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Lamivudine plasma levels in chronic hepatitis B patients

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ABSTRACT

Lamivudine has recently been registered for the treatment of chronic hepatitis B patients. The main therapeutic outcome in the studies on which the registration was based was a drop of HBV DNA below 10^7 genome equivalents/ml, the level of detection of the insensitive Abbott Genostics assay. However, as we have reported previously, with the use of sensitive PCR-based assays, individual differences in virological response to lamivudine can be detected.
As a first step in analysing the chain of events after oral intake of lamivudine we modified and validated a high-pressure liquid chromatography (HPLC) method to evaluate lamivudine plasma levels. Lamivudine levels in chronic hepatitis B patients who participated in a study on the efficacy of lamivudine were comparable to our reference curve, which was derived from eight chronic hepatitis B patients. From the reference curve, a mean area under the curve (AUC) of 4994 mcg/l.h (SD 1524), a mean t_{max} of 42 minutes (SD 11), and a mean C_{max} of 1.9 mg/l (SD 0.70) were calculated. Lamivudine exerts its action as the active triphosphate inside the hepatocyte after extensive handling. Therefore, additional steps in the pharmacokinetic process should be evaluated to explore the potential mechanisms that are responsible for the diversity in quantitative HBV DNA response to lamivudine.

INTRODUCTION

Lamivudine, the negative enantiomer of 2'-3' deoxy 3' thiacytidine, is a nucleoside analogue which has recently been registered for the treatment of chronically infected hepatitis B patients. In large phase III studies the favourable effect of this drug was shown on suppression of hepatitis B virus (HBV) DNA, a parameter expressing active viral replication, which is often followed by a decline in transaminases and improvement of liver histology.[1-3] The conclusions in these studies were based on the percentage of patients with a viral decline below the lower limit (approximately 10^7 genome equivalents/ml (geq/ml)) of the insensitive liquid hybridisation assays (Abbott Genostics, Abbott Laboratories, Abbott Park, IL). HBV DNA became undetectable in around 80% of patients measured with this test after six months of therapy.[1] However, if we look more carefully with more sensitive polymerase chain reaction (PCR)-based assays, individual differences in response to lamivudine become apparent.[4,5] Whereas some patients show a rapid decline in levels even below the threshold of the qualitative PCR assay (Roche Monitor, lower limit of detection 400 geq/ml), in others, the HBV continues to replicate actively even after six months of therapy. We previously reported on a cohort of long-term lamivudine-treated chronic hepatitis B patients in Rotterdam.[4] In the 19 patients in whom HBV DNA was still detectable by insensitive assays (Digene, liquid hybridisation assay, lower limit of detection 1.5 x 10^6 geq/ml) after six months of therapy, only three patients had a mutant virus that could explain this continuing active viral replication. Thus, ongoing active replication of the HBV must be based on some other phenomenon in the majority of patients.
Lamivudine is subject to several transport and activation steps from oral intake until incorporation into the pregenomic viral chain. Our hypothesis was that poor

uptake of lamivudine might be responsible for the suboptimal decline in HBV DNA in some patients.
In order to be able to address this issue, we modified and validated a high-pressure liquid chromatography (HPLC) method to measure lamivudine plasma levels in chronic hepatitis B patients. Moreover, we studied the availability of lamivudine in blood after a standard oral dose in chronic hepatitis B patients.

PATIENTS AND METHODS

Patients

In group A eight patients were evaluated for 24 hours after oral intake of a single dose of lamivudine 150 mg to obtain a lamivudine plasma reference curve. Patients fasted overnight and blood was withdrawn over a period of 24 hours at t=0, 15, 30, 45, 60 and 90 minutes and 2, 3, 4, 6, 8, 12, 18 and 24 hours after intake of lamivudine.
In group B nine patients, in whom the viral decline during lamivudine 150 mg therapy[6] was studied in detail, the lamivudine concentration in a serum sample taken six hours after start of lamivudine therapy was assessed. The pharmacokinetic reference curve was based on plasma samples. Therefore, the agreement between plasma and serum results was ascertained in 11 randomly selected patients on lamivudine who visited the outpatient clinic (group C).

High-pressure liquid chromatography of lamivudine in plasma and serum

Lamivudine in plasma and serum was assayed with an HPLC method slightly modified from Harker *et al.*[7] In short, the following procedure was used.
Sample extraction is performed using a solid-phase extraction method (Bond Elute Verify LRC; 10 cc/130 mg, Varian Inc., Harbor City, CA, USA), after activation of the column with subsequently 2 ml of methanol and 2 ml of acetic acid 1%. Next, a mixture of 1 ml of plasma and 1 ml of acetic acid 1% is applied to the column with a pressure of 5 mmHg for at least two minutes. The column is consecutively washed and dried with distilled water, methanol/acetic acid 10% (9:1) and distilled water again. Desorption is carried out four times with 0.5 ml of methanol/ammonia 25% (9:1) under a low vacuum. The four fractions are collected and evaporated to dryness with a gentle flow of nitrogen at 40°C and subsequently suspended in 300 µl of the mobile phase by vortex-mixing. Separation of the mixture is performed by HPLC, equipped with a BDS Hypersil C18 column (250 x 4.6 mm ID; 5 um), using a mixture of methanol (40 ml), acetonitrile (5 ml), glacial acetic acid (0.5 ml), and 0.1 M ammonium acetate in water (455 ml) as the mobile phase at a flow of 1 ml/min and at a temperature of 40°C. Quantification was based on UV detection at 270 nm, calibrated with a range of external standards in plasma, which were processed the same way.

Intra- and interassay variability

Eight calibration standards of lamivudine, with a concentration ranging from 0.1 mg/l to 7.5 mg/l, were analysed simultaneously six times (intra-assay variability), expressed as the average accuracy with percent of the deviation from the nominal concentration. The procedure was repeated on three separate days (interassay variability) expressed as a co-efficient of variation.

Correlation between lamivudine levels in plasma and serum

The concentrations of lamivudine in serum and plasma were compared by means of a linear plot, as well as a Bland and Altman plot.[8]

Modelling of pharmacokinetic data

From the 24-hour pharmacokinetic curves, the average area under the curve (AUC), the half-life of lamivudine (t1/2), t_{max}, and C_{max} were calculated. Lamivudine concentrations were fitted with the TOPFIT pharmacokinetic programme[9] using a one-, two- and three-compartment model using four weightings (1, $1/\sqrt{y}$, 1/y, and $1/y^2$). The Akaike criterion[10] was used to establish the best fit for our data.

Table 1
Patient characteristics

	GROUP A (N=8)	GROUP B (N=9)	GROUP C (N=9)
Age in years (median range)	37 (17-60)	28 (22-51)	29 (17-57)
Male/female	7/1	7/2	6/3
Cirrhosis	4	1	0 (n=8)
Additional medication	Patient 3: ferrofumarate Patient 5: furosemide, aldactone Patient 7: methotrexate	Patient 4: oral contraceptive	Patient 1: pantoprazole Patient 3: clinoril, cough medicine, doxazosine, losartan, atorvastatin, insulin Patient 4/9: paracetamol

RESULTS

Patient characteristics of group A, B and C are shown in *table 1*. In group A, 50% of the patients had an advanced stage of liver disease.

The lower limit of detection of the HPLC assay was determined at 0.005 mg/l and the lower limit of quantification at 0.1 mg/l. The higher limit of detection was arbitrarily determined at 7.5 mg/l. All calibration curves were linear between 0.1-7.5 mg/l with a variance between -15% to +10% in this range. A variety of drugs, which were co-administered frequently to these patients, did not interfere with the extraction and detection procedure.

The intra-assay variability showed an accuracy of 80 to 95%, which is comparable with data described in the literature (*table 2*).[7] The interassay variability was concentration dependent, 3 to 16.6% (*table 2*). Recovery of lamivudine in spiked plasma samples compared with non-processed standard solutions was 86% (± 6.7%).

The relation between the concentration of lamivudine in plasma and serum was linear as observed by a line with a slope of 0.997 and an intercept at (0.0). The Bland and Altman plot showed a mean of the difference between the serum and plasma level of 0.02 mg/l (SD ± 0.0411).

For group A, a mean AUC of 4994 µcg/l.h (SD 1524), a mean t_{max} of 42 minutes (SD 11), and a mean C_{max} of 1.9 mg/l (SD 0.70) were calculated (*figure 1*).

If we compare the six-hour serum concentration of lamivudine in group B (median 0.35 mg/l; range 0.28-0.52) with the same time point in group A (median 0.32 mg/l; range 0.15-0.48), these concentrations are within the same range.

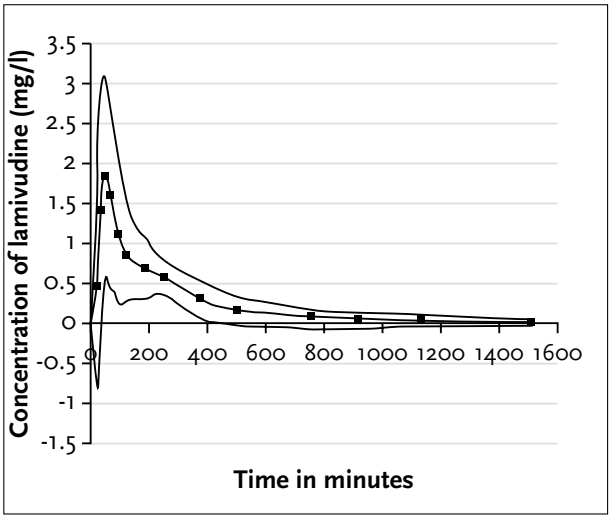

Figure 1
Pharmacokinetic reference curve (± 2 x SD) based on eight chronic hepatitis B patients treated with lamivudine 150 mg once a day (group A)

DISCUSSION

If the inhibitory effect of lamivudine on HBV replication is studied with a sensitive PCR-based assay with a dynamic range between 400-10^9 geq/ml, a wide variation in response between individual patients is observed. In a previous study, we showed that this could only in part be explained by the emergence of a mutation in the catalytic site of the polymerase gene of the HBV.[4] In this study we made a first step in further exploration of host-dependent mechanisms which might explain the variability of response to lamivudine.

Table 2
Intra- and interassay variability

	INTRA-ASSAY VARIABILITY			INTERASSAY VARIABILITY		
THEORETICAL VALUE (MG/L)	MEAN (N=6)	STANDARD DEVIATION	% COEFFICIENT OF VARIATION	MEAN (N=6)	STANDARD DEVIATION	% COEFFICIENT OF VARIATION
0.1	0.096	0.009	9.4	0.094	0.016	16.6
0.21	0.189	0.009	4.8	0.19	0.018	10.0
0.56	0.495	0.022	4.4	0.487	0.026	5.4
1.04	0.875	0.013	1.5	0.882	0.043	4.9
1.53	1.293	0.018	1.4	1.296	0.05	3.9
2.18	1.819	0.11	6.0	1.884	0.102	5.4
3.24	2.583	0.037	1.4	2.69	0.13	5.0
7.5	6.177	0.18	2.9	6.22	0.18	3.0

The pharmacokinetic process of any drug, including lamivudine, is characterised by a sequence of events: absorption, distribution, metabolism and elimination. Lamivudine is highly soluble, dissolves rapidly once in the stomach and is absorbed in the small intestine by passive diffusion. Food reduces the rate of absorption but not the extent: t_{max} is prolonged and c_{max} is reduced, but the AUC is not altered.[11,12] The absolute bioavailability is reported to be around 80%, with a mean volume of distribution of 1.3 l/kg, indicating considerable distribution into deeper tissues.[13]
In chronic hepatitis B patients, lamivudine acts in the liver, the target organ for viral replication. Lamivudine probably enters hepatocytes through active uptake by pyrimidine nucleoside transporters.[14,15] In the cytoplasm of the hepatocyte, lamivudine is phosphorylated to the mono-, di- and triphosphate by deoxycytidine kinase, cytidine monophosphate kinase and pyrimidine nucleoside diphosphate kinase, respectively. The diphosphate is present in highest concentrations inside the hepatocyte and the conversion of the diphosphate to the triphosphate is the rate-limiting step.[16] This extensive bioactivation makes the drug prone to individual differences between patients. Less than 10% of lamivudine is metabolised by the liver, only 5 to 10% of lamivudine is metabolised to a trans-sulphoxide metabolite and excreted in urine, while around 70% of the drug is excreted unchanged in urine.[17,18]
In this study, we modified and validated the HPLC assay for detection of lamivudine in plasma. Only few data on pharmacokinetics of lamivudine in compensated chronic hepatitis B patients have been published.[18] Our pharmacokinetic parameters are comparable to the published data.
Measurement of levels of lamivudine in daily practice may be useful for two purposes. If the level is within the normal range, this ascertains that patients have been compliant with therapy on the one hand and that on the other hand absorption, the first pharmacokinetic step, is adequate. As can be observed from our data, levels of lamivudine in plasma six hours after intake of lamivudine (group B) are in the same range as in patients in group A at six hours. These data, however, should be interpreted with caution, since group characteristics may vary. Recent studies have stressed the potential influence of co-administered drugs on lamivudine kinetics. This is either caused by the increase of phosphorylation of lamivudine (e.g. hydroxyurea, methotrexate)[19] or because of reduction of the excretion ratio of lamivudine in urine (e.g. trimethoprim).[20]
Our kinetic data show that the lamivudine concentrations in group B are well above the *in vitro* IC_{50} even five hours after the maximum concentration in plasma has been reached.[16] Plasma levels have been measured after intake of the first dose of lamivudine but these levels may change during long-term therapy. Previous data do not indicate that lamivudine accumulates during long-term application, but these data ascertain sufficient levels of lamivudine above the *in vitro* IC_{50} throughout the 24-hour period.[16] In contrast, in seven out of the eight patients in group A of our study, levels of lamivudine 24 hours after intake are undetectable. This may necessitate re-opening the discussion on twice daily dosing in patients with hepatitis B virus infection. The key question here will be how these plasma levels relate to the levels of the phosphorylated lamivudine inside the human hepatocytes. Half-life of lamivudine triphosphate in human lymphocytes infected with the human immunodeficiency virus (HIV) has been calculated to be substantially longer (10.5-15.5 hours) than lamivudine serum half-life.[21] Conflicting data on the half-life of lamivudine triphosphate in hepatocytes have been published: 3.6 to 8 hours in primary duck hepatocytes[22] versus 17 to 19 hours in HepG2 cell lines.[16] Therefore, research into human hepatocytes is needed both to address the dosing issue, as well as to better understand the differences between individual patients.
Absorption of lamivudine is a passive process and may therefore be the least important reason for variation in response to lamivudine between patients. In contrast, uptake of lamivudine in hepatocytes is an energy-driven active process, after which lamivudine is phosphorylated inside hepatocytes. Phosphorylation is mediated by host enzymes and the efficacy of the process from parent drug to active triphosphate and persistence of the active triphosphate in the hepatocyte may vary between individual patients due to genetic polymorphism. Therefore, to be able to explain differences in viral decline between patients infected with the same virus, patient-to-patient differences in conversion from lamivudine to phosphorylated lamivudine should be explored further.

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