ABSTRACT

Objective: Lennox–Gastaut syndrome (LGS) is mainly treated with antiepileptic drugs (AEDs) but using one AED is not sufficient to relieve all or even most patients. A combination of agents is usually preferred. In the current study, an isocratic, selective, sensitive, precise, and accurate reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed for the simultaneous determination of rufinamide (RUF), lamotrigine (LAM), clonazepam (CLO), valproic acid (VAL), and diazepam (DIA) which are commonly used in the management of LGS in their dosage forms using lacosamide as internal standard.

Methods: The method depends on using RESTEK C18 column (5 μm, 250 mm × 4.6 mm) and a mobile phase composed of acetonitrile:water (55:45, v/v), pH = 3.3 adjusted with phosphoric acid. The method was conducted in an isocratic mode with a flow rate of 1 ml/min and ultraviolet detection at 210 nm.

Results: The linearity range was 2–40 μg/ml for RUF and DIA, 0.5–40 μg/ml for LAM and CLO, and 36–180 μg/ml for VAL.

Conclusion: Statistical analysis revealed no significant difference between the results obtained and the official or reported ones for each cited drug. The method is simple to be easily implemented in quality control studies of the mentioned drugs in their pharmaceutical preparations.

Keywords: Rufinamide, Lamotrigine, Clonazepam, Valproic acid, Diazepam, High-performance liquid chromatography, Dosage form.

INTRODUCTION

Lennox–Gastaut syndrome (LGS) is a severe form of childhood epileptic encephalopathy with multiple etiologies, whether genetic, structural, metabolic, or unknown. LGS could be diagnosed by EEG, usually demonstrating high voltage, bifrontal 1.5–2.5 Hz spikes, and wave complexes. Several seizure types are associated with LGS including sudden tonic–atonic seizures (drop attacks), atypical absence (the most common), myoclonic seizures, generalized tonic–clonic seizures, and partial onset seizures. The optimum treatment for LGS has yet to be established [1–4]. Treatment is aimed at reducing seizure burden using the least number of medications while minimizing side effects. However, seizure freedom is rare, and multiple anticonvulsants are often required. Surveys have shown that valproate is often the preferred drug for initial therapy. Either lamotrigine (LAM) or topiramate or rufinamide (RUF) is often the second-line choice for monotherapy if valproate is not efficacious. BZDs have been used as parenteral or rectal agent (diazepam [DIA], lorazepam, and midazolam) in acute cases, while for chronic oral use, clonazepam [CLO], clorazepate, and nitrazepam were used. BZDs remain, in most guidelines, the treatment of choice for acute or subacute seizures [5–15].

On searching literature, it was found that many high-performance liquid chromatography (HPLC) methods were recently reported for the determination of rufinamide (RUF) [16–19], lamotrigine (LAM) [20–23], clonazepam (CLO) [24,25], diazepam (DIA) [26–28], and valproic acid (VAL) [29–32] in their dosage forms. No method was reported for the simultaneous determination of the five cited drugs yet. The aim of the current work was to develop a sensitive, selective, and precise chromatographic method able to separate and quantify the cited drugs in their dosage forms. This method could also be used in the assays of the cited drugs in biological fluids as it covers their therapeutic ranges.

EXPERIMENTAL

Instrumentation

An HPLC instrument (Agilent 1100 series) was equipped with an Agilent isocratic pump G1310A, Agilent ultraviolet (UV)-visible detector G1314A, an Agilent manual injector G1328B with (20 µl) injector loop and RESTEK C18 column (5 µm, 4.6 × 250 mm, made in USA). An Agilent syringe (50 µl, USA) and a Powersonic 405 ultrasonic processor (Human Lab INC - Hwaseong City, Korea) were employed. The pH measurements were carried out using a pH meter (Jenway, 3565, Essex, U.K.). The mobile phase was filtered through 0.45 µm nylon membrane filters (Sigma-Aldrich Co., Germany).

Materials and reagents

RUF (its purity was certified as 99.45%) and lacosamide, used as internal standard (IS) (Fig. 1f), were purchased from Wuhan Sunrise Technology Development Company, Wuhan, China. DIA, LAM, CLO, and VAL were supplied by the National Organization for Drug Control and Research, Egypt (certified to contain 99.91%, 99.98%, 99.53%, and 100.10%, respectively). Prepared Banzel® tablets were used because of its unavailability in the local market while Valium® ampoules (labelled to contain 10 mg of DIA per ampoule) were manufactured by Roche, Lamictal™ tablets (labelled to contain 25 mg of LAM per tablet, Batch No. AC0602) were manufactured by GlaxoSmithKline Pharmaceuticals, Apetral® tablets (labelled to contain 0.5 mg of CLO per tablet) were manufactured by APEX Pharma, and Depakine® tablets (labelled to contain 200 mg of sodium valproate per tablet equivalent to 173.49 mg
of VAL, Batch No. C13958) were manufactured by SANOFI and were purchased from the local market. Acetonitrile (HPLC grade) was supplied from Sigma-Aldrich, Germany. Double-distilled water was used after filtration through 0.45 µm nylon membrane filters. o-phosphoric acid (EL-Nasr Pharmaceutical Chemicals Co., Egypt) was prepared as 0.01 N aqueous solution.

Chromatographic conditions
Chromatographic separation was achieved on RESTEK C18 column (5 µm, 4.6 × 250 mm), applying isocratic elution using a mobile phase consisting of acetonitrile:water (55:45, v/v, adjusted with 0.01 N aqueous solution of o-phosphoric acid to pH = 3.3). The mobile phase was filtered through a membrane filter of 0.45 µm porosity and pumped through the column at a flow rate of 1 ml/min. Analysis was performed at ambient temperature, and the UV detector was set at 210 nm.

Stock and working solutions preparation
Standard stock solutions of (200 µg/ml) for RUF, LAM, CLO, DIA, and IS and also a stock solution of (9 mg/ml) for VAL were prepared in acetonitrile by transferring an accurately weighed amount of each drug in a series of 50 ml volumetric flask, adding 25 ml acetonitrile, then the mixture was sonicated and the flask was completed to volume with the same solvent. For the preparation of working solutions of 50 µg/ml for RUF, LAM, CLO, and DIA, 25 ml was transferred from the stock solution of each drug into a 100 ml volumetric flask and completed with acetonitrile to volume. Furthermore, two working solutions of 900 µg/ml and 100 µg/ml for VAL and IS, respectively, were prepared similarly.

Sample preparation
Twenty tablets of Depakine®, Apetryl®, Lamictal®, and Banzel® were separately weighed and finely powdered. A quantity of each powdered tablets equivalent to 10 mg of VAL (equivalent to 11.53 mg of sodium valproate), CLO, LAM, and RUF, respectively, was accurately weighed, 25 ml of acetonitrile was added, and each drug was extracted by sonication for 15 min. The volume was completed to 50 ml with acetonitrile; the solution was mixed well and filtered on dry funnel and dry filter paper discarding the first few milliliters to obtain a sample stock solution of 200 µg/ml. Further, dilution was carried out using acetonitrile to obtain sample working solutions of 50 µg/ml of CLO, LAM, and RUF and 90 µg/ml of VAL. Furthermore, one ampoule of valium was transferred to a 50 ml volumetric flask, and the volume was completed with acetonitrile to prepare a sample stock solution of (200 µg/ml) from which a sample working solution of 50 µg/ml of DIA was also prepared in the same manner.

General procedures and linearity
Accurately measured aliquots of RUF, DIA, LAM, and CLO standard solutions (50 µg/ml) equivalent to 20–400 µg of RUF and DIA and 5–400 µg of LAM and CLO, respectively, were transferred into a series of 10 ml volumetric flasks and completed to volume with acetonitrile. Furthermore, different aliquots were transferred from VAL standard solution (90 µg/ml) to produce solutions of the concentration range of 36–180 µg/ml. A volume of 20 µl of each solution was injected in triplicates into the chromatograph. The chromatographic conditions were adjusted as mentioned under section chromatographic conditions. The recorded AUPs × 10⁻³ were plotted versus the corresponding
concentrations of RUF, DIA, LAM, CLO, and VAL to obtain the calibration curves.

RESULTS AND DISCUSSION

Method development

The current work aimed to develop an accurate, sensitive, and precise chromatographic method to separate and simultaneously determine RUF, DIA, LAM, CLO, and VAL. A variety of mobile phases were investigated in the development of the present method where different proportions of methanol: Water and acetonitrile: Water and phosphate buffer: Acetonitrile at different pH was attempted as mobile phases, but it was found that the presence of buffer is not needed. The use of acidified water was satisfactory for the separation of the peaks, but adjusting the pH was a very important step due to the big differences in pKa of the cited drugs. Finally, a mobile phase composed of acetonitrile:water (55: 45, v/v), pH = 3.3 adjusted with phosphoric acid, was satisfactory to achieve the separation and resulted in symmetric peaks of the cited drugs with good retention times. The detection wavelength was selected to be 210 nm which is suitable for the determination of the cited drugs as it represents the maximum absorption wavelength of each drug. By adjusting all the chromatographic conditions, a good separation of RUF, DIA, LAM, CLO, and VAL using lacosamide as IS was achieved with the following retention times: 3.102, 8.365, 4.235, 4.879, 7.242, and 3.712 for IS, respectively (Fig. 2).

Method validation

The optimized chromatographic method was validated by evaluating linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), specificity, and system suitability parameters in accordance with the ICH guideline Q2 (R1) [33].

Linearity

The linearity was investigated at six concentration levels of the standard solutions of RUF, DIA, LAM, CLO, and VAL; each concentration was analyzed 3 times. The linearity was evaluated by linear regression analysis. The correlation coefficients were 0.9998 for RUF, DIA and VAL, 0.9999 for CLO, and 0.9992 for LAM, respectively. The analytical data of the calibration curves including standard deviations (SD) for the slope (Sb) and intercept (Sa), confidence limits of the slope, and intercept are summarized in Table 1.

Accuracy

The accuracy of the proposed method was tested by analyzing triplicate samples of standard solutions of each cited drug. The recovery percentages are stated in Table 2, and the results revealed the high accuracy of the proposed method. Furthermore, method accuracy was assessed as recovery obtained when spiking the sample solution with known concentrations of the cited drugs (standard addition technique) as shown in Tables 3-7.

Fig. 2: High-performance liquid chromatogram of rufinamide, lamotrigine, clonazepam, diazepam, valproic acid, and lacosamide (IS) in their laboratory prepared mixture

| Item                          | RUF | DIA | LAM | CLO | VAL |
|-------------------------------|-----|-----|-----|-----|-----|
| Retention time (tR) (min)     | 3.102 | 8.365 | 4.235 | 4.879 | 7.242 |
| Wavelength of detection (nm)  | 210 | 210 | 210 | 210 | 210 |
| Range of linearity            | 2–40 µg/ml | 2–40 µg/ml | 0.5–40 µg/ml | 0.5–40 µg/ml | 36–180 µg/ml |
| Regression equation           | PAR=0.1004 | PAR=0.1296 | PAR=0.1048 | PAR=0.1310 | PAR=0.0097 |
| Regression coefficient (r²)   | 0.9998 | 0.9998 | 0.9992 | 0.9999 | 0.9998 |
| LOD (µg/ml)                   | 0.5263 | 0.4226 | 0.1362 | 0.1258 | 5.4411 |
| LOQ (µg/ml)                   | 1.5948 | 1.2807 | 0.4127 | 0.3813 | 16.4881 |
| SD of the slope (Sb)          | 0.007 | 0.0009 | 0.0015 | 0.0007 | 0.0003 |
| Standard deviation of the intercept (Sa) | 0.0160 | 0.0199 | 0.0340 | 0.0159 | 0.0094 |
| Confidence limit of the slope | 0.1004±0.0020 | 0.1296±0.0025 | 0.1048±0.0042 | 0.1310±0.0020 | 0.0097±0.0002 |
| Confidence limit of the intercept | −0.078±0.0044 | 0.1078±0.0552 | 0.0880±0.0946 | 0.0612±0.0443 | 0.0172±0.0262 |
| Standard error of estimation  | 0.0238 | 0.0296 | 0.0538 | 0.0252 | 0.0095 |
| *Intraday % RSD               | 0.136±0.600 | 0.120–0.372 | 0.060–0.255 | 0.252–0.483 | 0.144–0.974 |
| *Intraday % RSD               | 0.153–0.456 | 0.084–0.774 | 0.129–0.779 | 0.431–0.658 | 0.166–0.312 |

*PAR: Peak area ratio, **LOD: 3.3*SD/slope, ***LOQ: 10*SD/slope. ****The intraday (n=3), average of three concentrations of 3, 15, and 35 µg/ml for RUF and DIA, 1.5, 15, and 35 µg/ml for LAM and CLO, and 45, 135, and 171 µg/ml for VAL repeated 3 times within the day. *****The interday (n=3), average of three concentrations of 3, 15, and 35 µg/ml for RUF and DIA, 1.5, 15, and 35 µg/ml for LAM and CLO, and 45, 135, and 171 µg/ml for VAL repeated 3 times in 3 successive days. RP-HPLC: Reversed-phase high-performance liquid chromatography. RUF: Rufinamide, LAM: Lamotrigine, CLO: Clonazepam, DIA: Diazepam, VAL: Valproic acid, LOD: Limit of detection, LOQ: Limit of quantification, SD: Standard deviations.

169
Table 2: Determination of RUF, DIA, LAM, CLO, and VAL in drug substance using the proposed RP-HPLC method

| Taken (µg/ml) | PAR | Taken (µg/ml) | PAR | Taken (µg/ml) | PAR | Found (µg/ml) | Recovery % |
|--------------|-----|--------------|-----|--------------|-----|---------------|------------|
| RUF          |     | DIA          |     | LAM          |     | CLO           |            |
| 4            | 0.322 | 0.623 | 1 | 0.192 | 0.191 | 54 | 0.541 | 3.99 | 3.98 | 0.99 | 0.99 | 54.00 | 99.63 | 99.38 | 99.20 | 99.10 | 100.00 |
| 6            | 0.525 | 0.879 | 4 | 0.512 | 0.583 | 81 | 0.802 | 6.01 | 5.95 | 4.05 | 3.98 | 80.91 | 100.12 | 99.18 | 101.15 | 99.58 | 99.89 |
| 8            | 0.718 | 1.131 | 12 | 1.351 | 1.623 | 108 | 1.058 | 7.93 | 7.90 | 12.05 | 11.92 | 107.30 | 99.11 | 98.69 | 100.43 | 99.35 | 93.35 |
| 16           | 1.527 | 2.179 | 18 | 1.983 | 2.410 | 117 | 1.149 | 15.99 | 15.98 | 18.08 | 17.93 | 116.68 | 99.92 | 99.88 | 100.46 | 99.61 | 97.33 |
| 24           | 2.317 | 3.210 | 24 | 2.587 | 3.202 | 126 | 1.236 | 23.86 | 23.94 | 23.85 | 23.98 | 125.65 | 99.40 | 99.74 | 99.35 | 99.90 | 97.72 |
| 32           | 3.113 | 4.238 | 32 | 3.421 | 4.294 | 153 | 1.487 | 31.78 | 31.87 | 31.80 | 31.85 | 151.53 | 99.33 | 99.59 | 99.38 | 99.54 | 99.04 |

RP-HPLC: Reversed-phase high-performance liquid chromatography, RUF: Rufinamide, LAM: Lamotrigine, CLO: Clonazepam, DIA: Diazepam, VAL: Valproic acid, SD: Standard deviations

Table 3: Determination of RUF in Banzel® tablets applying standard addition technique using the proposed RP-HPLC method

| Tablet (µg/ml) | PAR | Found (µg/ml) | Recovery % |
|---------------|-----|---------------|------------|
| Tablet        | Tablet and added | Added | Tablet and Added |
| Mean          | ±SD | ±RSD | Mean |

Table 4: Determination of DIA in Valium® ampoules applying standard addition technique using the proposed RP-HPLC method

| Ampoule (µg/ml) | PAR | Found (µg/ml) | Recovery % |
|----------------|-----|---------------|------------|
| Ampoule        | Ampoule and added | Added | Ampoule and Added |
| Mean           | ±SD | ±RSD | Mean |

Table 5: Determination of LAM in Lamictal® tablets applying standard addition technique using the proposed RP-HPLC method

| Tablet (µg/ml) | PAR | Found (µg/ml) | Recovery % |
|---------------|-----|---------------|------------|
| Tablet        | Tablet and added | Added | Tablet and Added |
| Mean          | ±SD | ±RSD | Mean |

RP-HPLC: Reversed-phase high-performance liquid chromatography, RUF: Rufinamide, DIA: Diazepam, LAM: Lamotrigine, SD: Standard deviations

Precision
The precision of the developed method was checked by analyzing three different concentrations of the cited drugs in triplicate during the same day (intraday precision) and on 3 consecutive days (interday precision). The results are presented in Table 1.

Specificity
Specificity was established by analyzing each drug in its dosage form separately showing no interference from excipients (Fig. 3). The use
Table 6: Determination of CLO in Apetryl® tablets applying standard addition technique using the proposed RP-PLC method

| Taken (µg/ml) | PAR | Tablet | Added | Tablet and added | Found (µg/ml) | Recovery % |
|--------------|-----|--------|-------|-----------------|---------------|------------|
| 2            | 1.6 | 0.321  | 0.530 | 1.98            | 3.58          | 1.60       |
| 2            | 2   | 0.321  | 0.582 | 1.98            | 3.98          | 1.99       |
| 5            | 5   | 0.715  | 1.370 | 4.99            | 9.99          | 5.00       |
| 6            | 6   | 0.715  | 1.495 | 4.99            | 10.95         | 5.96       |
| 10           | 8   | 1.360  | 2.397 | 9.92            | 17.63         | 7.92       |
| 10           | 12  | 1.360  | 2.928 | 9.92            | 21.88         | 11.97      |

Table 7: Determination of VAL in Depakine® tablets applying standard addition technique using the proposed RP-HPLC method

| Taken (µg/ml) | PAR | Tablet | Added | Tablet and added | Found (µg/ml) | Recovery % |
|--------------|-----|--------|-------|-----------------|---------------|------------|
| 36           | 36  | 0.366  | 0.713 | 35.96           | 71.73         | 35.77      |
| 36           | 45  | 0.366  | 0.801 | 35.96           | 80.80         | 44.85      |
| 72           | 54  | 0.712  | 1.229 | 71.63           | 124.93        | 53.30      |
| 72           | 72  | 0.712  | 1.398 | 71.63           | 142.35        | 70.72      |
| 90           | 72  | 0.884  | 1.576 | 89.36           | 160.70        | 71.34      |
| 90           | 90  | 0.884  | 1.746 | 89.36           | 178.23        | 88.87      |

Table 8: Determination of RUF, DIA, LAM, CLO, and VAL in laboratory prepared mixtures using the proposed RP-HPLC method

| Taken (µg/ml) | PAR | Table | Added | Table and added | Found (µg/ml) | Recovery % |
|--------------|-----|-------|-------|-----------------|---------------|------------|
| 2            | 0.121| 0.362 | 0.5   | 0.140           | 0.127         | 36         |
| 36           | 36  | 0.366 | 0.713 | 35.96           | 71.73         | 35.77      |
| 72           | 72  | 0.712 | 1.229 | 71.63           | 124.93        | 53.30      |
| 90           | 90  | 0.884 | 1.746 | 89.36           | 178.23        | 88.87      |

of phosphoric acid in the mobile phase liberates VAL from sodium valproate in its dosage form which results in a peak with the same retention time as VAL in drug substance (Fig. 3). The good recovery % and low SD proved the high specificity of the proposed method (Table 8).

**LOD and LOQ**
According to the ICH recommendations [33], the parameters LOD and LOQ were determined on the basis of SD of the response and slope of the regression equation, Table 1.

**System suitability**
The system suitability parameters with respect to the number of theoretical plates, resolution factor, tailing factor, capacity factor, and selectivity factor were displayed in Table 9.

**Statistics**
The proposed analytical method was compared with the reference methods of the cited drugs [19] using statistical analysis. The Student’s t-test and F-test were applied and revealed no significant difference between the experimental values obtained in the pure sample analysis by the newly developed method and that of the references methods (Table 10).

**CONCLUSION**
The proposed RP-HPLC method was accurate, precise, selective, and sensitive. It allows the simultaneous separation and determination of five anti-epileptic drugs: RUF, DIA, LAM, CLO, and VAL in their pharmaceutical dosage forms using lacosamide as IS. The validation of the developed method according to the ICH guidelines proved the applicability and great value of this method for routine analysis in quality control laboratories for the determination of the cited drugs in their pure form and their dosage forms.

**CONFLICT OF INTEREST**
All of the authors declare that they have no conflict of interest.
Table 9: System suitability tests of the proposed RP-HPLC method for the simultaneous determination of RUF, DIA, LAM, CLO, and VAL.

| Parameter | RUF   | IS     | LAM   | CLO   | VAL   | DIA   | Reference value |
|-----------|-------|--------|-------|-------|-------|-------|-----------------|
| N         | 14573 | 13315  | 12893 | 13541 | 19220 | 18288 | The higher the value, the more efficient the column is |
| R         | 3.02  | 3.37   | 3.68  | 12.53 | 4.92  | >2    |                 |
| T         | 0.95  | 0.87   | 0.94  | 0.90  | 0.77  | 0.94  | ≤2              |
| K’        | 2.102 | 2.712  | 3.235 | 3.879 | 6.242 | 7.365 | 1–10            |
| α         | 1.20  | 1.14   | 1.15  | 1.48  | 1.16  |       |                 |

N: Number of theoretical plates; R: Resolution factor; T: Tailing factor; K’: Capacity factor; α: Selectivity factor. RP-HPLC: Reversed-phase high-performance liquid chromatography, RUF: Rufinamide, LAM: Lamotrigine, CLO: Clonazepam, DIA: Diazepam, VAL: Valproic

Table 10: Statistical comparison between the proposed reversed-phase high-performance liquid chromatography method for the simultaneous determination of RUF, DIA, LAM, CLO, and VAL in drug substance and the reference methods

| Statistical term | Reference method for RUF¹ | RUF by RP-HPLC method | Reference method for DIA¹ | DIA by RP-HPLC method | Reference method for LAM¹ | LAM by RP-HPLC method | Reference method for CLO¹ | CLO by RP-HPLC method | Reference method for VAL¹ | VAL by RP-HPLC method |
|------------------|---------------------------|----------------------|---------------------------|----------------------|---------------------------|------------------------|---------------------------|------------------------|---------------------------|------------------------|
| Mean             | 99.45                     | 99.59                | 99.61                     | 99.11                | 99.38                     | 99.54                  | 99.58                     | 100.10                 | 100.10                   | 99.62                  |
| ±SD              | 0.797                     | 0.381                | 0.936                     | 0.432                | 0.927                     | 0.976                  | 0.796                     | 0.356                  | 0.269                     | 0.744                  |
| ±RSD             | 0.318                     | 0.155                | 0.382                     | 0.176                | 0.378                     | 0.325                  | 0.325                     | 0.145                  | 0.110                     | 0.304                  |
| % RSD            | 0.784                     | 0.382                | 0.937                     | 0.435                | 0.927                     | 0.796                  | 0.358                     | 0.270                  | 0.270                     | 0.743                  |
| n                | 6                         | 6                    | 6                         | 6                    | 6                         | 6                      | 6                         | 6                      | 6                         | 6                      |
| v                | 0.608                     | 0.415                | 0.876                     | 0.435                | 0.927                     | 0.633                  | 0.127                     | 0.072                  | 0.553                     | 0.130                  |
| (F=(*5.03)       | 4.19                      | 4.69                 | 4.19                      | 4.69                 | 4.19                      | 4.69                   | 4.19                      | 4.69                   | 4.19                      | 4.69                   |

¹Figures in parentheses are the theoretical t and F values at p=0.05. RP-HPLC: Reversed-phase high-performance liquid chromatography, RUF: Rufinamide, LAM: Lamotrigine, CLO: Clonazepam, DIA: Diazepam, VAL: Valproic

Fig. 3: High-performance liquid chromatography of rufinamide (a), lamotrigine (b), clonazepam (c), diazepam (d), and valproic (e) in their dosage forms

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