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Short communication

CYP3A4 activity in four different animal species liver microsomes using 7-benzyloxyquinoline and HPLC/spectrofluorometric determination

Narine Baririan [a], Jean-Pierre Desager [a], Martine Petit [b], Yves Horsmans [a,b,*]

[a] *Clinical Pharmacology Unit, Hôpital Universitaire St. Luc, Université Catholique de Louvain (UCL), Avenue Hippocrate, 10 MD/GAEN, 1200 Brussels, Belgium*
[b] *Department of Gastroenterology, Université Catholique de Louvain (UCL), Brussels, Belgium*

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Abstract

Some microplate-based direct assays with different fluorometric substrates have been developed, among which 7-benzyloxyquinoline (BOQ) has demonstrated the highest degree of selectivity for CYP3A subfamily. In our study, we firstly developed and validated an efficient, fast and cheap HPLC/spectrofluorometric analytical method to quantify 7-hydroxyquinoline (BOQ metabolite). Secondly, BOQ oxidation rate (1.95 ± 0.24 μM/mg protein/min) was compared to that of midazolam (MDZ) (1.4 ± 0.21 μM/mg protein/min), an other specific CYP3A probe. However, the difference did not reach statistically significance (test of Sign; $p = 0.125$, two tailed). Thirdly, the potential use of BOQ in other species than the rat (mouse, dog and monkey) was studied. The highest BOQ activity was observed in rat microsomes (3.75 μmol/mg protein/min) with lower P450 content (0.3 nmol/mg protein) compared to other species. Finally, the effect of CYP3A enzymes-selective inhibitor ketoconazole on the dealkylation of BOQ in control and dexamethasone (DM)-treated rat microsomes was studied. Ketoconazole inhibition potency was greater in control ($IC_{50} \approx 21.6$ μM) compared to DM induced ($IC_{50} \approx 32.3$ μM) microsomes. At concentrations greater than that considered to be enzyme-selective (e.g., 10–30 μM), ketoconazole inhibitory activity did not rise significantly, and at the maximal concentration tested (1000 μM) a nearly similar inhibition (76%) was observed than that at 50 μM concentration (68.2%).

Keywords: CYP3A(4); HPLC; 7-Benzyloxyquinoline; Midazolam; Ketoconazole

1. Introduction

Cytochrome P450 (CYP) enzymes are the main enzymes catalyzing drugs and other xenobiotics metabolism. It is now recognized that CYP3A subfamily is the most important of all drug-metabolizing enzymes and that CYP3A4 is the most mass-abundant isoform both in humans and animals [1]. A number of CYP substrates and inhibitors have been identified that allow the activity measurement of this isoform.

Cytochrome P450 activity assays which have fluorometric endpoints are advantageous in that they offer high sensitivity and are often direct and homogenous assays. Some microplate-based direct assays for the activity of the main cytochromes, with different fluorometric substrates have been developed [2–5]. The fluorescent plate detection is a high-throughput method, but the microplate reader is not available in all routine laboratories because of its high cost. The use of an HPLC/spectrofluorometric analysis for 7-benzyloxyquinoline (BOQ) metabolites has been performed to study the different sites of fixation of three CYP3A4 substrates (testosterone, 7-benzyloxy-4-trifluoromethylcoumarin (BFC) and BOQ), but this method has only been performed by Lu et al. [6].

(i) The first aim of the present study was to validate HPLC/spectrofluorometric analysis method for 7-hydroxyquinoline (the common metabolite of BOQ) detection and quantification, using BOQ as a fluorometric and highly specific substrate for liver CYP3A4.

* Corresponding author. Tel.: +32 2 7642837; fax: +32 2 7649029.
E-mail address: horsmans@gaen.ucl.ac.be (Y. Horsmans).

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Some fluorometric probes have been reported to be selective for CYP3A4 or rather for CYP3A subfamily: 3, 4-difluorobenzyl)-4-(4-methylsulfonylphenyl)-5,5-dimethyl-(5H) furane-2-one (DFB), dibenzylfluorescein (DBF), 2-(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (2TFBFC), 2,5-bis(trifluoromethyl)-7-benzyloxy-4-trifluoromethylcoumarin (BFBFC), BFC and BOQ [2–5]. BOQ has demonstrated the highest degree of selectivity for and the highest rate of metabolism by CYP3A, compared to other fluorometric probes [5,7]. BOQ metabolism has been potently inhibited by ketoconazole, and its dealkylase activity correlated with CYP3A4 marker activity, the V_{max} value for BOQ oxidation has been comparable to the rate of consumption of testosterone [5,7]. These results suggest that the fluorescent product of BOQ oxidation may be a suitable probe for CYP3A(4). This was also supported by determination of BOQ metabolism activity in rat liver microsomes which was compared to that of midazolam (MDZ), one of the most used probes for CYP3A or CYP3A4 [8].

(ii) Our second major objective was to compare CYP3A protein content and catalytic activity between different species using the in vitro metabolisation of different substrates (testosterone, midazolam, cyclosporine, quinine, dexamethazone ...) [1,9–11]. For the first time, the BOQ catalytic activity was determined in liver microsomes from different species (rat, mouse, dog and monkey).

(iii) Finally, the BOQ metabolism was studied in control and dexamethasone (DM)-induced rats liver microsomes, after CYP3A4 inhibition by ketoconazole.

2. Materials and methods

2.1. Chemicals

MDZ, flunitrazepam and NADPH were obtained from Hoffman-La Roche (Nutley, NJ, USA), magnesium chloride and phosphoric acid from Merck KGA (Darmstadt, Germany), quinidine from Sigma–Aldrich (GmbH, Germany), acetonitrile and triethylamine from SDS (Peypin, France), BOQ and 7-hydroxyquinoline from BD Gentest and ketoconazole from Johnson & Johnson (MSD, The Netherlands).

2.2. Liver microsomes and incubation reactions

Dog and monkey liver microsomes and DM standard-induced rat liver microsomes were provided by Lilly Laboratories (Mont St. Guibert, Belgium) and mice liver microsomes by UCB (Braine-l' Alleud, Belgium). The experimental protocol was approved by the Animals Ethics Committee of the Catholic University for rats liver microsomes. Rats microsomes were prepared from male Wistar rats obtained from a colony maintained at the animal room of Medical Faculty of UCL (Bruxelles, Belgium) and were 6 weeks old at the time of sacrifice. Rat microsomes were prepared by the method of Leclercq et al. [12]. All microsomal incubations were prepared on ice in tubes containing 25 μL microsomes suspension which was diluted with 300 μL of phosphate buffer (pH 7.4, 0.1 M). Hundred microliters of 100 μg/mL BOQ solution (or 150 μL of 167 μg/mL MDZ solution), 25 μL of 2 mM magnesium chloride solution, mixture was pre-incubated at 37 °C during 5 min. Than 50 μL NADPH 1.5 mg/mL was added, mixed and incubated at 37 °C for 30 min. The reaction was stopped with 100 μL of HPLC grade acetonitrile, mixed vigorously and kept on crushed ice. The internal standard (IS) as 100 μL of a solution at 20 μM of quinidine (or 30 μL flunitrazepam solution at 50 μg/mL, for MDZ determination) was mixed with the sample. After centrifugation, 20 μL of the supernatant was injected on the HPLC column. For the inhibition reaction, the different concentrations (from 0 to 1000 μM) of 50 μL ketoconazole solution were added to the incubation mixture and incubated by the same method as described above.

Microsomal cytochrome P450 content was determined according to the method of Omura and Sato with sodium dithionite as a reductant [13], a spectrophotometer Anthelie (Secomam®, France) operating from 420 to 490 nm was used to calculate the absorbance after the bubbling of carbon monoxide in the mixture.

2.3. HPLC/fluorometric analysis

The 7-hydroxyquinoline chromatographic analysis was performed using an isocratic HPLC Gilson® 307 (Food Chem, Gent University, Belgium) high pressure pump and a manual injector Rheodyne® 7125 (Cotati, CA, USA). The 7-hydroxyquinoline was separated from quinidine on a Lichrosorb RP8 5 μm column (25 cm × 4.6 mm, i.d.) with a quaternary solvent mixture (85.4% water, 13% acetonitrile, 1% triethylamine and 0.6% concentrated phosphoric acid, v/v/v/v) at a flow rate of 0.6 mL/min. A volume of 20 μL was injected; the retention time was 5.6 min for 7-hydroxyquinoline and 13 min for internal standard. The detection of 7-hydroxyquinoline was performed by a fluorometric detector from Perkin-Elmer LS 40 (Nova Biotech, El Cajon, CA, USA) operating at 358 nm (excitation) and 505 nm (emission).

The analytical method was validated according to AOAC International guidelines for validation of analytical methods [14]. The calibration curve was obtained at six concentration levels of 7-hydroxyquinoline (0, 0.25, 0.5, 1, 5 and 10 μM). The linearity was evaluated by the least-squares regression method with triple determination at each concentration level. The precision of the method was determined by intra-day and inter-day coefficients of variations (CV%), which was evaluated by analysing the five samples of each calibration curve concentrations on the same and five different days, respectively. The intra-day and inter-day CVs of responses were determined. The limit of detection (LOD) and the limit

of quantification (LOQ) were calculated by linear regression analysis of 10 times injected calibration curve concentrations responses (area/IS area). The common equations used were LOQ = $mb + 10sb$ and LOD = $mb + 3sb$, where mb is the slope of the regression equation and sb corresponds to the standard deviation (SD) amongst the responses. The coefficient of variation of the slopes and intercepts of five calibration curves were calculated to determine the reproductiveness.

The MDZ chromatographic analysis was performed using the capillary HPLC system consisted of a 7125 Rheodyne injector with a 20 μL loop (Cotati), a Kontron Instruments® model 325 pump (Milan, Italy) and a Kontron 335 UV detector equipped with a UZ capillary flow cell (LC Packings, Amsterdam). Separation and quantification of 1′-OH-MDZ and 4′-OH-MDZ were performed as described by Eeckhoudt et al. [15].

2.4. Data analysis

Results are expressed as means ± standard deviation. The statistical differences between the groups were tested using non-parametric two-related-samples test of Sign (SPSS Science, Chicago, USA). Statistical significance was admitted for a p-value <0.05. Least-squares linear regression analysis (SPSS Science, Chicago, USA) was used to determine the slope to calculate LOD and LOQ and intercept to calculate the reproductiveness.

3. Results and discussion

(1) Calibration curves for 7-hydroxyquinoline were linear ($r^2 > 0.95$) within the ranges studied, i.e. 0.1–10 μM. The coefficient of variation of the slopes of five curves was 6.6%, and all intercepts were close to zero. The intra-day CV of 7-hydroxyquinoline were 4.1% for the highest and 9.5% for the lowest concentration. The inter-day CV of 7-hydroxyquinoline were 5.8% for the highest and 7.15% for the lowest concentration. LOD and LOQ were found to be 0.1 and 0.03 μM, respectively.

The intra- and inter-day precision were good as indicated by the CVs which were smaller than 15% and smaller than 20% for LOD. In accordance with the guidance for Analytical Methods Validation this sensitive HPLC method can thus be used for the quantification of 7-hydroxyquinoline [14].

(2) Differences in MDZ and BOQ activities, occurring in V_{max} values in μmol of metabolite (fluorescent 7-OH-quinoline or 1′-OH-MDZ and 4′-OH-MDZ) per minute per milligram of protein (μM/mgP/min), were compared in microsomes of four different rats (A, B, C and D). Fig. 1 showed that BOQ oxidation rate was greater (1.95 ± 0.24) than MDZ oxidation rate (1.4 ± 0.21), without however reaching statistical significance of this difference (test of Sign; $n = 4$, $p = 0.125$, two tailed).

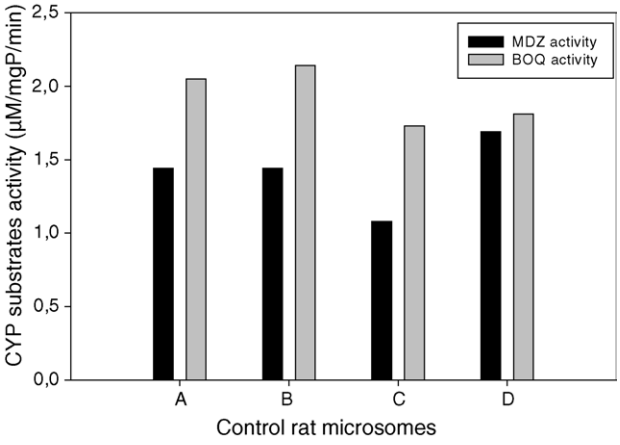

Fig. 1. BOQ and MDZ oxidation activities in four rats.

BOQ oxidation has already been compared to testosterone hydroxylation as a CYP3A4 substrate (6). However, one of the classical CYP3A probe is MDZ, which is known to be metabolised to 1′-OH-MDZ and 4′-OH-MDZ [16–18]. This probe was used as a reference in our study. MDZ metabolism is predominantly contributed to CYP3A4, however, CYP3A3 and CYP3A5 can also be largely involved, especially in rat microsomes [15]. Concerning BOQ oxidation, it has been demonstrated that CYP3A4 was the major enzyme involved, CYP1A2 and CYP3A5 roles were found to be negligible in rat microsomes [5]. Differences between the enzymes responsible for MDZ and BOQ metabolism can explain the observed difference in their activities. Probably because of a few number of rats studied ($n = 4$) the activities difference was not statistically significant.

(3) The potential use of BOQ in other species than the rat (mouse, dog and monkey) was studied (Table 1). The highest BOQ activity was observed in rat microsomes (3.75 μmol/mg protein/min) with lower P450 content (0.3 nmol/mg protein) compared to other species. When looking at the BOQ activity calculated per nanomoles of P450 content, the highest activity was observed also in rat microsomes (12.54 μmol/mg protein/min/nmol P450).

(4) The effect of ketoconazole, a selective inhibitor of CYP3A enzymes, on the dealkylation of BOQ in control and DM-treated rat microsomes are shown in Fig. 2. An approximate estimation of inhibitory concentration 50% (IC_{50}) value indicated that ketoconazole inhibition

Table 1
BOQ activity and P450 content in liver microsomes of different species

Species	BOQ activity (μmol/mg protein/min)	P450 content (nmol/mg protein)	BOQ activity per content of P450 (μmol/mg protein/min/nmol P450)
Rat	3.75 ± 1.08	0.3 ± 0.15	12.54
Mouse	6.6 ± 2.47	0.7 ± 0.3	9.54
Dog	1.95 ± 0.08	0.63 ± 0.09	3.12
Monkey	2.22 ± 0.27	1.17 ± 0.26	1.9

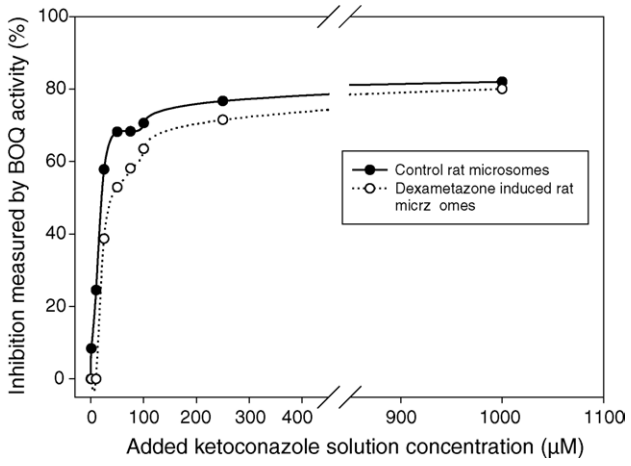

Fig. 2. Inhibition of BOQ oxidation by ketoconazole in normal and DM pre-treated rat microsomes.

potency was greater in control ($IC_{50} \approx 21.6\,\mu M$) compared to the DM-induced ($IC_{50} \approx 32.3\,\mu M$) microsomes. A low concentration of 1 μM ketoconazole inhibited BOQ metabolism only at 10%, whereas at concentration ranges generally considered enzyme-selective (e.g., 10–30 μM), inhibition was greater than 50% in control microsomes. At concentrations greater than 50 μM, ketoconazole inhibitory activity did not rise significantly, and at maximal concentration tested (1000 μM) a nearly similar inhibition (76%) was observed than that at the concentration of 50 μM (68.2%).

Ketoconazole is a well known selective and highly potent inhibitor and DM a known inducer of liver microsomal CYP3A4 activity [19]. Inhibitory effect of the single 2 μM concentration of ketoconazole in BOQ dealkylation has been studied by Stresser et al. [5]. We studied inhibitory effect of ketoconazole at different concentrations. At concentrations above its enzyme-selective concentration (>30 μM) saturation phenomenon was observed, consequently, the inhibitory effect remained stable. It has been previously demonstrated that the V_{max} and K_{cat} (turnover number) values for 7-hydroxyquinoline were higher for the control rat microsomes compared to those 3-methylcholantrene induced [20]. Similarly, in our experiment, higher ketoconazole concentrations are needed to achieve the same inhibition value in DM-treated microsomes, when compared to controls.

4. Conclusion

Although the number of developed CYP3A(4) activity probes, the need for a specific and simple substrate probe remains evident. The analytical method described in the present study showed satisfactory validation data in terms of specificity, linearity, sensitivity and precision. Compared to MDZ, BOQ was a good in vitro probe for CYP3A(4) in rat microsomes showing even a higher rate of oxidation than MDZ. In microsomes from different species, BOQ has demonstrated a large panel of activities, consequently, to use it like a CYP3A(4) probe in these species, future investigations will be devoted. BOQ metabolism was significantly (>50%) inhibited by ketoconazole enzyme-selective concentrations, and this inhibition was lesser in DM-induced microsomes, which provides arguments for the selectivity of BOQ for CYP3A(4).

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