ABSTRACT

Purpose: To adapt and validate an HPLC method for verapamil determination in blood and urine samples.

Materials/Methods: Identification of verapamil and its metabolites was made by means of gas-chromatography, using Agilent 7890B/5977A GC-MS system featuring a DB-1701 column. Quantification was done by means of liquid chromatography on Agilent 1260 series HPLC, equipped with Zorbax Extend-C18 column and both diode-array and fluorescent detection modules. Blood and urine specimens were taken from patients of the Clinic for intensive treatment of acute intoxications and toxicocollergies within the course of their treatment.

Results: GC-MS identification of verapamil and its metabolites was carried out after simple liquid-liquid extraction of samples without further chemical derivatization. Adapted HPLC method for quantification require isocratic conditions and mobile phase, consisted of phosphate buffer (pH 2.7; 10 mM) containing 1.5 ml L⁻¹ triethylamine – acetonitrile (70:30, v/v) at 20 °C, flow-rate 1.0 mL/min and FLD detection (excitation: 203 nm, emission: 320 nm). The method was demonstrated to be linear within the whole region of interest (4.6-4600 ng mL⁻¹) with excellent accuracy (101.7-102.2%) and inter-day precision (5.81%) as well as good analytical recovery (81.2%) and LOQ (7.0 ng mL⁻¹).

Conclusion: A precise and easy to use method for verapamil detection and quantification is developed. The method is applicable as a routine procedure in the Laboratory of analytical toxicology for both diagnosis clarification in cases of acute intoxications and therapeutic drug monitoring.

Keywords: verapamil, GC-MS, HPLC, acute intoxications, therapeutic drug monitoring

INTRODUCTION

Verapamil (Fig. 1) is an antihypertensive and anti-arrhythmic medication of calcium channel blocker family, used for the treatment of HBP [1, 2], angina pectoris [3] and supraventricular tachycardia [4], given orally or i.v. It is also known to possess anti-manic properties [5, 6], and although not as effective as valproate or lithium, for example, it could be the medication of choice in specific occasions due to its very low teratogenic profile [7].

Fig. 1. Chemical structure of verapamil. The applicable form is usually hydrochloride.

Common dosage corresponds to blood verapamil therapeutic concentration of 20-250 ng mL⁻¹, acute intoxications lead to values close to 1000 ng mL⁻¹, and levels above 2500 ng mL⁻¹ are considered comatose-fatal [8]. Side effects are reportedly common (11.3%) and include constipation (4.03%), dizziness (3.65%), headache (1.54%), and other (less than 1%); as a result in approximately 3% of cases, therapy may require premature discontinuation [9].

Taken in quantities above the toxic dose, verapamil may cause any of following principal effects: hypotension due to arterial vasodilatation, bradycardia and atrioventricular block and cardiogenic shock secondary to a negative inotropic effect; hyperkalemia and metabolic acidosis are also possible [10-16]. Recently, intravenous lipid emulsion (ILE) infusion therapy is repeatedly reported to be highly effective, especially in cases of massive overdoses [17-21].

After oral ingestion, verapamil is effectively absorbed (above 90%), but bioavailability is low (10-35%) due to its extensive first-pass metabolism; plasma protein binding is estimated to be 90% [22]. It undergoes a liver degradation involving CYP450 isoforms, mainly by oxidative dealkylation to more than 30 identified metabolites, notably partially active (approx. 20%) norverapamil (N-desmethylverapamil) [23-25]. Excretion of verapamil is by the kidney with an elimination half-life of 2.8-7.4 hours; only 3-4% of the initial drug is in unchanged form and roughly 70% as metabolites [22].
Although acute intoxications with calcium channel blockers in general and with verapamil, in particular, are rare, the mortality rate may exceed 10% [26]. That is why any laboratory of analytical toxicology should maintain appropriate techniques for verapamil determination. For identification purposes, GC-MS is considered a gold standard because of its versatility of toxicological applications as well as unambiguity of its results [27-28]. Quantification could also be done by gas chromatography, although liquid techniques, such as HPLC (preferably in tandem with fluorescence detection) are more convenient [25, 29-33]. Sample preparation routinely includes liquid-liquid extraction, and however, solid-phase extraction methods have also been proposed [34]. Critically studying the literature available, one can notice that developed methods for verapamil quantification are mostly optimized for precision and sensitivity and, therefore, require either complicated hyphenated equipment, arduous multi-step procedures and/or complex extract/eluent mixtures. As clinical work at toxicology units is often urgent, rapidity of results and simplicity of lab work may be of greater importance. Therefore a simple, yet effective procedure should be developed by optimization of the available techniques.

MATERIALS AND METHODS

Analytical identification of organic compounds relied on Agilent 7890B/5977A GC-MS system equipped with a DB-1701 column (30 m × 0.250 mm × 0.25 µm). The HPLC analysis was done onto Agilent 1260 Infinity system featuring Zorbax Extend-C18 column (150 mm × 4.6 mm × 5 µm) and DAD/FLD detection modules. Human blood and urine samples originated from controlled stationary patients of Naval Hospital – Varna. Deionized water (0.067-0.100 µS cm–1, TKA™ Pacific water purification system), HPLC grade solvents, and only analytical grade chemicals were used. Agilent OpenLAB (ChemStation edition, rev. C.01.05), MassHunter (rev. B.07.00), and spectral library NIST (ver. 2.0) software were used for chromatographic data acquisition and manipulation. Statistical processing was done by MS Excel™ and OriginPro® software.

RESULTS AND DISCUSSION

For paraclinical identification of verapamil intoxication, a 4 mL urine sample is required. The sample is processed by routine GC-MS screening procedure, including initial deproteinization (500 µL acetonitrile), followed by simple liquid-liquid extraction (4 mL ethyl acetate in alkaline conditions); organic (upper) layer is transferred, dried (100 mg anhydrous MgSO₄), and centrifuged (2 min at 4000 rpm). The solvent is evaporated to dryness (60°C under N₂ stream), and the residue is reconstituted in 50 µL of methanol. The GC-MS injection volume is 1 µL; operation conditions are given in Tabl. 1. Verapamil peak is always present in chromatograms of positive cases at Rₜ = 42.4 min, mass spectrum (EI, 70 eV): m/z (Iₚ, %): 303 (100), 304 (23), 58 (17), 151 (12). Depending on specific conditions (e.g. the extent of intoxication, time from the event and the measures taken), series of metabolites could be identified as well, most notably norverapamil at Rₜ = 43.6 min, mass spectrum (EI, 70 eV): m/z (Iₚ, %): 289 (100), 290 (21), 151 (19), 152 (12) and N-desalkylverapamil at Rₜ = 24.7 min, mass spectrum (EI, 70 eV): m/z (Iₚ, %): 57 (100), 164 (78), 290 (73), 247 (55), 70 (49).

Analytical quantification of verapamil is made by HPLC, applying external calibration approach; 0.5 mL blood serum/plasma sample is required. Sample preparation goes through alkalinization (500 µL 1 M NaOH), deproteinization (1.5 mL acetonitrile) and double liquid-liquid extraction (2 × 3 mL of ethyl acetate), desiccation

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of combined extracts (100 mg anhydrous MgSO₄), centrifugation (1 min at 4000 rpm), and evaporation (60°C under N₂ stream). The residue is reconstituted in 500 µL of the mobile phase followed by syringe filtration (0.22 lm, Nylon); 20 µL of filtrate are injected on the HPLC column. Liquid chromatography was done under isocratic conditions. Mobile phase consisted of phosphate buffer (pH 2.7; 10 mM) containing 1.5 mL L⁻¹ triethylamine – acetonitrile (70:30, v/v) at 20°C, flow-rate 1.0 mL/min and FLD detection (excitation: 203 nm, emission: 320 nm). We tried to use a UV-DAD detection (λ=222, 224, 226, 278, 298 nm) only to found this approach inadequate, as even at the highest concentration used the quality of the signal was not satisfactory. Retention times were between 3.9 and 4.1 min.

Validation of the method follows the recommendations of the Internatioanl Committee of Harmonization (ICH) as well as the United Nations Office on Drug and Crimes (UNODC) protocols for analysis of biological specimens. Stock verapamil solution (4.6 µg mL⁻¹) was prepared by diluting 1 mL of the original substance (2.5 mg mL⁻¹ verapamil.HCl) with a mobile phase. Ten standard solutions (4.6-4600 ng mL⁻¹) were prepared by progressive dilutions, analyzed, and results fitted linearly (Fig. 2). The model was linear over the whole concentration range (4.6-4600 ng mL⁻¹) with Pearson’s R²=0.9994. Inter-day precision was estimated to 0.55% at 920 ng mL⁻¹ and 5.8% at 11.5 ng mL⁻¹. Intra-day precision (one week) at 920 ng mL⁻¹ equals 13.8%. Accuracy was determined for low (101.7% at 23 ng mL⁻¹) and high (102.2% at 920 ng mL⁻¹) concentration zones. Spiking blank blood samples, 81.2% analytical recovery was determined. Limits of detection (LOD) and quantification (LOQ) are estimated to be 2.1 ng mL⁻¹ and 7.0 ng mL⁻¹, respectively. Stability of retention time was within 0.1 min over 10 days period. A single experimental cycle takes approximately 45 minutes altogether, including sample preparation, HPLC analysis and administrative information maintenance.

**CONCLUSION**

Rapid, stable and precise HPLC method for the quantitative determination of verapamil was developed, optimized and applied in clinical practice. The method is a useful asset for the Lab of Analytical toxicology as it helps in diagnostics and treatment processes. Identification of acute intoxications is done my GC-MS and targets verapamil itself as well as some of its metabolites.
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