STUDY OF THE INFLUENCE OF BILE SALTS AND LECITHIN ON DISTRIBUTION OF KETOCONAZOLE BETWEEN PLASMA AND METHYLENE CHLORIDE

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Abstract

The aim of the paper was to estimate the effect of increased amounts of bile salts and lecithin following postprandial changes in human plasma composition on the liquid/liquid extraction of a hydrophobic moiety.

The effect could be integrated in what is called "matrix food effect". Experiments concerned separation from plasma samples in methylene chloride by liquid/liquid extraction of the lipophilic drug ketoconazole (K) in presence of various amounts of sodium taurocholate bile salt (BS), lecithin (L) or their mixture. Determinations were performed at three concentration levels within the therapeutic range of ketoconazole.

The use of L increased the extraction yield of K from plasma. The effect of L on K extraction was not linear but rather parabolic: a significant increase occurred in the L concentration range 1-10 mg/mL and a return towards initial values in the 10-50 mg/mL domain.

The effect of BS on K extraction appeared to be rather fluctuant. At high analyte concentrations, the dependence BS concentration-K extraction yield was found to also be parabolic, but the effect was smoother than in the case of L.

The enrichment of plasma with both BS and L led to a similar behaviour to L, a parabolic evolution of K extraction as a function of BS+L mixture concentration being obtained. The addition of BS+L mixture led to an enhanced response over both the effect of L and the sum of separate effects of BS and L. The maximum effect corresponded to low-range concentrations of BS and L.

Rezumat

Scopul lucrării a fost de a estima efectul prezenței unor cantități crescute de săruri biliare şi lecitină, datorate modificărilor postprandiale ale compoziției plasmei umane, asupra extracției lichid/lichid a unui compus medicamentos hidrofob.

Efectul ar putea fi integrat în ceea ce se numește "efect de matrice alimentară". Experimentele au presupus separarea din plasmă a unui compus lipofil, ketoconazolul (K), prin extracție lichid/lichid cu clorură de metilen în prezența taurocolatului de sodiu (BS), lecitinei (L) sau a amestecului lor. Determinările au fost efectuate la trei niveluri de concentrație din domeniul terapeutic al ketoconazolului.

Rezultatele au confirmat faptul că L a crescut randamentul de extracție al K din plasmă. Efectul nu a fost liniar, ci mai degrabă parabolic: o creștere marcată s-a înregistrat la concentrații de L în intervalul 1-10µg/mL şi o revenire către valorile inițiale în intervalul 10-50 µg/mL.

Efectul BS asupra extracţiei K a fost destul de fluctuant: la concentrații mari ale K s-a înregistrat de asemenea o dependență parabolică, însă efectul a fost mult mai atenuat față de cel al L.

Amestecul de BS şi L a prezentat un efect similar cu cel al L, cu o tendinţă parabolică mai accentuată. Adaosul de BS a condus la un efect mai mare decât cel datorat doar prezenţei L. Efectul maxim a corespuns concentraţiilor scăzute de BS şi L (10-30µg/mL).

Keywords: Liquid/liquid extraction; lecithin; bile salts; distribution; matrix effects; transfer of lipophilic drugs.

Introduction

Bile salts, which are produced by the hepatic metabolism of cholesterol [1,2] are stored in the gall bladder after secretion by the liver [3,4]. When a meal is ingested, bile is excreted into the duodenum, extensively reabsorbed from the terminal ileum, and transported back to the liver via the portal vein. At hepatic level, bile salts undergo hepatocyte extraction and are reexcreted into bile, passing through the enterohepatic circulation several times before final excretion [5]. Thus, after food intake, BS levels in the hepatocyte will increase. The hepatic extraction rate of BS is ranged between 80 and 90% and usually remains constant during the fasting state and during digestion [6]. Consequently, a significant amount of BS can spill over into the systemic circulation. Therefore, after a meal, BS levels will not only increase in the portal vein and the liver, but also in the systemic circulation [7,8]. Fasting serum bile salts concentrations are reported to be usually below 5µM, whereas postprandial levels raise up to 15 µM [9-11]. As a consequence of this phenomenon, serum BS levels vary during the day following a rhythm dictated by the ingestion of meals. The concentration of total serum bile salts is significantly increased in patients with hepatobiliary disease, the use of postprandial levels of total serum bile salts being suggested as a liver function test [12-14]. The highest values of preprandial serum bile salts were found in case of viral hepatitis (up to 300 µM), but elevated levels (40-150 µM) occurred in other hepatic diseases, such as cirrhosis, neoplasia or extrahepatic obstruction. The postprandial:fasting ratio for bile salts levels ranged between 1.1 and 3 [7,9].

Bile acids and bile salts play a critical role in lipid assimilation by promoting emulsification [15,16], being responsible for postprandial lipemia, a physiological phenomenon characterized by a rise in triglyceride-rich lipoproteins after eating. Altrough often seen in patients with disturbances of triglyceride metabolism, obesity, diabetes or metabolic syndrome [17], exaggerated postprandial hyperlipemia (triglyceride values more than 400 mg/dL, total lipids concentration above 1000 mg/dL) has been observed even in fasting normolipidemic subjects [18].

The presence in postprandial plasma of both bile salts and lipids as well as their mixed-micelles produces a high variability of bioanalytical results with unwanted consequences on validation for the extractive and chromatographic methods used for the assay [19].

These effects have to be integrated in what is called "matrix food effect" in the standard methodologies for validation of bioanalytical methods. In fact, the latest EMA (European Medicines Agency) guidance on bioanalitycal method validation included the study of matrix effect as a required test when using mass spectrometric methods [20]. Ion suppression appears as one particular manifestation of matrix effects, which is associated with matrix influencing the extent of analyte ionization, with negative effect on sensitivity, precision and accuracy of the method. Although MS (mass spectrometry) techniques are influenced in an extensive manner by the biological matrix composition, other bioanalitycal methods are also being affected by plasma composition modifications, and little is known about their effect [21,22].

Bile salts are amongst the most important groups of naturally occurring surfactants. They form micellar aggregates that play an important role in the physiologically vital process of gastrointestinal digestion, absorption of lipids [23-25] and of lipophilic substances [2,26].

The micelles of bile salt have low aggregation numbers, typically 2–10 and are normally smaller than micelles formed by synthetic surfactants [23,27].

In the body, bile salts are often associated with phospholipids in mixed micelles, which are larger and more fluid than simple micelles and thus, may act as better solubilizing agents for lipophilic compounds [28,29].

The presence in the intestinal lumen of bile salts with surface-active properties and the ability to form mixed micelles contributes to improving the solubility and permeability of many pharmaceutically active substances [30,31]. Hence, the use of simple bile salt and mixed bile salt:fatty acid micellar systems as adjuvant to control drug lifetime and bioavailability, and to minimise undesirable side effects is a present concern in pharmaceutical technology [32,33].

As model drug for evaluation of such type of influences was chosen the lipophilic molecule ketoconazole, at similar concentrations with plasma levels in therapy.

Such a research is especially needed for a full validation of bioanalytical methods used in bioavalability and bioequivalence studies due to the fact that this kind of studies are usually longer than 24 hours and the collected samples are associated with two very different physiological

conditions: preprandial samples (after at least 10 hours of fasting) and postprandial samples (after ingestion of standard meals).

The experimental part of the present paper evaluates the influence of BS, L and their mixture on the yield of extraction of ketoconazole from plasma solutions at the interface with methylene chloride.

Materials and Methods

Reagents

Ketoconazole reference standard was purchased from Sigma. Physiological compounds - granular Lecithin, (Acros Organics) and Sodium taurocholate 97% (Sigma) were used. Trifluoroacetic acid was purchased from Scharlau. Methanol, acetonitrile, dichlormethane were all of analytical grade and purchased from Merck KGaA (Darmstadt, Germany).

HPLC Analysis

The analyses were carried out using a Waters Liquid Cromatograph (600E Multisolvent Delivery System, 717 Autosampler, 486 Tunable Absorbance Detector, Waters, Milford, MA, USA). The detector was set at 210 nm. The chromatographic separation was achieved on a Hypersil Gold, 5-µm 150 x 4 mm column (Thermo Scientific) using as mobile phase an isocratic mixture of acetonitrile: trifluroacetic acid (35:65 v/v) delivered at 1.0 mL/min flow rate. Fifty microliters of each sample were injected onto the chromatographic column.

Standard solutions

A stock solution of ketoconazole was prepared at the concentration of 100 µg/mL. Working solutions at concentrations of 1, 5, and 10 µg/mL were obtained by appropriate dilutions in blank plasma. All these solutions were stored at −20 ◦C prior to analysis.

Sample preparation

The standard plasma samples were allowed to thaw at room temperature and then prepared according to the extraction protocol.

Extraction procedure:

Three ketoconazole sample series were prepared in order to quantify the separate and combined effect of lecithin (L) and bile salts (BS) on K extraction:

- *L samples:* plasma samples spiked with various amounts of lecithin. Concentrations of 0, 1, 5, 10, 25, 50mg lecithin per mL of plasma were used in the experiments.
- *BS samples*: plasma samples spiked with various amounts of sodium taurocholate. Concentrations of 0, 1, 5, 10, 25, 50µg sodium

taurocholate per mL of plasma were used in order to study the influence of bile salts presence on bioanalytical evaluations.

- *BS+L samples:* plasma samples spiked with both lecithin and sodium taurocholate (Table I).

Table I

The L, BS and BS+L levels in the spiked plasma samples

Sample Code	*L samples*		*BS samples*		*BS+L samples*	
	BS (µg/mL)	L (mg/mL)	BS (µg/mL)	L (mg/mL)	BS (µg/mL)	L (mg/mL)
S0	-	0	0	-	0	0
S1	-	1	1	-	1	1
S2	-	5	5	-	5	5
S3	-	10	10	-	10	10
S4	-	25	25	-	25	25
S5	-	50	50	-	50	50

The extraction procedure is synthesized in figure 1:

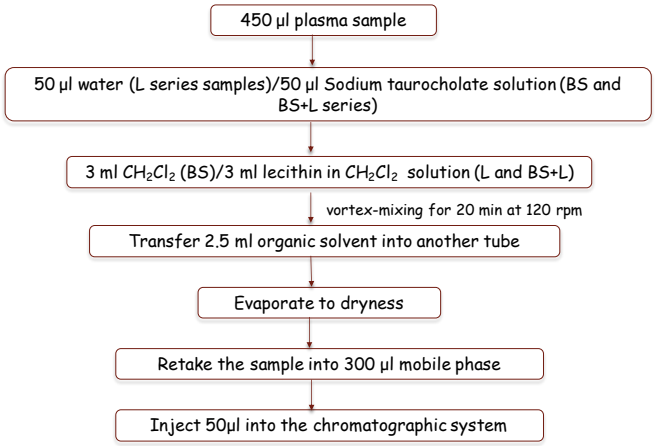

Figure 1

Extraction procedure of ketoconazole from plasma, in presence of BS and L

For both L and BS five concentration levels were used, in order to evaluate partition variations in the proximity of the CMC (critical micellar concentration) of the formed mixed micelles.

The concentration range used was 0-50mg/mL for L and 0-50µg/mL for BS.

The L concentration range was chosen to include normal preprandial total lipids amounts (5-8 mg/mL), postprandial hyperlipemia values, as well as different pathological situations (>10 mg/mL) for blood total lipids [17,18].

Plasma concentration of BS is much smaller (<5 μM in fasted and <15 μM in fed state) [9-11], but in cholestatic liver diseases their amount is dramatically increased, up to 100 fold [9]. The 0-50 μg/mL range chosen in our experiments (corresponding to 0-125 μM), includes all physiological and most of the pathological levels of BS in plasma.

The difference in range is due to a very small concentration of BS in postprandial plasma; hepatocytes extract bile acids very efficiently from sinusoidal blood, and little escapes the healthy liver into systemic circulation [34].

Results and Discussion

The organic phase was completely evaporated and residuum taken in all cases with the same volume of mobile phase. Consequently, the peak areas were proportional with the amount of ketoconazole extracted in CH_2Cl_2. It was considered unnecessary to transform areas in concentrations since all the effects were considered in comparison with the extraction in absence of bile salts and lecithin.

The results are presented in figure 2, for the three concentrations of ketoconazole (1, 5 and 10 mg/L).

As a rule, lecithin increased in all cases the extraction yield of K. Dependence of the effect on L concentration was not linear but biphasic: a significant increase of K extraction in the L concentraton interval 1-10 mg/mL and a return towards initial values in the 10-50 mg/mL interval (Figure 2).

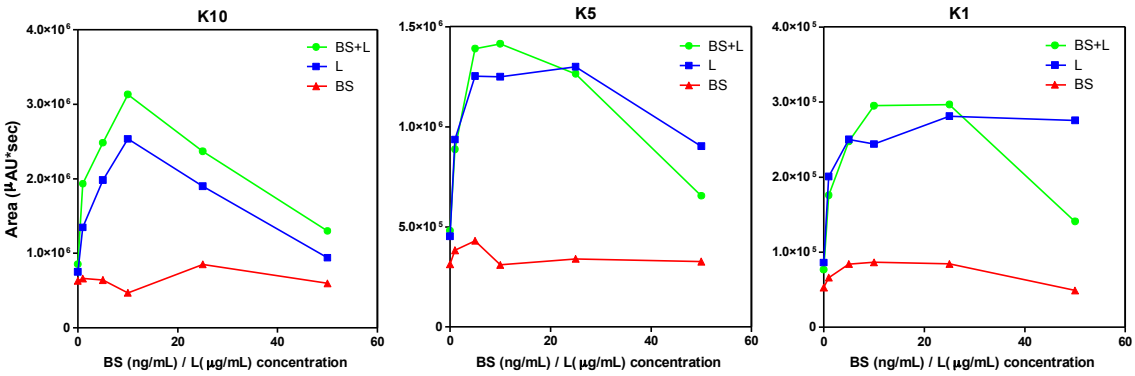

Figure 2

Extraction of ketoconazole from plasma in presence of variable amounts of bile salts and lecithin at three ketoconazole concentrations levels: 1 (K1), 5 (K5) and 10(K10) μg/mL

The effect of sodium taurocholate was rather fluctuant. At the lowest K concentration (1 µg/mL), the effect looked to be no more random but to follow a biphasic dependence, similar with the dependence of extraction yield in the case of lecithin.

The mixture of BS and L presented a similar effect to that of L, biphasic (approximately parabolic). The maximum effect corresponds to lower concentrations of BS and L (10 – 30 µg/mL, respectively 10 – 30 mg/mL, interval) (Figure 2). The result correlates to what was reported in literature: it has been observed that the solubility of lipophilic drugs increases when lecithin is added to a bile salt dissolution medium [35-37]. In an expected model for bile salt/lecithin mixed micelles, lecithin has the polar heads on the outer surface of the micelles; but their charge is partially compensated by the hydroxylic groups of the bile salts and consequently, the net superficial charge is smaller than in bile salt micelles, a factor that may affect the solubilizing capacity of mixed micelles [38]. Drug partition of nitrazepam was found to depend on the bile acid and addition of lecithin to bile salt micelles decreases the values of the partition coefficients in the mixed micelles at physiological pH [39].

Additivity of effects of L and BS was further verified. In the case of 5µg/mL and 10µg/mL K solutions, the sum of BS and L effects is lower than the effect of mixture (BS+L). Consequently it appeared that potentiation of the separate effects of the two surfactants occurred (Figure 3).

At smaller concentrations of K (1mg/L) the effects of BS and L seemed to be rather additive, excepting for the highest BS and L concentration.

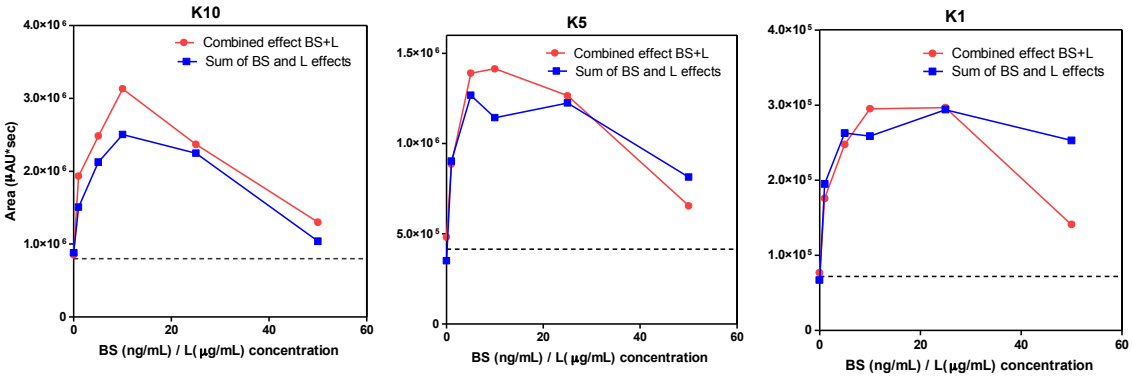

Figure 3
Additivity of BS and L effects. Dotted line (baseline) = mean ketoconazole area in plasma samples without BS or L

A comparison between curves corresponding to the three K concentration levels suggests a similar behaviour. For example, the curves representing the dependence of peak areas on the K concentration in the case of BS curves, normalized by dividing to K concentrations (1, 5, and 10) led practically superposed curves (Figure 4).

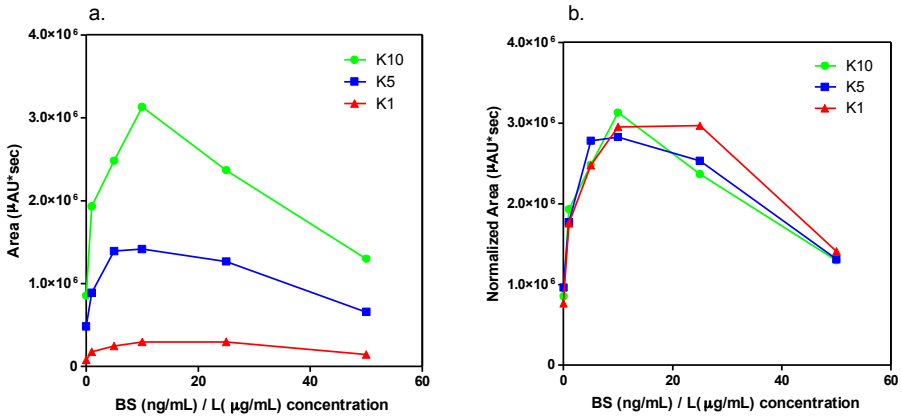

Figure 4

a. Dependence of ketoconazole extraction on BS and L concentration
b. Normalization vs. ketoconazole concentration (K1, (K5)/5, (K10)/10)

Representation of peak areas as function of K concentrations in the presence of BS proved clear linear dependences (Figure 5). In the case of mean areas the dependence was perfectly linear.

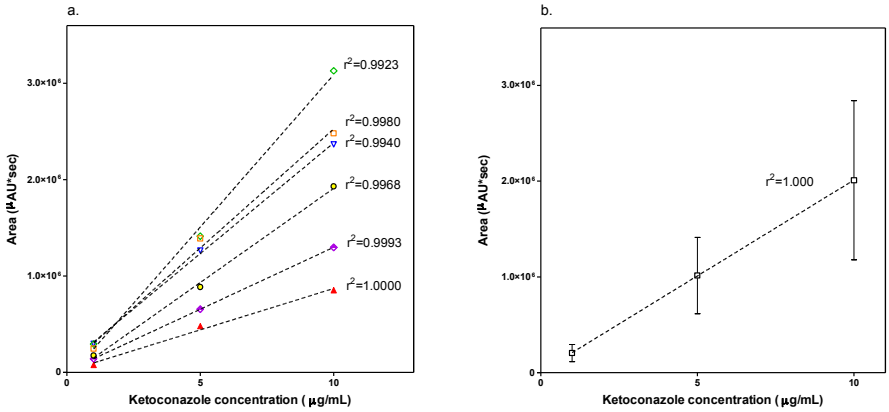

Figure 5

a. Peak areas for different BS concentrations (0, 1, 5, 10, 25, and 50 µg/mL) as function of K concentration; b. mean areas

A difficult problem remains the explanation for the biphasic aspect of the effect of BS, L and their mixture.

The process of distribution of K is very complex.

In the case of postprandial plasma samples there might be some concurrent distributions of the analyte as for example:

- transfer of active substance from the continuous phase (plasma) in the extraction solvent,
- transfer of substance from plasma in different nanostructured systems existing in plasma and in the organic phase.

The transfer from solution to nanostructured systems (which could be emulsion or multiple emulsion or even liposoms) as well as the transfer between continuous phases are determined by the partition coefficients between these thermodynamic phases, size of particles etc.

At equilibrium, ratios of these concentrations have to be equal to corresponding partition coefficients. It can be written

$$C_p = C_p^f + k_{o/m} C_m^p$$

$$C_o = C_o^f + k_{o/m} C_m^o$$

$$C_p^f / C_o^f = k_{p/o} \text{ etc.}$$

where C_o^f is the concentration of free K in organic phase, C_p^f is the concentration of free substance in plasma, $k_{o/m}$ is the partition coefficient of the active substance between the organic phase and micelles and C_m is the concentration of micelles.

But in all these equations, the dependence between total K concentration and concentration of micelles is linear, therefore it is not possible to explain the biphasic effect found experimentally.

Other possible phenomena could be associated with the critical micellar concentrations (CMC) of the surfactants. The mean of the CMC values for the egg-yolk L fractions was found 15.3 mg/mL and that for soybean lecithin was 15.8 mg/mL [40].

The value for soybean lecithin was slightly different from the one reported by Wu and Wang [41] where the cmc was found to be 13.6 mg/mL. To be sure, the CMC in plasma or in organic solvent are different from CMC determined in standard conditions, but it is reliable to think that they are not very far form it. Since the concentration interval for L was 1 – 50 mg/mL, most probable the CMC of L in both phases falls within this interval.

From the literature, it appears that the sodium taurocholate CMC lies in a broad range, between 1 and 12 mM [42]. There is also a debate on whether taurocholate has a CMC at all and whether increasing the concentration forms aggregates that gradually increase in size [43].

Making the transformation of units it was obtained that concentrations of BS used in this paper were much lower than critical micellar concentrations, but the CMC at interface could be significantly different. This could be the reason of the difference between the effect of BS and L.

Conclusions

Lecithin increased the extraction yield of ketoconazole from plasma. The effect was biphasic. In the range 1-10mg/mL lecithin produced a significant increase of the transfer of ketoconazole toward organic phase. At higher concentration levels of lecithin (10-50 mg/mL) the effect declined gradually towards the values obtained in the absence of lecithin.

The effect of sodium thaurocholate was rather fluctuant. The dependence was more clearly correlated to ketoconazole concentration in plasma samples. At the highest concentration of ketoconazole, the model of evolution was parabolic.

The mixture of thaurocholate and lecithin presented a similar effect to that of lecithin, with a more clear parabolic aspect. The addition of BS led to a greater effect than that of lecithin alone. The maximum effect corresponded to low concentrations of thaurocholate and lecithin (10 – 30 μg/mL and 10 – 30 mg/mL respectively).

In the case of thaurocholate the effect was an increase of yield with rise of the concentration of thaurocholate. Dependence on BS concentration was linear.

Effect of sodium thaurocholate-lecithin mixture was an increase of extraction yield. It appeared that the potentiation of the separate effects of the two surfactants occurred.

A comparison between curves corresponding to the three levels of K concentration suggests a similar behaviour. Normalization of starting from K concentration in the case of BS curves, practically led to superposed curves.

The postprandial increase in BS and L plasma concentration can have a significant impact on the bioanalytical evaluations of hydrophobic compounds, inducing a high variability on the extraction process of the analyte. Reducement of matrix effect impact on quantification could be

achieved by using the internal standard, provided that matrix effect is similar for both analyte and selected internal standard.

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