cdot s_a$ $a \le 8.53$ %	
	s_a	2.651	3.650	3.228	3.003	3.295	3.577	4.208	3.558		
	RSD_0	3.136	4.319	3.819	3.554	3.899	4.233	4.979	4.210	≤ 9.93 %	≤ 7.02 %
	R_c	0.9986	0.9975	0.9980	0.9983	0.9979	0.9976	0.9967	0.9976	≥ 0.9830	≥ 0.9915
Procedure 3	b	1.014	1.020	1.011	1.015	1.028	1.032	1.030	1.030		
	s_b	0.024	0.033	0.029	0.027	0.030	0.032	0.037	0.032		
	a	–3.384	–3.680	–3.131	–3.398	–4.501	–3.904	–4.110	–4.172	$a \le 2.015 \cdot s_a$ $a \le 8.53$ %	
	s_a	2.656	3.655	3.192	2.993	3.323	3.633	4.180	3.578		
	RSD_0	3.142	4.325	3.776	3.541	3.932	4.299	4.946	4.234	≤ 9.93 %	≤ 7.02 %
	R_c	0.9986	0.9974	0.9980	0.9983	0.9979	0.9975	0.9967	0.9976	≥ 0.9830	≥ 0.9915

Table 3

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
			blood			*urine*			
			run 1	run 2	run 3	run 1	run 2	run 3	
Procedure 1	within-run	$\overline{RR}^k$	100.87	101.03	100.47	100.90	100.79	100.66	–
		δ^k	0.87	1.03	0.47	0.90	0.79	0.66	≤ 6.40 %
		RSD_{RR}^k	3.94	5.19	2.37	4.23	3.55	3.69	–
		Δ_{RR}^k	7.66	10.09	4.61	8.22	6.90	7.17	≤ 14.14 %
	between-run	$\overline{RR}^{intra}$	100.79			100.78			–
		δ^{intra}	0.79			0.78			≤ 6.40 %
		RSD_{RR}^{intra}	3.94			3.88			–
		Δ_{RR}^{intra}	6.80			6.69			≤ 14.14 %
		RR^{sample}	101.93			101.71			–
		δ^{sample}	1.93			1.71			≤ 6.40 %
		RSD_{RR}^{sample}	5.03			2.95			–
		Δ_{RR}^{sample}	9.03			5.30			≤ 20.00 %
Procedure 2	within-run	$\overline{RR}^k$	100.75	100.98	100.67	100.94	101.11	100.87	–
		δ^k	0.75	0.98	0.67	0.94	1.11	0.87	≤ 6.40 %
		RSD_{RR}^k	3.71	4.82	3.21	4.38	4.90	4.20	–
		Δ_{RR}^k	7.21	9.37	6.24	8.51	9.52	8.16	≤ 14.14 %
	between-run	$\overline{RR}^{intra}$	100.80			100.98			–
		δ^{intra}	0.80			0.98			≤ 6.40 %
		RSD_{RR}^{intra}	3.95			4.53			–
		Δ_{RR}^{intra}	6.82			7.82			≤ 14.14 %
		$\overline{RR}^{sample}$	105.72			102.08			–
		δ^{sample}	5.72			2.08			≤ 6.40 %
		RSD_{RR}^{sample}	4.27			1.90			–
		Δ_{RR}^{sample}	7.67			3.42			≤ 20.00 %
Procedure 3	within-run	$\overline{RR}^k$	100.75	100.98	100.67	100.95	101.12	100.86	–
		δ^k	0.75	0.98	0.67	0.95	1.12	0.86	≤ 6.40 %
		RSD_{RR}^k	3.72	4.81	3.19	4.42	4.94	4.17	–
		Δ_{RR}^k	7.23	9.35	6.20	8.59	9.60	8.10	≤ 14.14 %
	between-run	$\overline{RR}^{intra}$	100.80			100.98			–
		δ^{intra}	0.80			0.98			≤ 6.40 %
		RSD_{RR}^{intra}	3.94			4.54			–
		Δ_{RR}^{intra}	6.79			7.83			≤ 14.14 %
		$\overline{RR}^{sample}$	106.32			101.95			–
		δ^{sample}	6.32			1.95			≤ 6.40 %
		RSD_{RR}^{sample}	4.64			2.14			–
		Δ_{RR}^{sample}	8.34			3.84			≤ 20.00%

Table 4

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

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

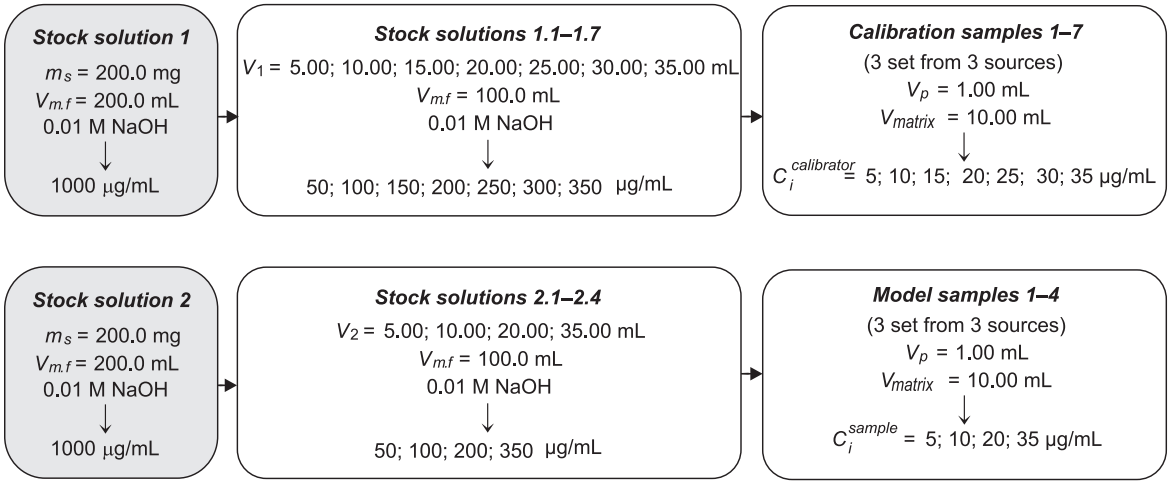
Experimental part

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 IMID 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 – Analitika-Chrom® (Analitika 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 $LiClO_4$ – 0.005 M $HClO_4$;
- 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 RSD_{nom} calculated towards the nominal value of peak area S_{nom} should not exceed:

$$RSD_{nom} = \frac{s}{S_{nom}} \cdot 100\ \% \le \frac{0.1 \cdot \max \Delta_{As} \cdot \sqrt{n}}{t(95\ \%;\ n-1)} = \begin{Bmatrix} 1.47\ \%;\ n=3 \\ 1.88\ \%;\ n=4 \\ 2.22\ \%;\ n=5 \\ 2.52\ \%;\ n=6 \end{Bmatrix};$$

$$S_{nom} = S_{min} = \overline{S}_{25\,\%}$$

where: $\max\Delta_{As}$ – is the extreme relative uncertainty of the procedure of analysis, $\max\Delta_{As}$ = 20 % [20]; $\overline{S}_{25\,\%}$ – 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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