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Short communication

A highly selective RP-HPLC/UV method for therapeutic drug monitoring of structurally diverse antiepileptic drugs in human plasma

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Introduction

Epilepsy is one of the most prevalent neurological disorders, affecting approximately 0.6-1% of the population in developed countries and significantly impacting quality of life, cognitive function, and overall patient wellbeing. Long term pharmacotherapy remains the cornerstone of epilepsy management, with antiepileptic drugs (AEDs) being used to achieve seizure control and reduce the risk of complications. Therapeutic drug monitoring (TDM) therefore plays a critical role in optimizing AEDs therapy, by enabling individualized dose adjustment based on measured drug concentrations in biological fluids, most commonly plasma or serum. Among these AEDs are levetiracetam (LEV), lamotrigine (LTG), carbamazepine (CBZ), and its active metabolite carbamazepine-10,11-epoxide (CBZ-EP). These compounds differ significantly in their physicochemical and pharmacokinetic properties, making simultaneous determination analytically challenging. Reliable and accessible bioanalytical methods are therefore critical for routine clinical TDM (Martinho et al., 2024).

The aim of this study was to develop and validate a simple and rapid RP-HPLC/UV method for the simultaneous determination of LEV, LTG, CBZ, and CBZ-EP in human plasma suitable for routine clinical application.

Materials and methods

Chromatographic analyses were performed on Agilent 1260 Infinity III HPLC-UV, controlled by Open Lab 2.8 software. The separation of the AEDs and nitrazepam as internal standard (IS) was performed on a Zorbax Eclipse Plus Phenyl-Hexyl column (150 mm x 4.6, 3.5 µm; Agilent Technologies, Inc., USA) with gradient elution at a temperature of 40°C. Mixture of methanol (MeOH) and 25mM phosphate buffer (pH 5.1) was used as the mobile phase, with a flow rate of 1.0 mL/min, injection volume 20 µL and detection at 210 nm.

Two extraction procedures were optimized and evaluated: solid-phase extraction (SPE) using Bond Elut HLB cartridges (Agilent Technologies, Inc., USA) and protein precipitation (PP) using cold acetonitrile (ACN) at 4°C. Stock standard solutions of LTG, LEV, CBZ, CBZ-EP and IS were prepared in MeOH with concentrations of 500 µg/mL, 500 µg/mL, 500 µg/mL, 200 µg/mL and 500 µg/mL, respectively. The stock solutions were stored at a temperature of -20°C. Working standard solutions covering the therapeutic plasma concentration range were prepared by dilution of the stock solutions. The

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samples were prepared for SPE in 500 µL plasma from healthy volunteers and for PP in 100 µL plasma from healthy volunteers.

Results and discussion

Initially, chromatographic optimization was performed using a Zorbax Extend-C18 column (4.6 × 150 mm; 5 µm). Isocratic and gradient elution with different mobile phases containing phosphate buffer of varying molarities (15-50 mM), different pH values (3.0, 4.5 and 5.1) and mixtures with MeOH or ACN were investigated. Although satisfactory separation of CBZ, CBZ-EP, LTG, and IS was achieved, insufficient selectivity for LEV was observed. Optimal separation for all analytes was accomplished using the Zorbax Eclipse Plus Phenyl-Hexyl column (4.6 × 150 mm; 3.5 µm), which demonstrated improved baseline stability, enhanced retention and good resolution for all analytes. MeOH was selected as the organic modifier due to the improved retention and selectivity compared to ACN. After evaluating several detection wavelengths, 210 nm was selected as optimal because it provided adequate sensitivity for LEV, which lacks a strong chromophore, while maintaining satisfactory absorbance for LTG, CBZ and CBZ-EP, despite their stronger absorbance at higher wavelengths (~305 for LTG, ~285 for CBZ and CBZ-EP) (Kharouba and Mahmoud, 2023). Nitrazepam was selected as the IS due to its suitable physicochemical properties, stable extraction recovery and favourable chromatographic behaviour under the optimized analytical conditions.

During optimization of the PP, MeOH, ACN and salting-out with $ZnSO_4$ were evaluated as precipitation agents, under different centrifugation conditions. MeOH and $ZnSO_4$-based salting-out showed poor yield and were excluded. PP with cold ACN as a simple and economical method provided satisfactory results. To achieve sample concentration and avoid ACN as the final sample solvent, precipitated samples were evaporated to dryness under nitrogen and reconstituted with 50% MeOH. This approach improved extraction recovery compared to direct analysis of ACN extracts. Optimized PP with cold ACN was retained as an alternative extraction procedure when SPE is not available. SPE was performed using HLB cartridges because of their combined hydrophilic and lipophilic properties, suitable for the extraction of structurally diverse analytes. Optimization of the SPE steps revealed that washing with 1 mL of 5% MeOH in water caused loss of LEV. Therefore, the washing step was performed with only 1 mL of water. In order to concentrate the sample, an evaporation step (under nitrogen) of the eluate was introduced. However, SPE without evaporation resulted in slightly higher yields and faster preparation. Therefore, this extraction procedure was selected as the first choice for sample preparation. According to the ICH M10 Guideline, method validation was performed using blank plasma from healthy volunteers, spiked with AEDs and IS. The validated method was subsequently applied to a clinical sample from a patient under AEDs therapy, yielding reliable and reproducible results.

Conclusion

A simple, selective, accurate, and precise RP-HPLC/UV method was successfully developed for the simultaneous determination of LEV, LTG, CBZ, and CBZ-EP in human plasma. The proposed method is characterized by straightforward sample preparation, accessible instrumentation and an analytical range suitable for routine TDM. In addition, the method with PP requires only a small plasma volume, making it particularly suitable for vulnerable patient populations, including paediatric, pregnant, elderly, and critically ill patients, in whom minimal blood sampling is preferred. The applicability of the method in clinical practice was confirmed through successful analysis of plasma samples obtained from a patient with epilepsy receiving a combination of antiepileptic therapy.

References

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