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ARTICLES

Simultaneous Determination of Zidovudine and Lamivudine in Plasma Samples Using Miniaturized Homogenous Liquid–Liquid Extraction and High-Performance Liquid Chromatography[1]

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Abstract—A simple and effective miniaturized homogenous liquid–liquid extraction technique coupled with a gradient HPLC method for the determination of lamivudine and zidovudine in the human plasma samples was developed. Separation of these drugs was performed on a C_{18} stationary phase by using a mixture of acetonitrile and water as mobile phase. Extraction of drugs was achieved based on salting-out phenomenon. Variables affecting the extraction efficiency, such as solvent type and its volume, type of salt and its concentration and sample pH have been fully evaluated and optimized. These drugs were successfully extracted by acetonitrile as extracting solvent with sodium sulfate as salting-out agent. Under the optimized experimental conditions calibration curves showed good linearity ($r^2 > 0.9938$) and precision (RSD < 6.7%) in the working concentration ranges. The limits of detection for lamivudine and zidovudine were 0.006 and 0.003 μg/mL, respectively. The limits of quantification for lamivudine and zidovudine were 0.02 and 0.01 μg/mL, respectively. The recoveries were in the range of 88.0–100.0% with RSD values less than 7.5%. The method was successfully applied to extract and determine lamivudine and zidovudine in human plasma.

Keywords: lamivudine, zidovudine, homogenous liquid–liquid extraction, HPLC, plasma

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Acquisition both qualitative and quantitative chemical information about an analyte depends on sample preparation method and analysis instrument. Also, the quality of obtained information related to the matrix in which the analyte of interest is determined. Therefore, choice of appropriate sample preparation technique is an important step in analysis. The next step is to select the analytical method and optimize the conditions for analytes extraction.

A drawback of the use of water-immiscible organic solvents in liquid–liquid extraction (**LLE**) is their low dielectric constants. Therefore, they are unable to extract the water-soluble compounds that may require extraction at very low or high pH values. For this reason, attention to more polar and water miscible solvents such as acetonitrile, tetrahydrofuran, isopropanol and methanol lead to introduce an efficient extraction method namely salting-out homogeneous liquid–liquid extraction (**HLLE**) [1–7]. In salting-out HLLE method, addition of an inorganic salt into a mixture of water and a water-miscible organic solvent results in separation of the organic solvent from the mixture and the formation of a two-phase system [8]. The salting-out HLLE technique is simple, fast, inexpensive, and results in extracts containing solutes in an organic solvent that can be evaporated and reconstituted into a small volume of suitable solvent for preconcentration and analysis with HPLC or gas chromatography [9–11]. Since the extracts in salting-out HLLE are compatible with analysis instruments, the extract can be injected directly into the chromatographic systems.

Lamivudine and zidovudine were quantified using various techniques simultaneously and in combination with other antiviral drugs in different matrixes [12–19]. In order to extract and quantify these compounds, different sample preparation techniques and detection systems were used. Most of these methods employ LLE or solid-phase extraction (**SPE**) techniques for sample preparation, and detection is achieved by tandem mass spectrometry (**MS/MS**). Compared to salting-out extraction technique, these methods are time-consuming, expensive and potentially damaging to the environment.

The aim of this study was to develop and validate an analytical procedure for the determination of lamivudine and zidovudine in human plasma using the miniaturized homogeneous liquid–liquid extraction

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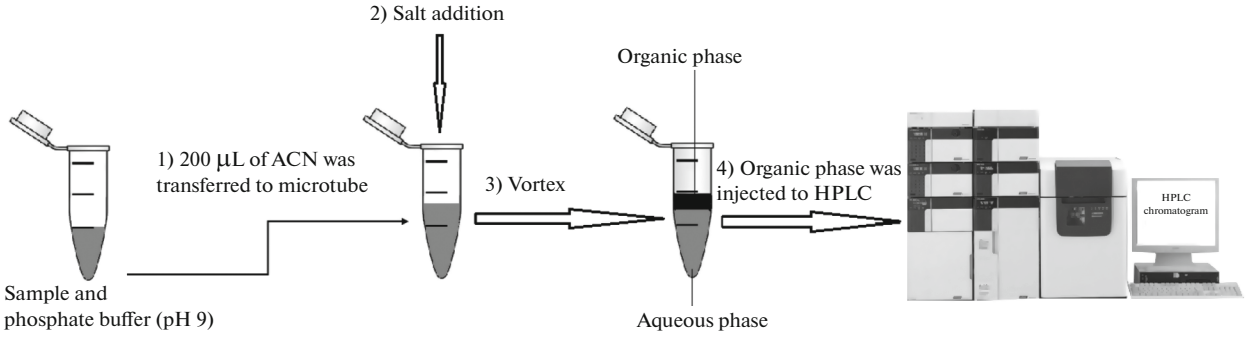

Fig. 1. Schematic diagram of the proposed miniaturized homogenous liquid–liquid extraction procedure.

(**MHLLE**) technique. To the best our knowledge, it is the first MHLLE report for the determination of these analytes. The proposed analytical method was optimized, validated and applied to the quantification of these analytes in human plasma.

EXPERIMENTAL

Chemicals and materials. Pure zidovudine (**ZDV**) and lamivudine (**LAM**) powders (working standard) were obtained from Hetero (Heyderabad, India). Acetonitrile (**ACN**, HPLC grade), ethanol, acetone, tetrahydrofuran (**THF**), isopropanol (**IPA**), sodium carbonate, sodium chloride, sodium sulfate, sodium dihydrogen phosphate, sodium hydroxide and orthophosphoric acid were purchased from Merck (Darmstadt, Germany). All chemicals were analytical grade and used without further purification. Deionized water was supplied using a Milli-Q system (Millipore, USA).

Chromatographic conditions. The HPLC system (Shimadzu, Kyoto, Japan) consisting of a quaternary pump (LC-10ATvp), UV-Vis detector (SPD-M10Avp), vacuum degasser and system controller (SCL-10Avp) was used. A manual injector with a 10 μL sample loop was applied for loading the sample. Class VP-LC workstation was employed to acquire and process chromatographic data. A reversed-phase C_{18} analytical column (Shim-Pack VP-ODS, 250 × 4.6 mm i.d., 5 μm, Shimadzu, Japan) was used.

The mobile phase consisted of water and acetonitrile. Gradient elution was carried out with 10% acetonitrile for 7 min and increased up to 30% within 5 min. Then it returned to initial conditions within 4 min to be kept in this ratio for 4 min. Prior usage the mobile phase, water and acetonitrile were degassed separately using a Millipore vacuum pump. The UV detector was set at 265 nm. Flow rate and column oven were set at 1.0 mL/min and ambient temperature.

Standard solution preparation. Standard stock solutions were prepared by dissolving each analyte in methanol with concentration of 100 μg/mL. Working standard solutions at different concentrations were prepared freshly by mixing the appropriate volumes of the stock solutions and diluting with deionized water.

Sample preparation. A 1.0 mL of the plasma (containing ZDV and LAM) was placed in a 10 mL screw-capped polyethylene tube with a conical bottom. Plasma was deproteinized with 1 mL sulfosalicylic acid (4%, w/v). Afterwards the tube was centrifuged at 11000 rpm for 5 min. Then, 500 μL of the clear supernatant was subjected to extraction process.

Miniaturized homogenous liquid–liquid extraction (MHLLE) procedure. 500 μL of sample or standard solution and 400 μL of phosphate buffer (pH 9) were transferred into a 2 mL microtube. Then 200 μL of acetonitrile and 0.25 g of sodium sulfate as extracting solvent and salting-out agent were added, respectively. The mixture was vortexed using a vortex mixer (Dragon Lab MX-S, Beijing, China) at 1500 rpm for 2 min. After few minutes the layers are clearly separated. Finally, 10 μL of organic phase was withdrawn and injected into the HPLC system for analysis. The schematic diagram of extraction process is described in Fig. 1.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions. Firstly, separation of lamivudine and zidovudine was performed using isocratic elution with a mixture of acetonitrile and water (10 : 90, v/v) as mobile phase. In this state, zidovdine was more retained on the column and analysis time increased. For this reason, isocratic elution was replaced with gradient. On the other hand, due to increase of column and HPLC pump lifetime, buffer was not used as mobile phase.

The extraction efficiency of MHLLE depended on various parameters such as: the solvent type and its volume, salt type and its concentration and sample pH. The influence of these parameters was investigated and optimized to obtain their optimum values.

Optimization of extraction conditions. Various pure and mixed organic solvents such as: ACN, THF, EtOH, IPA, ACN–THF (50 : 50), acetone, acetone–ACN and acetone–THF were used as extracting

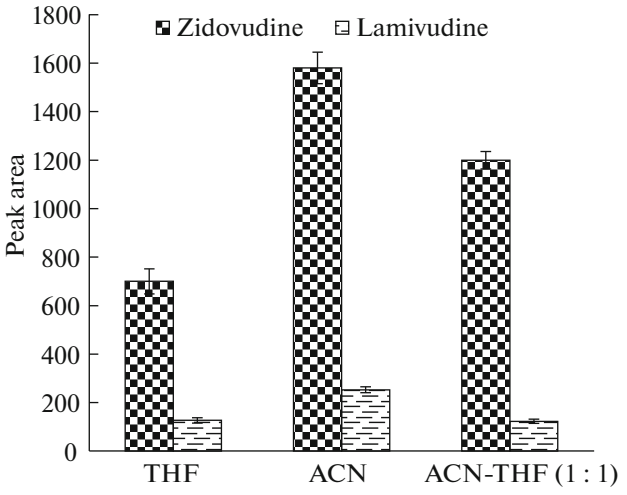

Fig. 2. Effect of extracting solvent on the extraction efficiencies of LAM and ZDV. Extraction conditions: volume of extracting solvent, 200 μL; sample pH, 9; salt concentration, 23% (w/v); salt, sodium sulfate.

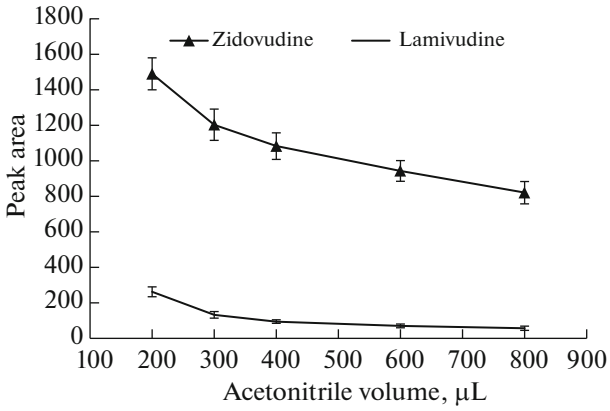

Fig. 3. Effect of extracting solvent volume on the extraction efficiencies of LAM and ZDV. Extraction conditions: extracting solvent, acetonitrile; sample pH, 9; salt concentration, 23% (w/v); salt, sodium sulfate.

solvents. All these solvents and their mixtures are miscible with aqueous solution. Phase separation was not occurred using EtOH and IPA as extracting solvents. On the other hand, acetone peak is overlapped with lamivudine. Therefore, EtOH, IPA, acetone and their mixtures were removed from the solvent list. The effect of solvent type on the extraction efficiencies of LAM and ZDV is shown in Fig. 2. According to these results, ACN was selected as the appropriate extracting solvent.

The volume of extracting solvent influences the signal intensities of analytes. In low volumes, extracting solvent is unable to extract the analytes from aqueous phase completely. On the other hand, high volumes of extracting solvent leads to increase of organic phase volume which dilute analytes in the organic phase. Therefore, volume of extracting solvent was investigated in the range of 200–800 μL. As can be seen from Fig. 3, increase of extracting solvent volume reduces the analytes signals. The reason for this phenomenon is attributed to analytes dilution in the organic phase. Finally, 200 μL was chosen as the optimum extracting solvent volume.

The pH of aqueous solution has a vital influence on the extraction efficiency of analytes with acidic or basic functional groups. On the other hand, pH can affect phase separation and volume of recovered organic solvent [20–22]. For these reasons, the effect of sample pH on the extraction of analytes was studied in the range of 4–10. The obtained results (Fig. 4) revealed that the extraction efficiencies of target analytes varied in the studied pH range. Low extraction efficiencies of ZDV and LAM in acidic and neutral pHs can be attributed to protonation of amine groups, which produced ionic species. At alkaline pHs, ZDV and LAM are in neutral forms and easily transferred into the organic phase. Therefore, pH 9 was selected as the best value for subsequent experiments.

The addition of an inorganic salt into a mixture of water and a water-miscible organic solvent leads to separation of the two phases. Also, salting-out effect can be used to enhance the extraction efficiencies of target analytes from aqueous phase into the organic phase. This behavior was attributed to decrease of analytes solubility in aqueous phase in presence of salt. Type of salt and its concentration are important parameters in salting-out phenomenon. Three sodium salts including carbonate, sulfate and chloride were investigated. Among these salts, sodium sulfate showed maximum extraction efficiency for two analytes (Fig. 5). Therefore, different concentrations of sodium sulfate were used in the range of 18 to 27% (w/v). As can be observed from the results in Fig. 6,

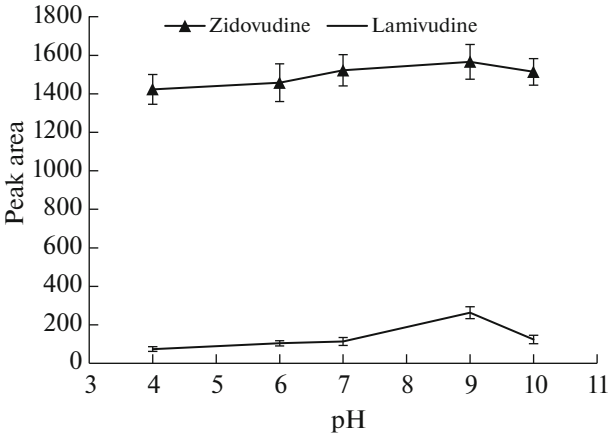

Fig. 4. Effect of sample pH on the extraction efficiencies of LAM and ZDV. Extraction conditions: extracting solvent, acetonitrile; volume of extracting solvent, 200 μL; salt concentration, 23% (w/v); salt, sodium sulfate.

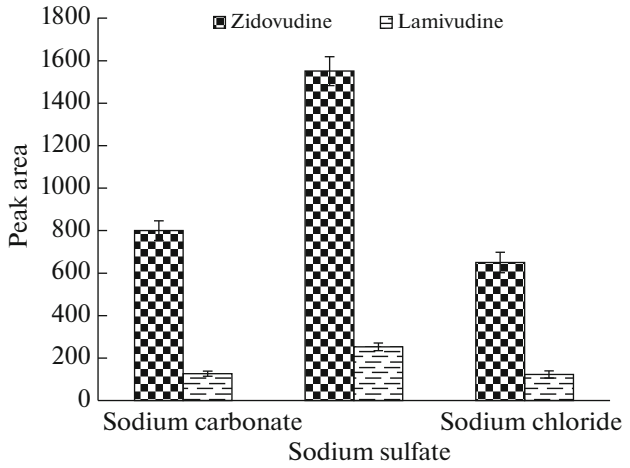

Fig. 5. Effect of salting-out agent on the extraction efficiencies of LAM and ZDV. Extraction conditions: extracting solvent, acetonitrile; volume of extracting solvent, 200 μL; sample pH, 9; salt concentration, 23% (w/v).

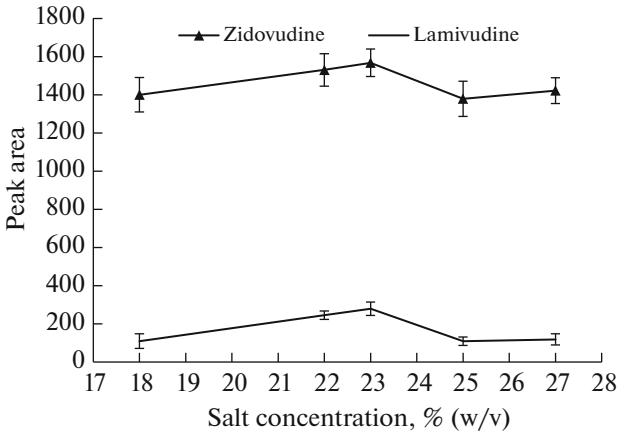

Fig. 6. Effect of salt concentration on the extraction efficiencies of LAM and ZDV. Extraction conditions: extracting solvent, acetonitrile; volume of extracting solvent, 200 μL; sample pH, 9; salt, sodium sulfate.

23% (w/v) was selected as the optimum salt concentration for subsequent experiments.

Method evaluation. The developed MHLLE method was validated in terms of linearity, limit of detection (**LOD**), limit of quantitation (**LOQ**), precision, accuracy and selectivity. The analytical figures of merit of the proposed analytical technique are summarized in Table 1. All experiments were performed using spiked plasma samples.

Precision and accuracy data were obtained using spiked real samples containing each analyte in three concentration levels. Relative standard deviation values for ZDV and LAM were in the range of 4.6–6.6 and 5.5–6.7%, respectively (Table 2). In order to validate the method accuracy, the recovery tests were performed by the analysis of the plasma samples spiked with three different concentrations of each analyte. Relative recovery values of ZDV and LAM were in the range of 88–99 and 95–100%, respectively.

Figure 7 shows the chromatograms of analytes mixture obtained under the optimized extraction conditions. Comparison of chromatograms of analytes mixture before and after extraction indicates that the analytes were concentrated using the proposed method.

Several parameters of the proposed method were compared with those of reported in the literature (Table 3). The results show that most of these methods used the SPE and LLE methods as main sample preparation methods. The SPE method has several steps which leads to spending more time. Also, it is more expensive than the proposed method. Solvents which used LLE are not compatible with HPLC systems. Therefore, in LLE methods evaporation of extracting solvent and reconstitution of the residue in appropriate solvent are essential. On the other hand, most methods used the MS/MS detection system which is not a routine in any laboratory. These limitations in sample preparation and detection led to the development of a suitable method for measuring the ZDV and LAM in the plasma samples. In comparison with reported methods, analytical parameters of the proposed method such as LODs, LOQs, RSDs and recoveries are satisfactory.

CONCLUSIONS

In the present work, a simple analytical procedure for the simultaneous determination of zidovudine and

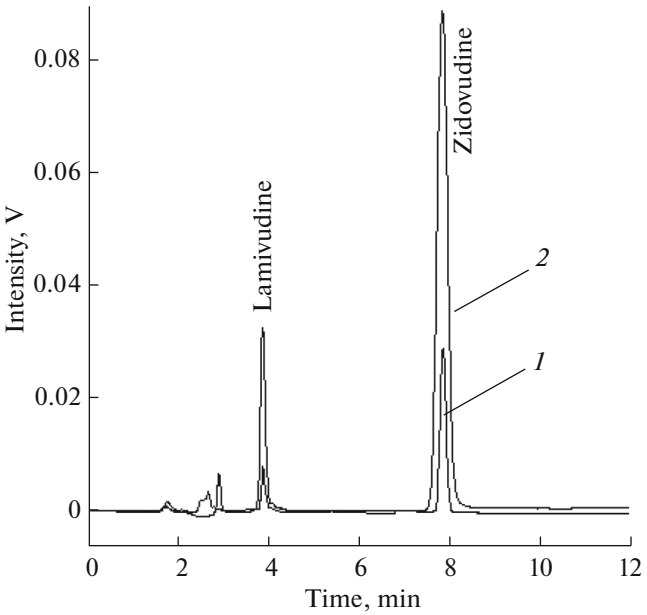

Fig. 7. Chromatograms of standard solution before (*1*) and after extraction process (*2*).

Table 1. Some of analytical parameters for the proposed method

Analyte	LOD, μg/mL	LOQ, μg/mL	R^2	Slope	Linear range, μg/mL
Zidovudine	0.003	0.01	0.9938	99.264	0.01–40
Lamivudine	0.006	0.02	0.9947	49.468	0.02–40

Table 2. Precision and accuracy data for the proposed method using plasma samples

Analyte	Concentration, μg/mL	RSD, %		Recovery, % ($n = 3$)
		within day ($n = 5$)	between days ($n = 15$)	
Zidovudine	0.01	4.59	5.65	89 ± 2
	0.1	4.61	5.82	95 ± 2
	10	5.57	6.61	98 ± 2
Lamivudine	0.02	5.49	6.52	95 ± 3
	0.2	5.51	6.72	98 ± 2
	20	5.50	5.80	100 ± 2

Table 3. Comparison of several figures of merit of proposed method with previously reported methods for simultaneous determination of lamivudine and zidovudine

Analyte	Matrix	Sample preparation	Detection	LOD, μg/mL	LOQ, μg/mL	RSD, %	Recovery, %	Reference
Zidovudine	Human plasma	SPE	MS/MS	N/R[a]	0.005	1.6–10.1	93.8–110.8	[12]
		SPE	ESI[b]-MS/MS	0.005	0.015	2.2–8.9	104.0–112.0	[14]
		Automated SPE	UV, 260 nm	0.08	0.13	3.2–5.5	70.0–75.0	[15]
		Protein precipitation, evaporation and reconstitution	MS/MS	N/R	0.02	5.12–10.6	98.0–107.0	[16]
		LLE	UV, 270 nm	N/R	0.05	0.29–2.1	94.0–99.5	[23]
		LLE	UV, 270 nm	N/R	0.08	1.06–4.37	85.0–106.8	[24]
		SPE	UV, 260 nm	N/R	0.015	1.5–2.0	99.0–101.0	[25]
		MHLLE	UV, 265 nm	0.003	0.01	4.5–6.6	88.0–98.2	This work
Lamivudine	Human plasma	SPE	MS/MS	N/R	0.005	1.6–10.1	93.8–110.8	[12]
		SPE	ESI-MS/MS	0.003	0.01	1.9–8.7	95.0–99.0	[14]
		Automated SPE	UV, 260 nm	0.069	0.11	4.9–6.3	69.0–72.0	[15]
		Protein precipitation, evaporation and reconstitution	MS/MS	N/R	0.02	3.66–8.36	99.7–111.0	[16]
		LLE	UV, 270 nm	N/R	0.05	0.51–1.7	97.0–99.7	[23]
		LLE	UV, 270 nm	N/R	0.08	0.82–13.3	87.0–112.8	[24]
		SPE	UV, 260 nm	N/R	0.015	1.4–2.3	92.0–98.0	[25]
		MHLLE	UV, 265 nm	0.006	0.02	5.4–6.7	95.0–99.8	This work

[a] N/R—not reported.
[b] ESI—ion electrospray ionization.

lamivudine in human plasma using MHLLE and HPLC was developed. Experimental parameters which can influence the efficiency of the proposed method were investigated and optimized. Compared to other reported methods, the proposed method offers several advantages such as simplicity, rapidity, low cost and appropriate performance in real samples analysis. To the best our knowledge, this is the first report of a MHLLE method for simultaneous determination of zidovudine and lamivudine in human plasma. In this study acetonitrile was used as the extraction solvent which it is compatible with HPLC instrument. Unlike the LLE method, in the proposed method evaporation of extracting solvent and reconstitution of residue in appropriate solvent are removed.

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