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Article

A Rapid and Sensitive HPLC–DAD Assay to Quantify Lamotrigine, Phenytoin and Its Main Metabolite in Samples of Cultured HepaRG Cells

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Abstract

A sensitive and fast high-performance liquid chromatography–diode-array detection assay was developed and validated for the simultaneous quantification of 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH), phenytoin (PHT) and lamotrigine (LTG) in samples of cultured HepaRG cells. Chromatographic separation of analytes and internal standard (IS) was achieved in ~15 min on a C_{18}-column, at 35°C, using acetonitrile (6%), methanol (25%) and a mixture (69%) of water–triethylamine (99.7:0.3, v/v; pH 6.0), pumped at 1 mL/min. The analytes and IS were detected at 215 or 235 nm. Calibration curves were linear with regression coefficients >0.994 over the concentration ranges of 0.1–15 µg/mL for HPPH; 0.15–30 µg/mL for PHT and 0.2–20 µg/mL for LTG. The method showed to be accurate (bias value of ±10.5 or ±17.6% in the lower limit of quantification, LLOQ) and precise (coefficient variation ≤8.1 or ≤15.4% in the LLOQ), and the absolute recovery of the analytes ranged from 62.5 to 96.9%. HepaRG cells have emerged as a very promising *in vitro* model to evaluate metabolic, drug interaction and/or pharmacokinetic studies, and this methodology will be suitable to support subsequent studies involving the antiepileptic drugs PHT and LTG.

Introduction

The *in vitro* studies play a crucial role during the discovery of drug candidates (1). In fact, the high-throughput approaches for the early assessment of potential drug candidates are important tools, providing a key direction in the choice of the most promising chemical series to pursue (2). Hence, the need of early predictive data became essential for the development of several *in vitro* approaches to study drug disposition and pharmacokinetic properties. Among these *in vitro* tools are those which permit to assess the metabolite profiling and the potential for cytochrome P450 (CYP) drug interactions in various species, including human-based *in vitro* models (1, 3, 4). Undoubtedly, the CYP isoenzymes mediate the metabolism of the majority of drugs available today. Actually, serious clinical drug–drug interactions could be prevented by knowledge of the potential for inhibition and/or induction of CYP isoenzymes in early stages of drug discovery programs. Even the regulatory authorities widely accept the use of *in vitro* data for assessment of the potential for drug–drug interactions in the development of new drugs (2). Accordingly, several *in vitro* metabolism models have been developed and are now available to study the hepatic drug metabolism (5). However, it should be highlighted that only those models that involve human liver cells have the capacity to express the complete metabolic pathways similar to what occurs in men (6, 7). Nevertheless, despite the primary human hepatocytes are considered the "gold standard" model for these purposes, some pitfalls that have to be taken into account (e.g., scarce and erratic availability, poor stability of functions in culture, limited

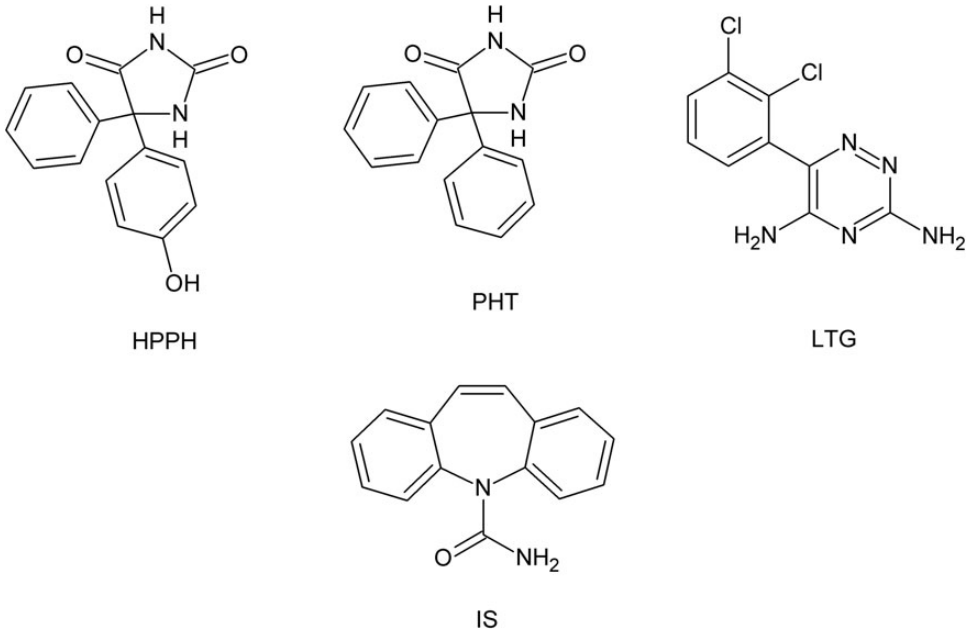

Figure 1. Chemical structures of HPPH, PHT, LTG and carbamazepine, which was used as internal standard (IS).

growth activity and life span) make them not as appropriate as would be desired for high-throughput screening (5, 7–12). Hence, other models have been proposed as an alternative to primary human hepatocytes like the immortalized human hepatic tumor cell lines such as HepG2, Huh-7 and HepaRG cells (8, 9, 13, 14). In fact, it is believed nowadays that the development of HepaRG cells, a new human cell-line derived from a hepatocellular carcinoma, constitutes a promising achievement to improve the evaluation of hepatic drug metabolism under *in vitro* conditions (8). Indeed, among several other advantages, the HepaRG cell line possesses the metabolic capacity characteristic of primary human hepatocytes and the indefinite proliferation property of hepatoma cell lines, making them a useful *in vitro* tool to study the hepatic kinetics of drugs and to foresee drug–drug interactions (5, 15, 16).

Thus, aiming at evaluating the potential for drug–drug interactions involving the antiepileptic drugs (AEDs) lamotrigine (LTG) and phenytoin (PHT) (Figure 1), a rapid, simple, sensitive, accurate and reliable analytical assay was herein developed, which enable the quantitative determination of LTG, PHT and its main metabolite in samples of cultured HepaRG cells. Although PHT is extensively *para*-hydroxylated to the inactive metabolite 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH) (Figure 1), mainly through CYP2C9 and CYP2C19 isoenzymes, LTG undergoes extensive metabolism to an inactive glucuronide metabolite (17). Therefore, this high-performance liquid chromatography assay with diode-array detection (HPLC–DAD) is a useful bioanalytical tool to support future *in vitro* metabolic, drug interaction and/or pharmacokinetic studies in HepaRG cell cultures incubated with PHT and/or LTG in the presence of other chemical entities (e.g., new drug candidates) to be tested.

Experimental

Reagents and cells

PHT, HPPH and carbamazepine (CBZ), used as internal standard (IS), were purchased from Sigma-Aldrich (St Louis, MO, USA). LTG was gently provided by Bluepharma (Coimbra, Portugal). Methanol and acetonitrile, both of HPLC gradient grade, were purchased from Fisher Scientific (Leicestershire, UK) and the ultra-pure water (HPLC grade, >18 MΩ cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Triethylamine was acquired from Merck KGaA (Darmstadt, Germany), the 85% orthophosphoric acid from Panreac Química SA (Barcelona, Spain) and ethyl acetate were purchased from Fisher Scientific (Leicestershire, UK). HepaRG cells (lot no. #48588) were obtained from Life Technologies—Invitrogen™ (through Alfagene, Portugal). The cell culture components including Williams' E medium, fetal bovine serum, hydrocortisone hemisuccinate, dimethyl sulfoxide and trypsin–EDTA were purchased from Sigma-Aldrich (St Louis, MO, USA).

Stock solutions, calibration standards and quality control samples

Stock solutions of PHT (30 mg/mL), HPPH (15 mg/mL) and LTG (10 mg/mL) were individually prepared by dissolving the appropriate amount of each compound in methanol. To obtain the corresponding working solutions, the stock solutions were then adequately diluted in water–methanol (50:50, v/v). Then, stock and working solutions of the individual compounds were properly mixed to afford six combined spiking solutions with final concentrations of 1.5, 3, 15, 60, 180 and 300 μg/mL for PHT, 1, 2, 5, 20, 80 and 150 μg/mL for HPPH and 2, 4, 15, 50, 125 and 200 μg/mL for LTG. Each one of these combined solutions was daily used for spiking aliquots of supplemented Williams' E medium to prepare six calibration standards in the concentration ranges of 0.15–30 μg/mL for PHT, 0.1–15 μg/mL for HPPH and 0.2–20 μg/mL for LTG. The stock solution of the IS was also prepared in methanol (2 mg/mL), and the working solution (200 μg/mL) was obtained after diluting an appropriate volume of the stock solution with water–methanol (50:50, v/v). The stock, working and combining solutions were stored at 4°C and protected from light, with exception of the IS working solution which was daily prepared.

Quality control (QC) samples at three representative concentration levels, representing the low (QC_1), medium (QC_2) and high (QC_3) ranges of the calibration curves, were independently prepared in

supplemented Williams' E medium. With this purpose, aliquots of supplemented Williams' E medium were spiked to attain final concentrations of 0.45, 15 and 27 µg/mL for PHT, 0.3, 7.5 and 13.5 µg/mL for HPPH and 0.6, 10 and 18 µg/mL for LTG. One other QC sample at the concentration of the lower limit of quantification (QC_{LLOQ}) was also prepared.

Apparatus and chromatographic conditions

Chromatographic analysis was carried out using an HPLC system (Shimadzu LC-2010A HT Liquid Chromatography) coupled with a DAD (Shimadzu SPD-M20A). All instrumental parts were automatically controlled by LC solution software (Shimadzu, Kyoto, Japan). The chromatographic separation of LTG, HPPH, PHT and IS was carried out at 35°C on a reversed-phase LiChroCART® Purospher Star column (C_{18}, 55 mm × 4 mm; 3 µm particle size) purchased from Merck KGaA (Darmstadt, Germany). An isocratic elution was applied using acetonitrile (6%), methanol (25%) and a mixture (69%) of water–triethylamine (99.7:0.3, v/v; pH 6.0), pumped at 1 mL/min. The mobile phase was filtered through a 0.2-µm filter and degassed ultrasonically for 15 min before use. The injection volume was 20 µL, and the wavelengths of 215 and 235 nm were selected for detection of the compounds.

Sample preparation and extraction

Each aliquot (200 µL) of supplemented Williams' E medium was added to 20 µL of IS working solution, 300 µL of acetonitrile and 1 mL of ethyl acetate. Then, this mixture was vortex-mixed for 30 s and centrifuged at 13,500 rpm (3 min). The organic layer was transferred to a glass tube, being the aqueous layer re-extracted twice more with ethyl acetate (1 mL each time) using the conditions previously described. The combined organic phases were evaporated to dryness under a gentle nitrogen stream at 45°C and then reconstituted with 100 µL of mobile phase. Finally, an aliquot (20 µL) of the final sample was injected into the chromatographic system.

Method validation

The international accepted recommendations for bioanalytical method validation (18–20) were followed to the validation of the developed method. Accordingly, the acceptance criteria proposed for specific validation parameters including selectivity, linearity, precision, accuracy, limits of quantification and recovery were considered.

Selectivity

To reproduce in the best way what happens in the real metabolic and pharmacokinetic studies, samples of supplemented Williams' E medium collected from cultured HepaRG cells were analyzed and compared with samples of the simple supplemented Williams' E medium. Under real experimental conditions, HepaRG cells were maintained in the Williams' E medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine and 5×10^{-5} M hydrocortisone hemisuccinate during 15 days. Afterward, cells were then maintained in the same culture medium but supplemented with 2% dimethyl sulfoxide (differentiation medium) for >15 days to obtain differentiated HepaRG cell cultures. In both phases, HepaRG cell cultures were maintained at 37°C in a humidified air incubator with 5% CO_2, and the medium was renewed every 3 days. Subsequently, the differentiated HepaRG cells were seeded at a high density (4.5×10^5 cells/cm^2) in 96-well plates and maintained during 48 h. After this period, the cells were exposed to 200 µL of supplemented Williams' E medium for 12 h. The total volume of each well was collected to an Eppendorf, and 300 µL of acetonitrile was immediately added.

Calibration curve

The linearity of the developed method for each analyte (PHT, HPPH and LTG) was evaluated in the concentration ranges previously defined using calibration curves prepared with six spiked supplemented Williams' E medium calibration standards and assayed on three different days ($n = 3$). Calibration curves were constructed by plotting the analyte–IS peak area ratio versus the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis using $1/x^2$ as weighting factor for all analytes, taking the plots and the sums of absolute percentage of relative error into account. This weighting factor yielded the best fit of peak area ratios versus concentration for all compounds (21).

Limits of quantification

The lower limit of quantification (LLOQ) is defined as the lowest concentration of the calibration curve, which can be measured with adequate inter/intraday precision and accuracy (18, 19). This parameter was evaluated by analyzing supplemented Williams' E medium samples prepared in replicates. The precision, expressed as percentage of coefficient of variation (CV), cannot exceed 20%; whereas accuracy, expressed by the deviation from nominal concentration value (bias), must be within ±20%.

Precision and accuracy

To investigate the interday precision and accuracy of the assay, QC samples analyzed on three consecutive days ($n = 3$) at the four concentration levels (QC_{LLOQ}, QC_1, QC_2 and QC_3) representative of the calibration range were used. On the other hand, the intraday precision and accuracy were assessed analyzing five sets of the QC samples in a single day ($n = 5$). Taking into account the acceptance criterion defined by the bioanalytical method validation guidelines, the intra- and interday precision (expressed as percentage of CV) must be ≤15% (or 20% in the LLOQ) and intra- and interday accuracy (expressed as percentage of *bias*) must be within ±15% (or ±20% in the LLOQ) (18, 19).

Recovery

Three QC samples (QC_1, QC2 and QC_3) were used to test the absolute recovery of the analytes from the samples submitted to the treatment previously described in the "Sample preparation and extraction" section. The recovery was calculated comparing the analytes' peak areas from extracted QC supplemented Williams' E medium samples with those obtained after direct injection of nonextracted solutions at the same nominal concentrations ($n = 5$). Likewise, the calculation of the ratio of IS peak areas in extracted samples and nonextracted solutions, evaluated at the concentration used in sample analysis, was used to define its absolute recovery.

Results

Method validation

The previously described chromatographic conditions enabled the separation of HPPH, PHT and LTG in spiked supplemented Williams' E culture medium samples. The order of elution of the compounds was the following: LTG, HPPH, PHT and CBZ (IS), with a running time of 15 min. Figure 2 shows the representative chromatograms of the analysis of blank and spiked supplemented Williams' E culture medium samples.

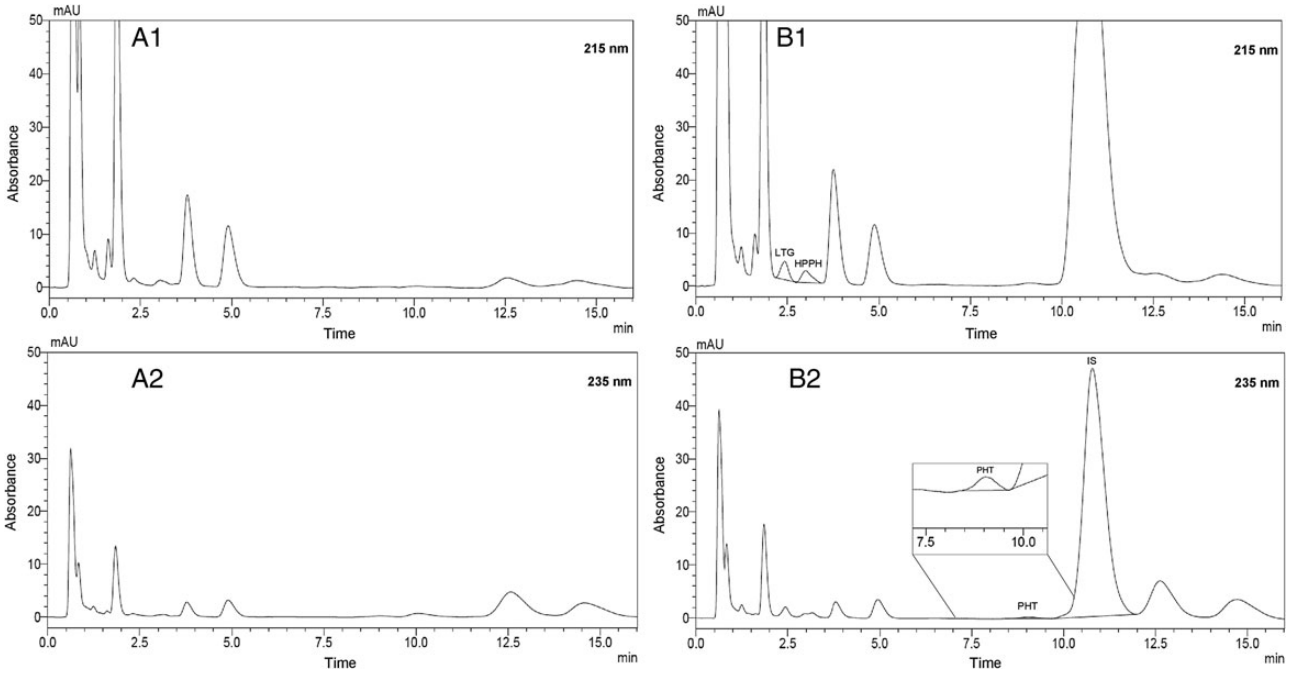

Figure 2. Typical chromatograms of extracted supplemented Williams' E medium samples obtained by the HPLC–DAD assay developed: blank supplemented Williams' E medium at 215 nm (A1) and 235 nm (A2) and supplemented Williams' E medium spiked with the analytes at the lower limit of quantification of the calibration ranges at 215 nm (B1) and 235 nm (B2). HPPH, 5-(4-hydroxyphenyl)-5-phenylhydantoin; IS, internal standard; LTG, lamotrigine; PHT phenytoin.

Table I. Mean Calibration Parameters Obtained for HPPH, PHT and LTG in Supplemented Williams' E Culture Medium ($n = 3$)

Analyte	Calibration parameters		
	Concentration range (μg/mL)	Equation[a]	r^2
HPPH	0.1–15	$y = 0.01288x + 0.0001$	0.995
PHT	0.15–30	$y = 0.0248x + 0.0007$	0.994
LTG	0.2–20	$y = 0.0172x + 0.0016$	0.997

[a]y represents the analyte–IS peak area ratio; x represents the analyte concentration (μg/mL).

Selectivity

Taking into account the chromatographic similarity of the blank samples of supplemented Williams' E medium collected from cultured HepaRG cells and those of simple supplemented Williams' E medium, the latter was chosen as matrix to the development and validation of this HPLC–DAD methodology.

Calibration curves and LLOQs

Over the concentration ranges established for all of the analytes (PHT, HPPH and LTG), the calibration curves obtained in supplemented Williams' E culture medium were linear ($r^2 \geq 0.994$; Table I), showing a consistent correlation between analyte–IS peak area ratios and the corresponding nominal concentrations in supplemented Williams' E culture medium. Regarding the wide calibration range and to compensate for heteroscedasticity, the calibration curves were subjected to weighted linear regression analysis using $1/x^2$ as the weighting factor for all analytes. The choice of this factor took into account the plots and the sums of absolute percentage relative error as statistical criteria. Table I summarizes the regression equations of the calibration curves and the corresponding determination coefficients (r^2) achieved for each analyte. The LLOQs were experimentally defined as 0.1 μg/mL for HPPH, 0.15 μg/mL for PHT and 0.2 μg/mL for LTG with acceptable precision (CV ≤ 15.4%) and accuracy (*bias* varied from −17.6 to 12.0%).

Precision and accuracy

Table II contains the data for intra- and interday precision and accuracy obtained from QC supplemented Williams' E medium samples at the four different concentration levels (QC_{LLOQ}, QC_1, QC_2 and QC_3). It is worthy to mention that all of the data fulfilled the acceptance criteria. Therefore, the overall inter- and intraday CV values ≤8.1% (or 15.4% in the QC_{LLOQ}), and the overall inter and intraday *bias* values ranging from −9.4 to 10.5% (or −17.6–12.0% in the QC_{LLOQ}) clearly demonstrate that the HPLC–DAD method herein described is reliable, accurate and reproducible.

Recovery

Five repeated analysis ($n = 5$) at the three concentration levels (QC_1, QC_2 and QC_3) for HPPH, PHT and LTG were used to determine the overall absolute recovery of each analyte. Table III exhibits the absolute recovery data. As noted, the absolute mean recoveries ranged from 62.5 to 96.9% with CV values <10.9% for all analytes (PHT, HPPH and LTG). On the other hand, the absolute recovery value of the IS (CBZ) was 82.4% with a CV value of 9.9%.

Discussion

Epilepsy is a serious chronic neurological disorder affecting ~50 million people worldwide (22–24). LTG and PHT are two AEDs commonly used in clinical practice. LTG has been widely used in epilepsy treatment due to its broad spectrum of activity, and it is also effective as a mood stabilizer agent (25, 26). In turn, the therapeutic value of PHT could be attested by its inclusion in the World Health

Table II. Inter- and Intraday Precision (% CV) and Accuracy (% *bias*) Values Obtained for HPPH, PHT and LTG in Samples of Supplemented Williams' E Culture Medium at the Concentrations of the Lower Limit of Quantification (*) and at the Low (QC_1), Middle (QC_2) and High (QC_3) Concentrations Representative of the Calibration Ranges

Analyte	Nominal concentration (μg/mL)	Interday		Intraday	
		Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)
HPPH	0.1*	1.8	12.0	15.4	0.6
	0.3	3.3	1.5	0.5	1.5
	7.5	3.6	−10.0	4.4	−9.2
	13.5	4.7	−3.2	0.3	5.0
PHT	0.15*	7.9	−16.2	3.7	−17.6
	0.45	3.6	0.1	1.9	−2.5
	15	3.5	−9.4	4.3	−8.5
	27	8.1	−1.3	0.4	10.5
LTG	0.2*	3.9	−4.1	3.9	1.2
	0.6	4.1	−5.5	1.8	−0.2
	10	3.2	−6.6	5.3	−3.8
	18	6.1	1.8	0.2	1.8

CV, coefficient of variation; *bias*, deviation from nominal concentration value.

Table III. Absolute Recovery of HPPH, PHT and LTG in Supplemented Williams' E Culture Medium

Analyte	Nominal concentration (μg/mL)	Absolute recovery (%)[a]	Precision (% CV)
HPPH	0.3	85.8 ± 9.4	10.9
	7.5	80.4 ± 3.9	3.9
	13.5	62.5 ± 1.4	2.2
PHT	0.45	85.1 ± 8.9	10.5
	15	80.1 ± 3.5	4.4
	27	66.2 ± 3.4	5.2
LTG	0.6	96.9 ± 6.2	6.4
	10	85.2 ± 2.3	2.7
	18	82.4 ± 2.5	3.0

[a]Mean ± standard deviation, $n = 5$.

Organization's list of essential medicines, which contains the most important medication needed in a basic health system (27).

Even though interactions may occur under AED polytherapy regimens, such drugs are commonly prescribed for long periods of time making possible the cotherapy and, consequently, the occurrence of drug–drug interactions with other kind of therapeutic agents used for usual comorbidities (28, 29). Actually, several comorbid health conditions are common among people with epilepsy, mainly psychiatric disorders (e.g., depression, psychosis, attention deficit hyperactivity, anxiety and panic disorder), increasing the likelihood of coprescription (24). This fact added to the narrow therapeutic index of several AEDs and their marked effects on the activity of CYP isoenzymes (inhibition or induction) (28) make the patients with epilepsy especially susceptible to complex and unpredictable pharmacokinetic and also pharmacodynamic interactions (28, 30–32).

As it is well known, PHT is extensively involved in drug interactions with other AEDs (felbamate, oxcarbazepine and valproic acid), and also with many other drugs such as antidepressants (fluoxetine, fluvoxamine, imipramine, paroxetine, sertraline, trazodone and viloxazine), antimicrobials (chloramphenicol, fluconazole, isoniazid, miconazole and sulfaphenazole), antineoplastic drugs (doxifluridine, fluorouracil, tamoxifen and tegafur) and several compounds from miscellaneous classes such as allopurinol, amiodarone, azapropazone, cimetidine, chlorpheniramine, dextropropoxyphene, diltiazem, disulfiram, omeprazole, tacrolimus and ticlopidine (28, 33). On the other hand, although there are fewer interactions described in the literature involving LTG, the AED valproic acid and the antidepressant sertraline have been found to increase its serum concentrations (28).

Nowadays, it is well recognized the value of the early identification of potential drug interactions in the development process of new drug candidates (34). In this context, *in vitro* methodologies are being increasingly used during preclinical drug development for the prediction of drug–drug interactions, providing useful data to extrapolate to human (35–38). Indeed, several *in vitro* models have demonstrated the ability to identify relevant drug interactions. For instance, the metabolic interactions between valproic acid and LTG (39), and those involving the PHT and the selective serotonin reuptake inhibitors, norfluoxetine or paroxetine (33), have been identified and studied in *in vitro* conditions. Therefore, bearing in mind the valuable characteristics of HepaRG cells, this *in vitro* model arises as a valuable tool to foresee drug–drug interactions (5, 16).

Thus, bioanalysis emerged as a critical tool in the process of drug discovery and development, being essential for pharmacokinetic/pharmacodynamics characterization of a drug compound. In fact, a plethora of assays has been continuously developed for novel chemical entities to support the various stages of discovery and development programs, involving quantitative bioanalytical methods for the measurement of parent compounds and their metabolites in several biological samples (40, 41). However, the degree of development and quality of the bioanalytical assays used tend to increase as the lead drug candidates progress to more advanced stages. Accordingly, the levels of analytical acceptance criteria become stricter in the latter stages, being the results confirmed by appropriate validation assays, which attest its reliability, robustness and accuracy (40–42). This aspect explains the scarcity of HPLC methodologies fully validated in the literature to support *in vitro* studies. Notwithstanding, the HPLC–DAD assay herein reported to quantify LTG, PHT and its main metabolite was extensively validated taking into account the international criteria of the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) guidelines.

Apart from other methodologies like, for example, gas chromatography (43), several HPLC assays have been developed for the simultaneous determination of PHT and LTG in human plasma or urine using mainly DAD or ultraviolet detection (30, 44–50). For instance, a HPLC–DAD assay, which enables the simultaneous quantification of PHT and LTG in human plasma, has been previously developed

by our group (50). Nevertheless, the PHT metabolite (HPPH) was not considered in such a bioanalytical assay; moreover, it is desirable the availability of less expensive, faster and simpler analytical methodologies to support drug interaction screening studies, and thus, the microextraction by packed sorbent used therein for sample preparation was not considered to be the most easy-to-use approach in this case. On the other hand, although the differences in terms of selectivity and sensitivity between DAD and mass spectrometry detectors used by other authors are incontestable (51) for screening purposes, the current technique employing a more accessible and cheaper DAD detector is considered to be appropriate. Taking also into account the LC–MS-MS method published by Kadi *et al.* (51), which was developed to the simultaneous determination of LTG and PHT in human plasma, it is also evident that our HPLC–DAD assay requires a more environmentally friendly chromatographic mobile phase because of the different nature of the column stationary phase employed (reversed-phase versus normal-phase liquid chromatography). Additionally, it should not be neglected the lack of HPLC assays fully validated for the quantification of LTG or/and PHT in culture media or even in supernatants of cell cultures. Actually, the HPLC–DAD method herein described is the first one, which permits the quantification of LTG, PHT and its main metabolite in samples of cultured HepaRG cells. In fact, we intended to develop a robust technique, which will provide results with a high level of confidence that could be properly interpreted.

Conclusion

In recent years, HepaRG cells emerged as a very promising model to evaluate the hepatic drug metabolism under *in vitro* conditions. Consequently, analytical methodologies to support the studies conducted with this model are extremely relevant. Thus, the reported HPLC–DAD method, validated according to the international requirements of EMA and FDA, will be suitable to support future metabolic, drug interaction and pharmacokinetics studies involving PHT and LTG in cultured HepaRG cells.

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References

1. Zhang, D., Luo, G., Ding, X., Lu, C.; Preclinical experimental models of drug metabolism and disposition in drug discovery and development; *Acta Pharmaceutica Sinica B*, (2012); 2: 549–561.
2. Walsky, R.L., Boldt, S.E.; In vitro cytochrome P450 inhibition and induction; *Current Drug Metabolism*, (2008); 9: 928–939.
3. Bajpai, M., Esmay, J.D.; In vitro studies in drug discovery and development: an analysis of study objectives and application of good laboratory practices (GLP); *Drug Metabolism Reviews*, (2002); 34: 679–689.
4. Ferreira, A., Rodrigues, M., Silvestre, S., Falcão, A., Alves, G.; HepaRG cell line as an in vitro model for screening drug–drug interactions mediated by metabolic induction: amiodarone used as a model substance; *Toxicology in Vitro*, (2014); 28: 1531–1535.
5. Anthérieu, S., Chesné, C., Li, R., Camus, S., Lahoz, A., Picazo, L., *et al.*; Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells; *Drug Metabolism and Disposition*, (2010); 38: 516–525.
6. Guillouzo, A., Guguen-Guillouzo, C.; Evolving concepts in liver tissue modeling and implications for in vitro toxicology; *Expert Opinion on Drug Metabolism & Toxicology*, (2008); 4: 1279–1294.
7. Jossé, R., Aninat, C., Glaise, D., Dumont, J., Fessard, V., Morel, F., *et al.*; Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies; *Drug Metabolism and Disposition*, (2008); 36: 1111–1118.
8. Andersson, T.B.; The application of HepRG cells in evaluation of cytochrome P450 induction properties of drug compounds; *Methods in Molecular Biology*, (2010); 640: 375–387.
9. Aninat, C., Piton, A., Glaise, D., Le Charpentier, T., Langouët, S., Morel, F., *et al.*; Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells; *Drug Metabolism and Disposition*, (2006); 34: 75–83.
10. Szabo, M., Veres, Z., Baranyai, Z., Jakab, F., Jemnitz, K.; Comparison of human hepatoma HepaRG cells with human and rat hepatocytes in uptake transport assays in order to predict a risk of drug induced hepatotoxicity; *PLoS One*, (2013); 8: e59432.
11. Turpeinen, M., Tolonen, A., Chesne, C., Guillouzo, A., Uusitalo, J., Pelkonen, O.; Functional expression, inhibition and induction of CYP enzymes in HepaRG cells; *Toxicology in Vitro*, (2009); 23: 748–753.
12. Donato, M.T., Castell, J.V.; Strategies and molecular probes to investigate the role of cytochrome P450 in drug metabolism: focus on in vitro studies; *Clinical Pharmacokinetics*, (2003); 42: 153–178.
13. Lübberstedt, M., Müller-Vieira, U., Mayer, M., Biemel, K.M., Knöspel, F., Knobeloch, D., *et al.*; HepaRG human hepatic cell line utility as a surrogate for primary human hepatocytes in drug metabolism assessment in vitro; *Journal of Pharmacological and Toxicological Methods*, (2011); 63: 59–68.
14. Pernelle, K., Le Guevel, R., Glaise, D., Stasio, C.G.-D., Le Charpentier, T., Bouaita, B., *et al.*; Automated detection of hepatotoxic compounds in human hepatocytes using HepaRG cells and image-based analysis of mitochondrial dysfunction with JC-1 dye; *Toxicology and Applied Pharmacology*, (2011); 254: 256–266.
15. Guillouzo, A.; New perspectives in the use of human hepatocytes in the preclinical drug development process; *Annales Pharmaceutiques Françaises*, (2008); 66: 288–295.
16. Darnell, M., Schreiter, T., Zeilinger, K., Urbaniak, T., Söderdahl, T., Rossberg, I., *et al.*; Cytochrome P450-dependent metabolism in HepaRG cells cultured in a dynamic three-dimensional bioreactor; *Drug Metabolism and Disposition*, (2011); 39: 1131–1138.
17. Patsalos, P.N., Berry, D.J., Bourgeois, B.F.D., Cloyd, J.C., Glauser, T.A., Johannessen, S.I., *et al.*; Antiepileptic drugs-best practice guidelines for therapeutic drug monitoring: a position paper by the subcommission on therapeutic drug monitoring, ILAE Commission on Therapeutic Strategies; *Epilepsia*, (2008); 49: 1239–1276.
18. Food and Drug Administration (FDA). (2001) Guidance for industry: bioanalytical methodvalidation. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf (accessed July 20, 2015).
19. European Medicines Agency (EMA). (2011) Guideline on bioanalytical method validation. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf (accessed July 20, 2015).
20. Shah, V.P., Midha, K.K., Findlay, J.W., Hill, H.M., Hulse, J.D., McGilveray, I.J., *et al.*; Bioanalytical method validation—a revisit with a decade of progress; *Pharmaceutical Research*, (2000); 17: 1551–1557.
21. Almeida, A.M., Castel-Branco, M.M., Falcão, A.C.; Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods; *Journal of Chromatography B*, (2002); 774: 215–222.
22. Zhao, M., Alleva, R., Ma, H., Daniel, A.G.S., Schwartz, T.H.; Optogenetic tools for modulating and probing the epileptic network; *Epilepsy Research*, (2015); 116: 15–26.
23. Zhang, C., Kwan, P., Zuo, Z., Baum, L.; The transport of antiepileptic drugs by P-glycoprotein; *Advanced Drug Delivery Reviews*, (2012); 64: 930–942.

24. Savage, N.; Epidemiology: the complexities of epilepsy; *Nature*, (2014); 511: S2–S3.
25. Lees, G., Leach, M.J.; Studies on the mechanism of action of the novel anticonvulsant lamotrigine (Lamictal) using primary neurological cultures from rat cortex; *Brain Research*, (1993); 612: 190–199.
26. Werz, M.A.; Pharmacotherapeutics of epilepsy: use of lamotrigine and expectations for lamotrigine extended release; *Therapeutics and Clinical Risk Management*, (2008); 4: 1035–1046.
27. World Health Organization. (2013) WHO model list of essential medicines. http://apps.who.int/iris/bitstream/10665/93142/1/EML_18_eng.pdf?ua=1 (accessed September 1, 2015).
28. Perucca, E.; Clinically relevant drug interactions with antiepileptic drugs; *British Journal of Clinical Pharmacology*, (2006); 61: 246–255.
29. Patsalos, P.N., Perucca, E.; Clinically important drug interactions in epilepsy: general features and interactions between antiepileptic drugs; *Lancet Neurology*, (2003); 2: 347–356.
30. Bugamelli, F., Sabbioni, C., Mandrioli, R., Kenndler, E., Albani, F., Raggi, M.; Simultaneous analysis of six antiepileptic drugs and two selected metabolites in human plasma by liquid chromatography after solid-phase extraction; *Analytica Chimica Acta*, (2002); 472: 1–10.
31. Johannessen, S.I., Landmark, C.J.; Antiepileptic drug interactions—principles and clinical implications; *Current Neuropharmacology*, (2010); 8: 254–267.
32. Brodie, M.J., Mintzer, S., Pack, A.M., Gidal, B.E., Vecht, C.J., Schmidt, D.; Enzyme induction with antiepileptic drugs: cause for concern? *Epilepsia*, (2013); 54: 11–27.
33. Schmider, J., Greenblatt, D.J., von Moltke, L.L., Karsov, D., Shader, R.I.; Inhibition of CYP2C9 by selective serotonin reuptake inhibitors in vitro: studies of phenytoin *p*-hydroxylation; *British Journal of Clinical Pharmacology*, (1997); 44: 495–498.
34. Löscher, W.; Current status and future directions in the pharmacotherapy of epilepsy; *Trends in Pharmacological Sciences*, (2002); 23: 113–118.
35. Lin, J.H., Lu, A.Y.; Role of pharmacokinetics and metabolism in drug discovery and development; *Pharmacological Reviews*, (1997); 49: 403–449.
36. Fabre, G., Combalbert, J., Berger, Y., Cano, J.P.; Human hepatocytes as a key in vitro model to improve preclinical drug development; *European Journal of Drug Metabolism and Pharmacokinetics*, (1990); 15: 165–171.
37. Costa, A., Sarmento, B., Seabra, V.; An evaluation of the latest in vitro tools for drug metabolism studies; *Expert Opinion on Drug Metabolism & Toxicology*, (2014); 10: 103–119.
38. Wienkers, L.C., Heath, T.G.; Predicting in vivo drug interactions from in vitro drug discovery data; *Nature Reviews Drug Discovery*, (2005); 4: 825–833.
39. Rowland, A., Elliot, D.J., Williams, A., Mackenzie, P.I., Dickinson, R.G., Miners, J.O.; In vitro characterization of lamotrigine N2-glucuronidation and the lamotrigine-valproic acid interaction; *Drug Metabolism and Disposition*, (2006); 34: 1055–1062.
40. Pandey, S., Pandey, P., Tiwari, G., Tiwari, R.; Bioanalysis in drug discovery and development; *Pharmaceutical Methods*, (2010); 1: 14–24.
41. Korfmacher, W.A.; Bioanalytical support for both in vitro and in vivo assays across drug discovery and drug development; *Encyclopedia of Drug Metabolism and Interactions*, (2011); 0: 1–18.
42. Srinivas, N.R.; Applicability of bioanalysis of multiple analytes in drug discovery and development: review of select case studies including assay development considerations; *Biomedical Chromatography*, (2006); 20: 383–414.
43. Queiroz, M.E.C., Silva, S.M., Carvalho, D., Lanças, F.M.; Determination of lamotrigine simultaneously with carbamazepine, carbamazepine epoxide, phenytoin, phenobarbital, and primidone in human plasma by SPME-GC-TSD; *Journal of Chromatographic Science*, (2002); 40: 219–223.
44. Serralheiro, A., Alves, G., Fortuna, A., Rocha, M., Falcão, A.; First HPLC-UV method for rapid and simultaneous quantification of phenobarbital, primidone, phenytoin, carbamazepine, carbamazepine-10,11-epoxide, 10,11-trans-dihydroxy-10,11-dihydrocarbamazepine, lamotrigine, oxcarbazepine and licarbazepine in human plasma; *Journal of Chromatography B*, (2013); 925: 1–9.
45. Patil, K.M., Bodhankar, S.L.; Simultaneous determination of lamotrigine, phenobarbitone, carbamazepine and phenytoin in human serum by high-performance liquid chromatography; *Journal of Pharmaceutical and Biomedical Analysis*, (2005); 39: 181–186.
46. Asadi, M., Dadfarnia, S., Haji Shabani, A.M., Abbasi, B.; Simultaneous extraction and quantification of lamotrigine, phenobarbital, and phenytoin in human plasma and urine samples using solidified floating organic drop microextraction and high-performance liquid chromatography; *Journal of Separation Science*, (2015); 38: 2510–2516.
47. Meyler, M., Kelly, M.T., Smyth, M.R.; New method for the determination of four antiepileptic drugs in human plasma by high performance liquid chromatography; *Chromatographia*, (1993); 36: 27–32.
48. Khoschsorur, G.A., Frühwirth, F., Halwachs-Baumann, G.; Simple and rapid HPLC method for simultaneous determination of multiple antiepileptic drugs in human serum; *Chromatographia*, (2001); 54: 345–349.
49. Rani, S., Malik, A.K., Singh, B.; Novel micro-extraction by packed sorbent procedure for the liquid chromatographic analysis of antiepileptic drugs in human plasma and urine; *Journal of Separation Science*, (2012); 35: 359–366.
50. Ferreira, A., Rodrigues, M., Oliveira, P., Francisco, J., Fortuna, A., Rosado, L., *et al.*; Liquid chromatographic assay based on microextraction by packed sorbent for therapeutic drug monitoring of carbamazepine, lamotrigine, oxcarbazepine, phenobarbital, phenytoin and the active metabolites carbamazepine-10,11-epoxide and licarbazepine; *Journal of Chromatography B*, (2014); 971: 20–29.
51. Kadi, A.A., Kassem, M.G., Makeen, H.A., Alhazmi, H.A.; Simultaneous determination of phenytoin and lamotrigine in human plasma using hydrophilic interaction liquid chromatography–triple quadrupole mass spectrometry; *Digest Journal of Nanomaterials and Biostructures*, (2013); 8: 1113–1122.