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Research Paper

Investigation of the Drug–Drug Interaction Between α-Lipoic Acid and Valproate via Mitochondrial β-oxidation

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Purpose. To investigate the potential drug–drug interaction (DDI) between lipoic acid (LA) and valproate (VA) via the mitochondrial β-oxidation pathway in rats.
Methods. *In vitro* mitochondrial assays were performed to compare the biotransformation of VA to valproyl-CoA (VA-CoA), in the absence and presence of LA. *In vitro* microsomal and protein binding assays were performed to elucidate their potential DDI at the microsomal metabolism and distribution levels. A pharmacokinetic study was conducted in Lister Hooded rats to ascertain the *in vivo* DDI between LA and VA.
Results. LA was shown to decrease significantly ($p<0.05$) the *in vitro* formation of VA-CoA in a concentration-dependent manner. Our *in vitro* assay results confirmed that there was minimal interaction between LA and VA in microsomal metabolism and protein binding. Based on the pharmacokinetic data, the absolute bioavailability of VA was determined to be 1.3 in the presence of LA.
Conclusions. Our study demonstrated for the first time that there is a potential DDI between LA and VA at the mitochondrial β-oxidation level. While further clinical study is essential, our preliminary finding suggested that medical practitioners need to be prudent when managing epileptic patients who are co-administered with both VA and LA.

KEY WORDS: β-oxidation; drug–drug interaction; lipoic acid; mitochondrial; valproate.

INTRODUCTION

Lipoic acid (LA), a naturally occurring dithiol compound (Fig. 1A), is an essential cofactor for mitochondrial enzymes (1). Besides its enzymatic role, *in vitro* and *in vivo* studies suggested that LA is a powerful micronutrient with diverse antioxidant and pharmacologic properties (2). As an antioxidant, LA acts as a scavenger of free radicals, chelates transition metal ions and intensifies cytosolic glutathione and vitamin C levels (2,3). Pharmacologically, LA improves glycemic control, alleviates diabetic neuropathy and mitigates toxicities associated with heavy metal poisoning (2). More recently, LA was investigated to manage patients with metabolic syndrome (4) and post-traumatic epilepsy (5). Currently, LA is broadly used in Germany for the treatment of diabetic neuropathy and is also marketed as an adjunct in the treatment of fungal infection, metal poisoning and liver disorders (6). The main metabolic pathways of LA include β-oxidation, *S*-methylation and glycine conjugation (7,8), with mitochondrial β-oxidation playing a paramount role. In the light of its extensive metabolism, we speculated the possibility of drug–drug interaction (DDI) between LA and concomitant medications. However, no study investigating the effect of LA on the disposition of other drugs has been reported.

Valproate (VA; Fig. 1B) is an antiepileptic drug used widely in the treatment of primary generalized seizures, severe absence and myoclonic seizures, and partial seizures (9). Other possible pathological conditions that are treated with VA include manic disorders (10), bipolar affective disorders, schizophrenia and migraine (11). Recently, VA has also been explored in the management of certain types of cancer (12) and human immunodeficiency virus infection (13). Clinically, VA is metabolized predominantly by hepatic mitochondrial β-oxidation (14) as well as glucuronidation, and to a certain extent by cytochrome P450 (CYP450)

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ABBREVIATIONS: ATP, Adenosine triphosphate; AUC, Area under concentration-time curve; CoA, Coenzyme A; DDI, Drug–drug interaction; DTNB, 5,5′-Dithio-bis-2-nitrobenzoic acid; EDTA, Ethylene diamine tetraacetic acid; ESI, Electrospray ionization; f_u, Fraction of drug unbound; HLM, Human liver microsomes; IC_{50}, Concentration that inhibited 50% of the control activity; LA, Lipoic acid; LC/MS/MS, Liquid chromatography tandem mass spectrometry; MRM, Multiple reaction monitoring; NADPH, Reduced nicotinamide adenine dinucleotide phosphate; PIS, Product ion scan; RLM, Rat liver microsomes; RLMIT, Rat liver mitochondria; $t_{1/2}$, Half-life; UPLC, Ultra-performance liquid chromatography; VA, Valproate; VA-CoA, Valproyl-Coenzyme A.

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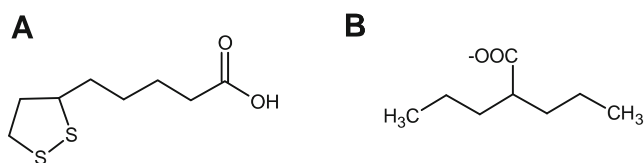

Fig. 1. Chemical structures of **A** lipoic acid and **B** valproate.

enzymes (15). Its β-oxidation biotransformation involves the activation of VA to VA-CoA by a medium-chain acyl-CoA (Coenzyme A) synthetase, followed by several steps of oxidation to form 3-keto-valproyl-CoA (Fig. 2) (16). Presumably, any inhibition along the β-oxidation pathway, for instance by a competing substrate, may influence the disposition of VA. This was evident in the DDI between felbamate and VA, in which felbamate caused a reduction in VA clearance via β-oxidation inhibition (17).

Since both LA and VA are metabolized predominantly by the hepatic mitochondrial enzymes, we hypothesized that LA may potentially inhibit the metabolism of VA via the β-oxidation pathway. With the narrow therapeutic window of VA (50–100 μg/ml), patients are highly predisposed to clinical toxicity in the event when the metabolism of VA is inhibited. Tremor, irritability, confusion and decreases in fibrinogen are commonly experienced at VA concentrations exceeding 80 to 100 μg/ml (18). In addition, an elevation of the level of VA also implies inhibition of other elimination routes, notably phase II glucuronidation (19). As LA and VA may be co-administered for the management of various medical conditions, an elucidation of the metabolic interaction between these drugs is clinically important. A thorough review of literature to date revealed no reports that evaluate the DDI between LA and VA.

In the present work, the potential metabolic inhibition by LA on VA was investigated *in vitro* by monitoring the formation of VA-CoA in rat liver mitochondria (RLMIT). Pharmacokinetic study in Lister Hooded rats was also performed to ascertain the presence and significance of their *in vivo* DDI.

MATERIALS AND METHODS

Chemicals and Reagents

Tris buffer, coenzyme A (CoA), magnesium chloride, adenosine triphosphate (ATP), LA, VA (sodium salt), α-methylbutyric acid, verapamil and citrate synthase assay kit were obtained from Sigma-Aldrich (St Louis, MO, USA). Formic acid, glycerol and tetra-sodium pyrophosphate were purchased from Merck (Darmstadt, Germany). MicroBCA protein assay kit, Coomassie plus—the better Bradford assay kit, T-PER tissue protein extraction reagent and mitochondria isolation kit were purchased from Pierce (Rockford, IL, USA). Pooled human liver microsomes (HLM) and NADPH regenerating system were purchased from BD Gentest (Woburn, MA, USA). HPLC-grade methanol and acetonitrile were purchased from Tedia Company Inc. (Fairfield, OH, USA). Ammonium acetate was obtained from VWR International Ltd. (Leicestershire, UK). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents used were of analytical grades.

Isolation of RLMIT

Adult male Lister Hooded rats (400–500 g), supplied by Harlan, UK, were starved for 18 h and sacrificed by decapitation. Subsequently, 8 g of the removed livers was washed with 160 ml of phosphate buffered saline, cut and

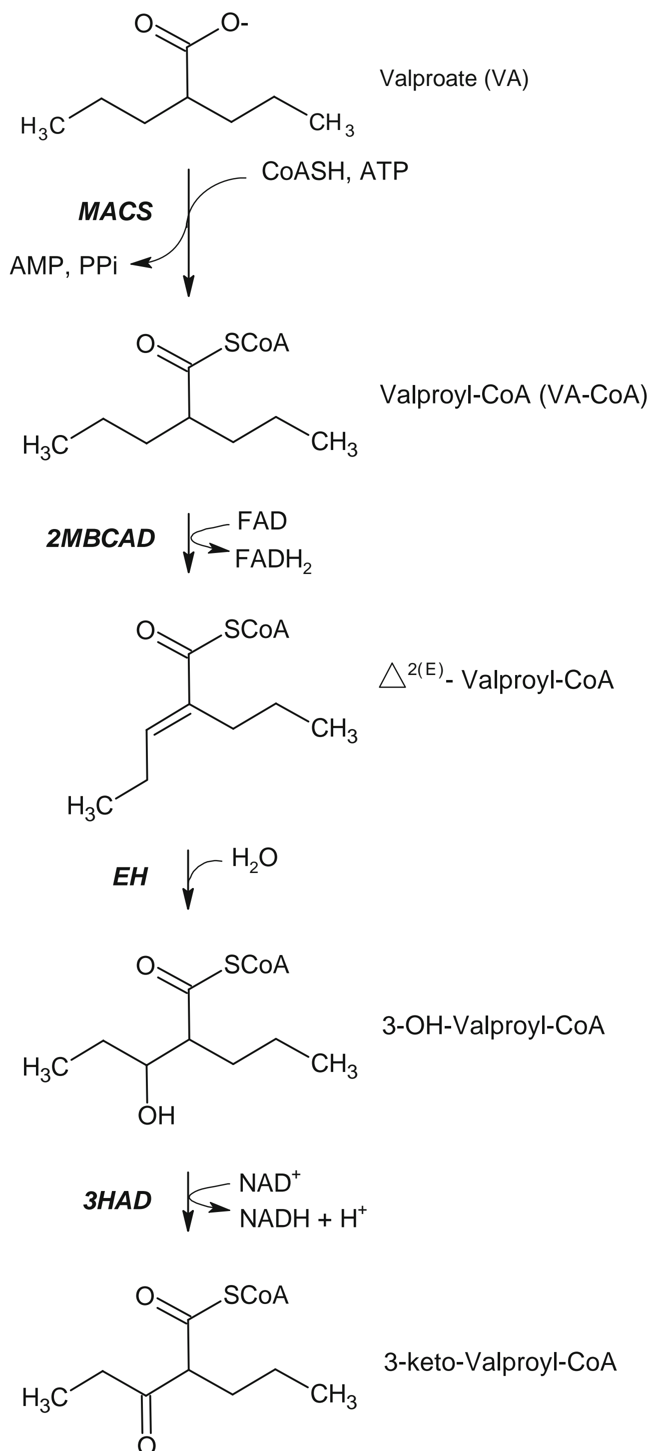

Fig. 2. Schematic representation of mitochondrial β-oxidation of VA. *MACS* Medium-chain acyl-CoA synthetase; *2MBCAD* 2-methyl-branched chain acyl-CoA dehydrogenase; *EH* 2-enoyl-CoA hydratase; *3HAD* 3-hydroxyacyl-CoA dehydrogenase (16).

rinsed into 32 ml of reagent A. Low speed homogenization was performed on ice using a hand-held blender, and 32 ml of reagent C was added. The homogenate was centrifuged (700×*g* for 10 min at 4°C) and the obtained supernatant was centrifuged at 3,000×*g* for 15 min (4°C). The resultant pellet was re-suspended in wash buffer and subjected to a surface wash. After a final centrifugation at 12,000×*g* for 5 min, the mitochondrial pellet was suspended in wash buffer with gentle mixing. Reagents A and C were provided by the mitochondrial isolation kit. The RLMIT suspension was frozen at −80°C until further use. Mitochondrial protein concentration was quantified by means of the MicroBCA protein assay kit, using bovine serum albumin as a reference substance and wash buffer as the diluent.

Citrate Synthase Enzyme Assay

Citrate synthase activity of the isolated RLMIT was measured on a UV/visible plate reader (Tecan® Infinite M200, Männedorf, Switzerland) and the formation of CoA was monitored using a coupled reaction with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) (20). To determine the intactness of the inner mitochondrial membrane, the assay was modified in accordance with the manufacturer's instructions. The reaction was initiated by the addition of 0.5 mM of oxaloacetate to an incubation mixture containing assay buffer, 0.3 mM acetyl CoA and 0.1 mM DNTB in a final volume of 200 µl. In a separate well, citrate synthase, a positive control, was added in place of the mitochondrial sample. Each experiment was performed in triplicates. Incubations were carried out at 25°C and the baseline endogenous level of CoA was measured prior to the incubation. The citrate synthase activity was calculated using the molar absorption coefficient of the reaction product (ε= 13.6 mM^{-1} cm^{-1}) at 412 nm.

In Vitro Metabolism of VA in Intact RLMIT and Inhibitory Effect of LA on VA-CoA Formation

Incubations (n=3) were performed at 37°C in a final volume of 200 µl containing isolated RLMIT (1 mg/ml) suspended in 50 mM Tris buffer (pH 8), in the presence of 1.2 mM CoA, 10 mM magnesium chloride and 5 mM ATP. After 8 min of pre-incubation, the reaction was started by the addition of 50, 100 and 150 µM of VA.

Inhibition studies were performed with and without the inclusion of LA at concentrations of 0, 1, 15 and 50 µM. The final organic solvent content in each incubation mixture did not exceed 0.5% (*v*/*v*). Reactions were terminated at 60 min by addition of two volumes of cold acetonitrile and high-speed vortex mixing for 20 s After removal of the protein precipitate by centrifugation (11,337×*g* for 30 min at 4°C), 500 µl of supernatant from each set was collected into clean eppendorf tube and evaporated to dryness at 25°C under a gentle flow of nitrogen gas (TurboVap LV, Caliper Life Science, Hopkinton, MA, USA). The residue was reconstituted with 50 µl of mobile phase, vortex-mixed and centrifuged at 11,337×*g* for 10 min. Subsequently, the supernatant was subjected to LC/MS/MS analysis. For the negative control, inactivated RLMIT was used. The inactivation was achieved by allowing RLMIT to degrade at 37°C for 60 min, followed by the addition of two volumes of cold acetonitrile. For the positive control, 100 µM of α-methylbutyric acid was used in place of LA.

Isolation of Rat Liver Microsomes (RLM)

Adult male Lister Hooded rats (400–500 g) were starved for 18 h and sacrificed by decapitation. Subsequently, the livers were removed, minced and rinsed into three volumes of ice-cold homogenizing buffer (10 mM EDTA, 150 mM potassium chloride, 0.1 M Tris–Cl, pH 7.4). After a low-speed homogenization on ice, the homogenate was centrifuged at 12,500×*g* for 15 min at 4°C. The resultant supernatant was ultra-centrifuged at 105,000×*g* for 70 min at 4°C and the obtained pellet was re-suspended in ice-cold pyrophosphate buffer (10 mM EDTA, 0.1 M sodium pyrophosphate, pH 7.4) with 3–5 s of homogenization on ice. With the uniform homogenate, the 105,000×*g* ultra-centrifugation was repeated for 45 min. The microsomal pellet was finally suspended in ice-cold microsome buffer (10 mM EDTA, 20% glycerol, 0.05 M Tris–Cl, pH 7.5) with gentle homogenization. Microsomal protein concentration was determined using Coomassie plus—the better bradford assay kit, using bovine serum albumin as a reference substance and T-PER Tissue Protein Extraction Reagent as the diluent. The RLM suspension was frozen at −80°C until further use.

In Vitro Metabolic Stability of LA and VA in RLM and HLM

RLM and HLM incubations (n=3) were performed at 37°C in a final volume of 500 µl containing microsomes (0.85 mg/ml) suspended in 50 mM phosphate buffer (pH 7.4), in the presence of NADPH regenerating system (consisting of 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM magnesium chloride and 0.4 U/ml glucose-6-phosphate dehydrogenase). After 8 min of pre-incubation, the reaction was initiated by the addition of either LA or VA (2–5 µM). Verapamil was chosen as a positive control and added as a substrate in a separate experiment. Negative control was performed in which the substrates were incubated in the absence of microsomes. The final organic solvent content in each incubation mixture did not exceed 0.5% (*v*/*v*). Seventy-five microliters of the mixtures were removed at appropriate times (0, 5, 15, 30, 45 and 60 min) and transferred to two volumes of cold acetonitrile to quench the reaction. After centrifugation at 11,337×*g* for 30 min at 4°C, aliquots of the supernatants were subjected to LC/MS/MS analysis.

In Vitro Inhibition of LA on Plasma Protein Binding of VA

Plasma from Lister Hooded rats was spiked with VA to a final concentration of 60 µg/ml. The protein binding study was conducted in the absence and presence of LA at 10 µg/ml. The final organic solvent content did not exceed 0.5% (*v*/*v*). Samples were incubated with constant shaking at 37°C for 1 h, transferred to ultrafiltration devices (30000 MWCO Hydrosart, Vivascience, Hannover, Germany) and centrifuged at 8,000×*g* for 30 min. For each unit loaded with a plasma sample, a partner ultrafiltration unit was loaded with control plasma. Following filtration, control plasma was spiked with VA to a concentration of 60 µg/ml. All experiments were

performed in triplicates. The concentration of VA in ultrafiltrate (C_{UF}) and control plasma (C_T) were analyzed by LC/MS/MS. The fraction of drug unbound (f_u) was determined using the formula: f_u=ZC_{UF}/C_T. To evaluate chemical stability of VA in plasma, aliquots of plasma were removed for quantification prior to and after incubation.

In Vivo Pharmacokinetic Study

Male Lister Hooded rats (446–491 g) were housed individually in clear cages in a pathogen-free environment at 20±1°C, 45±5% humidity and 12 h light/dark cycle. Food and water were available *ad libitum*. All animals were 21 weeks old when used for the pharmacokinetic study. Each animal had indwelling cannulae implanted in the left femoral vein and right jugular vein 2 days before the experiments. All experiments were conducted in compliance with the guidelines of the Institutional Animal Care and Use Committee (protocol number: 050046) and the National Advisory Committee for Laboratory Animal Care and Research.

VA (50 mg/kg) was administered through the femoral vein cannula by constant-rate infusion over 60 min to the rats in the control group (n=3). Both VA (50 mg/kg) and LA (50 mg/kg) were infused simultaneously in a similar way to the rats in the test group (n=3). One hundred twenty microliters of blood samples were collected in heparinized tubes via the jugular vein cannula before dosing and at 15, 30, 45, 60, 65, 75, 90, 120, 150, 180, 240, 300 and 360 min post-dosing. All samples were then diluted with equal volume of water and frozen at −80°C until analysis.

Sample Preparation for *In Vivo* Pharmacokinetic Study

Calibration standards of VA were prepared in ethanol at concentrations of 50, 100, 200, 500, 800, 1,000, 2,000 and 5,000 ng/ml, each in duplicates. Quality control (QC) samples were prepared in ethanol at concentrations of 80, 400 and 2,400 ng/ml, representing low, medium and high concentration QC samples, respectively. Subsequently, to portions of 50 μl of each calibration standard and QC sample, 100 μl of blank blood (diluted 1:1 with water) was added. To 100 μl of each collected blood sample, 50 μl of ethanol was added. Thereafter, 200 μl of 10% (*w*/*v*) trichloroacetic acid was added to precipitate the protein. The mixtures were vortex-mixed and allowed to stand for 20 min at 4°C. After centrifugation at 11,337×*g* for 20 min at 4°C, each supernatant was removed and filtered again using ultrafiltration devices at 8,000×*g* for 45 min. Finally, the filtered samples were subjected to ultra performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) analysis.

UPLC/Q-ToF MS/MS Analysis of VA-CoA

For the accurate mass measurement, a sample consisting of 150 μM of VA incubated in RLMIT for 60 min at 37°C was used. The sample was analyzed using an ACQUITY UPLC system (Waters, Milford, MA, USA) interfaced with a quadrupole, orthogonal acceleration time-of-flight tandem mass spectrometer (Q-ToF MS) equipped with ESI source (Q-Tof Premier™, Waters, Manchester, UK). The UPLC/Q-ToF MS system was controlled by MassLynx 4.1 software (Waters). Chromatographic separations were performed on an AQUITY UPLC BEH C_{18} 1.7 μm 50×2.1 mm i.d. column (Waters). The column heater and autosampler were kept 60°C and 4°C, respectively. The mobile phases consisted of solvent A (10 mM ammonium acetate) and solvent B (methanol). The optimized elution conditions were: gradient of 5% to 95% solvent B (0–1.80 min), isocratic at 95.0% solvent B (1.80–2.14 min) and isocratic at 0.1% solvent B (2.15–2.50 min). The flow rate was 0.7 ml/min. The eluent was splitted post-column into the ESI source using a T-splitter so as to enhance the MS sensitivity.

The Q-ToF MS system was tuned for optimum sensitivity and resolution in electrospray positive ionization mode (ESI +ve) mode using leucine enkephalin (50 pg/μl infused at 5 μl/min). The Q-ToF/MS/MS analysis was operated in "V" mode and the optimized conditions were as followed: capillary voltage 3,300 V, sampling cone 44 V, source temperature 100°C, desolvation temperature 350°C, cone gas flow 0 l/h, desolvation gas flow 500 l/h, collision energy 22 eV, MCP detector voltage 1,800 V, pusher voltage 915 V, pusher voltage offset −0.70 V and puller voltage 640 V. The precursor mass was set at 894.2 Da and continuum data were acquired for each sample from 250–915 Da with a 0.2 s scan time and a 0.02 s interscan delay. Prior to analysis, the system was calibrated in ESI +ve mode using 0.5 M sodium formate solution infused at a flow rate of 4 μl/min. All analyses were acquired using an independent reference spray via the LockSpray™ interface to ensure high mass accuracy and reproducibility; the $[M+H]^+$ ion of leucine enkephalin (2 ng/μl infused at 4 μl/min) was used as the reference lock mass (*m*/*z* 556.2771). The LockSpray™ was operated at a reference scan frequency, reference cone voltage and collision energy of 10 s, 30 V and 16 V, respectively.

LC/MS/MS Analysis

For the *in vitro* analysis of VA-CoA, VA and LA, an Agilent 1200 HPLC system (Santa Clara, CA, USA) was interfaced with a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP MS) equipped with TurboIonSpray electrospray ionization (ESI) source (QTRAP 3200, Applied Biosystems, Foster City, CA, USA). Chromatographic separation was achieved using an XBridge C_{18} column (50×2.1 i.d. mm, 3.5 μm, Waters, Milford, MA, USA) at 60°C. For VA-CoA, the isocratic mobile phases consisting of 55% 10 mM ammonium acetate and 45% methanol, was delivered at 0.55 ml/min. The total run time was 3 min. For VA and LA, the mobile phases consisting of 0.1% acetic acid (pH 4; solvent A) and acetonitrile (solvent B) were delivered at 0.7 ml/min. The optimized elution conditions were: linear gradient 40–95% solvent B (0–1.90 min), isocratic at 95% solvent B (1.90–2.50 min) and isocratic at 40% solvent B (2.51–5.00 min). The injection volume was 5 μl and the MS conditions were summarized in Table I.

The LC/MS/MS was also employed to determine the *in vitro* occurrence of other β-oxidation metabolites of VA. The expected metabolites screened were namely, $\Delta^{2(E)}$-valproyl-CoA, 3-OH-valproyl-CoA and 3-keto-valproyl-CoA and the associated MRM transitions were *m*/*z* 892.0 to 428.0, 910.0 to 428.0 and 908.0 to 428.0, respectively.

Table I. MS Conditions for the Detection of VA-CoA, VA and LA

Parameter	Value		
	VA-CoA	VA	LA
ESI mode	+ve	−ve	−ve
MRM transition	894.0 to 387.0	142.9 to 142.9	205.0 to 171.0
Curtain gas (psi)	20	15	15
IonSpray voltage (V)	5,500	−4,500	−4,500
Source temperature (°C)	600	600	600
Gas 1 (psi)	55	55	55
Gas 2 (psi)	55	60	60
Entrance potential (V)	8	−4	−4
Collision cell exit potential (V)	6	–1	–1
Collision energy (V)	45	−10.50	−14
Declustering potential (V)	45	−42	−30
Collision cell entrance potential (V)	30.28	−17.02	−19.32

For the *in vivo* analysis of VA, an ACQUITY UPLC system (Waters) was used to detect the lower levels of VA in blood. Chromatographic separation was achieved using an ACQUITY UPLC BEH C_{18} 1.7 µm 50×2.1 mm i.d. column (Waters) at 60°C. The mobile phases consisting of 0.1% acetic acid (pH 4; solvent A) and acetonitrile (solvent B) were delivered at 0.6 ml/min. The optimized elution conditions were: linear gradient 40–60% solvent B (0–1.45 min), isocratic at 100% solvent B (1.46–1.99 min) and isocratic at 40% solvent B (2.00–2.50 min). The MS conditions were similar to the *in vitro* method.

Data Analysis

For the *in vitro* studies, statistical differences between groups were analyzed using one-way analysis of variance and two-tailed independent *t* test where appropriate (SPSS 15.0, Chicago, IL, USA). Statistical significance was established when $p<0.05$. For the HLM and RLM metabolic stability studies, the half-life ($t_{1/2}$) of each drug was determined using GraphPad Prism 4 (San Diego, CA, USA). For the pharmacokinetic study, noncompartmental method was adopted for the analysis (WinNonlin 1.0, Scientific Consulting Inc., Cary, NC, USA). The area under the curve of VA was computed from 0 to 6 h ($AUC_{0\rightarrow 6\ h}$). The absolute bioavailability of VA in systemic circulation following co-intravenous infusion of VA and LA was calculated as $F=[(AUC_{0\rightarrow 6\ h})_{VA+LA}/(AUC_{0\rightarrow 6\ h})_{VA\ alone}]\times[Dose_{VA\ alone}/Dose_{VA+LA}]$. As the same dose of VA was used in both control and test groups, direct comparison of AUC was adopted without dose normalization.

RESULTS AND DISCUSSIONS

Citrate Synthase Activity in RLMIT

In order to establish that enzymatic activity in the isolated RLMIT was not compromised, citrate synthase activity was determined. Being an exclusive marker enzyme of the mitochondrial matrix (20,21), its activity reflects the general level of enzymatic activity in the organelle. Based on our assay, the total citrate synthase activity was 44.1 mM/min/mg protein. Our assay also indicated a 72% of intact mitochondria suggesting the suitability of the isolated RLMIT for use in the subsequent mitochondrial studies.

Optimization of the *In Vitro* Metabolism of VA in RLMIT

Preliminary experiments were performed to optimize the incubation conditions with regards to the choice of buffer. The suitability of Tris and SEM buffer (250 mM sucrose, 1 mM EDTA and 5 mM NaMOPS), the two routinely used buffers in mitochondrial assays, were assessed. Contrary to an earlier study that suggested a significant loss of β-oxidation activity due to Tris buffer (22), we found that Tris buffer resulted in optimal formation of VA-CoA as compared to SEM. Despite such contradiction, the clear formation of VA-CoA clearly justified the adoption of Tris buffer in all subsequent incubations.

The pH of the reaction mixture was also closely monitored as it was initially observed that precipitation of mitochondrial protein was likely, considering the acidity of the various components in the mixture. The organic content in each mixture was kept to a minimum (<0.5%) in order not to affect the enzymatic activity. Using the optimized assay conditions, VA-CoA was formed readily over time, reaching its maximum level at 60 min. For all subsequent inhibition studies, the incubation time was kept at 60 min.

α-Methylbutyric acid, which is structurally similar to VA, was previously reported to inhibit the mitochondrial formation of VA-CoA competitively (22). It was used as a positive control to validate the suitability of our developed assay in detecting an inhibition of VA-CoA formation. On the other hand, the negative control was designed to determine the matrix effect of the incubation medium on LC/MS/MS analysis. As no formation of VA-CoA was expected, any peak observed at the retention time of VA-CoA was attributed to matrix effect and accounted for in all data analysis.

MS and LC Optimization for Analysis of VA-CoA

As the authentic standard of VA-CoA was not commercially available, preliminary MS optimization was performed

using CoA, as it was likely to possess similar compound-dependent parameters and fragmentation pattern as VA-CoA. ESI +ve mode was found to produce higher sensitivity as compared to the negative mode. This concurred with previous work of Burns *et al.* (23) who used ESI +ve mode for the characterization of CoA.

To further ascertain the formation of VA-CoA, its accurate mass was measured using Q-ToF MS. In our Q-ToF MS experiment, accurate mass measurement was rendered possible by the simultaneous but independent acquisition of reference ions of leucine enkephalin via the LockSpray™ interface. Using MassLynx, the potential calculated masses, mass accuracy (mDa and ppm), i-FIT (the likelihood that the isotopic pattern of the elemental composition matches a cluster of peaks in the spectrum) values and elemental compositions associated with the measured mass of VA-CoA and its fragments were generated and studied. Using a mass tolerance of 12 ppm, the experimentally determined masses/mDa/ppm/i-FIT/ elemental composition of VA-CoA ($[M+H]^+$) and its fragments were determined and summarized in Table II. Our results demonstrated that the mass accuracy measurements were consistently high for VA-CoA and its most abundant product ions (less than 5 RMS ppm). The identity of VA-CoA was further confirmed by comparing its fragmentation pattern to that of CoA (*m*/*z* 768) which generated two characteristic product ions at *m*/*z* 428 and 261 (Fig. 3). As shown in Fig. 4A, the collision-induced dissociation (CID) of VA-CoA occurred at the same P–O and C–O bonds yielding the corresponding product ions at *m*/*z* 428 and 387. These characteristic fragmentation patterns of CoA and VA-CoA were also found to be reproducible in the QTRAP MS analyses (insets of Fig. 3 and 4A, respectively). Finally, it was observed that VA-CoA was not formed in VA-deprived RLMIT incubations. Collectively, we confirmed that VA-CoA was formed in all RLMIT experiments in our study.

With samples extracted from the incubation mixtures at 0 and 60 min, multiple reaction monitoring (MRM) experiment was performed in QTRAP MS to detect VA-CoA using transitions of *m*/*z* 894 to 428 and *m*/*z* 894 to 387. In general, the latter transition conferred a higher signal-to-noise ratio and was selected as the MRM transition for all subsequent analyses. To further improve the signal intensity, optimization of the compound-dependent parameters of VA-CoA was carried out. The optimized values were documented in the "MATERIALS AND METHODS" section.

In addition, the effects of solvents and additives on the ESI +ve MS detection of CoA were investigated and evaluated. It was observed that the inclusion of ammonium acetate were essential for the ionization of CoA while the inclusion of formic or acetic acid resulted in strong ion suppression. This was an interesting observation worthy of discussion, as it was widely accepted that acids facilitate the protonation of basic analytes in the positive-ion mode. The reason for the discrepancy remained to be determined, although a plausible explanation was that the acidic medium resulted in the formation of multiply charged ions and hence, the signal intensity of $[M+H]^+$ was reduced. Meanwhile, the use of methanol resulted in much higher sensitivity than acetonitrile, which was not unexpected since its lower surface tension and dielectric constant might have promoted ion evaporation (24). In summary, ammonium acetate and methanol were determined to be the best in aiding ionization and were hence selected as the mobile phase for the analysis of VA-CoA. With further adjustment of the eluent composition and condition, a distinct and well-resolved peak was obtained. A sensitive and specific LC/MS/MS method for quantifying VA-CoA was therefore developed.

Identification of Other β-oxidation Metabolites of VA

In this study, we also determined the presence of other β-oxidation metabolites that may be formed downstream of VA-CoA. An analysis of the various CoA esters (Fig. 2) by LC/MS/MS in both QTRAP MS and Q-ToF MS revealed a complete absence of these metabolites. Our results were consistent with former studies (16) which showed that VA-CoA is the predominant species present during the mitochondrial metabolism of VA and that other metabolites were not formed in appreciable amounts under the incubation conditions. Hence, we confirmed that the formation of VA-CoA was a suitable marker to measure the biotransformation of VA via the β-oxidation pathway.

Inhibition of LA on the Metabolism of VA in RLMIT

Using the optimized assay conditions and detection methods, the inhibition potential of LA on the metabolism of VA was investigated in RLMIT. The amount of VA-CoA formed over the 60-min incubation period was measured based on its integrated chromatographic peak area. To examine the effect of varying concentrations of LA on the

Table II. The Experimentally Determined Masses (*m*/*z*), mDa, ppm, i-FIT and Elemental Composition of VA-CoA ($[M+H]^+$) and its Product Ions After Collision Induced Dissociation in (CID) in Q-ToF MS

Experimentally determined *m*/*z*	mDa	Ppm	i-FIT	Elemental composition
894.2267 (VA-CoA)	−0.8	−0.9	2.1	$C_{29}H_{51}N_7O_{17}P_3S$
508.0051	1.5	3.0	ND	$C_{10}H_{17}N_5O_{13}P_3$
467.1988	0.7	1.5	1.0	$C_{19}H_{36}N_2O_7PS$
428.0372	0.0	0.0	1.5	$C_{10}H_{16}N_5O_{10}P_2$
410.0291	2.4	5.9	ND	$C_{10}H_{14}N_5O_9P_2$
387.2307	−1.1	−2.8	1.0	$C_{19}H_{35}N_2O_4S$
330.0602	−0.1	−0.3	ND	$C_{10}H_{13}N_5O_6P$
285.1605	−3.2	−11.2	0.5	$C_{14}H_{25}N_2O_2S$

ND Not determined due to low isotopic peak sensitivity

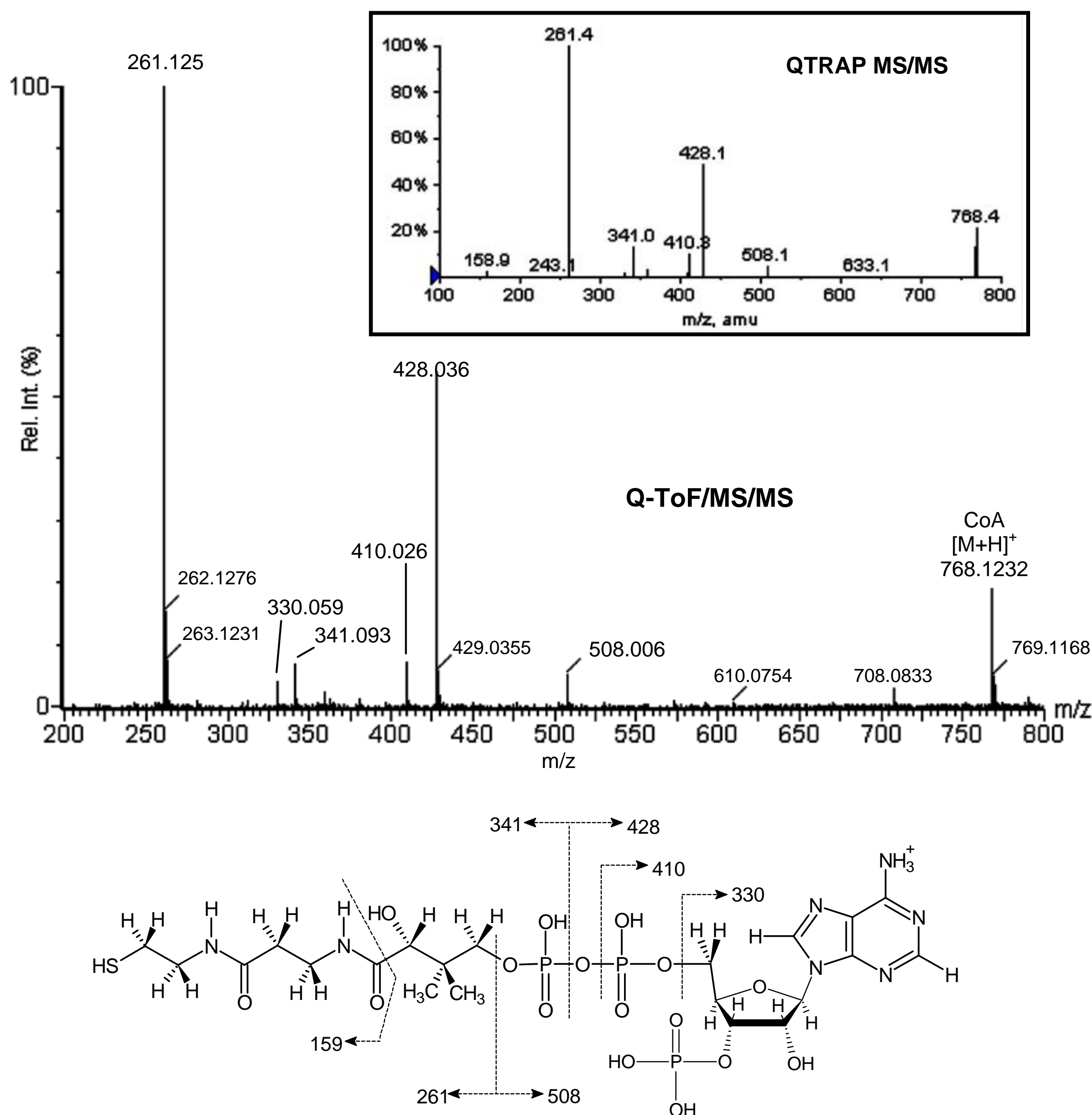

Fig. 3. QTof MS/MS and QTRAP MS (*inset*) mass spectrum of CoA (*m/z* 768). Putative fragmentation patterns are displayed at the *bottom* of the PIS spectrum.

formation of VA-CoA, the mean areas were compared. The concentrations of LA and VA were chosen to approximate C_{max} attained after a single 600 mg dose of lipoic acid (clinically used for diabetic neuropathy) (25) and the unbound steady-state plasma VA concentration achieved in humans administered with therapeutic dose of the drug (15), respectively. For all triplicate data of each set, the standard deviations (SDs) were calculated to be less than 23%.

As depicted in Fig. 5, the inhibition on the formation of VA-CoA by LA was clearly significant and concentration-dependent. Such significant differences ($p<0.05$) substantiated our hypothesis that LA inhibit the metabolism of VA via the β-oxidation pathway. While it was not our aim to extrapolate these observations to humans, our data suggested that such inhibition may be clinically significant as it occurred within the therapeutic plasma concentrations of both LA (<14 μM, C_{max} value arising from 600 mg/day dose indicated for diabetic neuropathy) and VA (unbound steady state concentration <105 μM). It was further supported by the low estimated IC_{50} values of LA at 7.5, 9.5 and 10 μM for concentrations of VA at 50, 100 and 150 μM, respectively. Collectively, these results clearly showed that LA was a potent inhibitor of the β-oxidation of VA and exhibited its inhibitory effect at clinically-relevant concentrations. With mitochondrial β-oxidation being the most important route of biotransformation of VA in man, it underscored the likelihood that LA may significantly impede the metabolic clearance of the drug from the body. The clear inhibition of VA-CoA formation by α-methylbutyric acid, the positive control, validated the assay and rendered the inhibition data credible.

In addition, one also has to bear in mind that such strong inhibition of LA on β-oxidation holds potential toxicological implication. There lies a possibility that LA may interfere with endogenous fatty acid metabolism, culminating in hepatic steatosis. Although there is no report thus far on its clinical toxicity, the likelihood that chronic doses may lead to hepatic injury cannot be ruled out. This speculation offers grounds for future research on the long-term toxicology of LA.

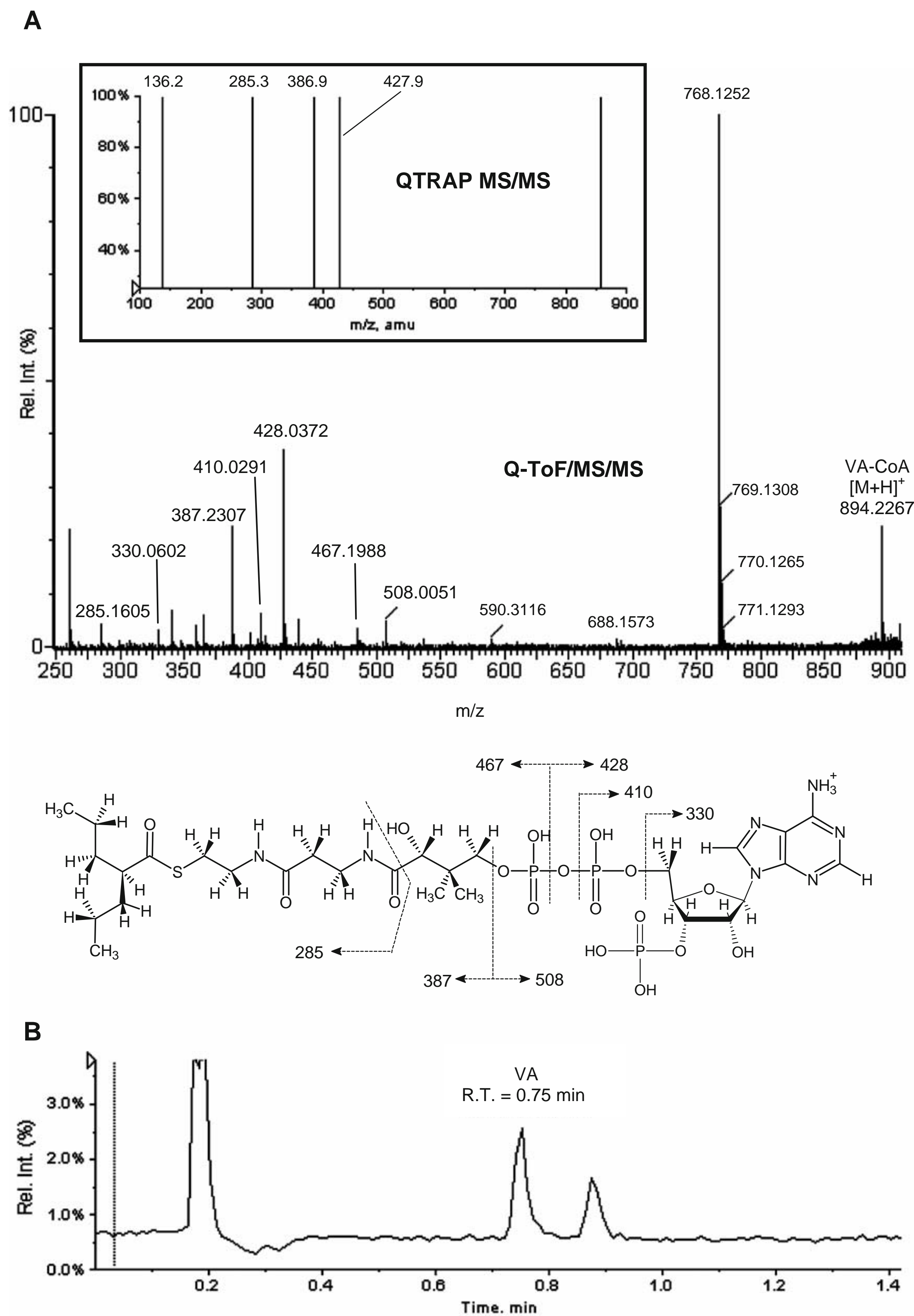

Fig. 4. **A** QTof MS/MS and QTRAP MS (*inset*) mass spectra of VA-CoA (*m*/*z* 894). Putative fragmentation patterns are displayed at the *bottom* of the PIS spectrum. **B** UPLC/MS/MS chromatogram of blank blood spiked with VA at 50 ng/ml.

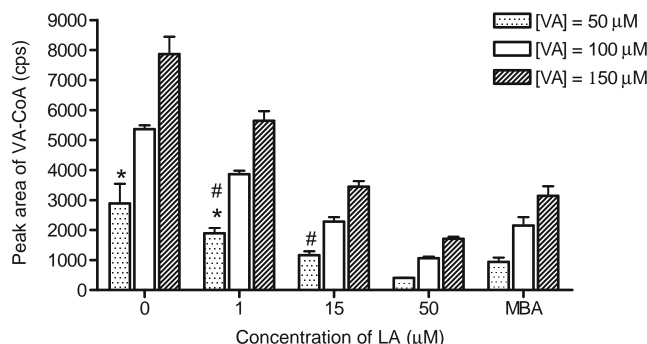

Fig. 5. Plot of integrated peak area (cps) of VA-CoA against concentration (μM) of inhibitors. VA was tested as a substrate at 50, 100 and 150 μM while LA was investigated as an inhibitor at 0, 1, 15 and 50 μM. α-methylbutyric acid (MBA) was the positive control (100 μM). Each measurement represents mean±SD (n=3). Statistical differences (p<0.05) were observed for all except for [VA]=50 μM at 0 and 1 μM LA (indicated with *asterisk*) and [VA]=50 μM at 1 and 15 μM LA (indicated with *pound sign*).

In Vitro Metabolic Stability of LA and VA in RLM and HLM

Kinetic analyses of LA and VA metabolism were examined in the microsomes to ascertain if these two drugs interact at the hepatic CYP450 enzyme level. The substrate concentrations were kept minimal (2–5 μM) to ensure first-order kinetics. Protein concentration was also kept low to reduce non-specific binding in microsomes. The half-lives ($t_{1/2}$) of the negative controls exceeded 60 min, showing negligible chemical degradation of the compounds under the incubation conditions.

It was evident from the long half-lives in both RLM and HLM that LA and VA were metabolized by the rat microsomal CYP450 enzymes to an insignificant extent (Table III). Therefore any DDI between LA and VA at the CYP450 level in rats was unlikely.

In Vitro Inhibition of LA on Plasma Protein Binding of VA

Although the main objective of our study was to investigate DDI at the metabolism level, interaction between LA and VA at the protein binding level was also investigated to acquire a complete understanding of their pharmacokinetic relationship in Lister Hooded rats. Plasma protein binding of VA was determined by the ultrafiltration method (26–28) and the concentrations of LA and VA were selected to approximate plasma levels of both drugs (when dosed at 50 mg/kg) in Lister Hooded rats (based on previous data obtained in our laboratory).

Table III. Half-lives ($t_{1/2}$) of LA and VA in HLM and RLM

	$T_{1/2}$ of degradation in microsomes (min)	
Compound	RLM	HLM
LA	41	>60
VA	>60	>60
Verapamil (positive control)	8	33

Analyzing the VA concentrations prior to and after incubation allowed the evaluation of the stability of VA in plasma at 37°C. No significant decline of VA was observed over the 60-min incubation period, demonstrating that VA was chemically stable under the incubation conditions. Hence any decrease in the amount of VA after ultrafiltration, observed in relation to the control, would be ascribed to the binding of VA to plasma proteins. Results from the assay determined the fraction unbound (f_u) of VA in rat plasma to be 0.39±0.02. As the value resembled closely to the reported value of 0.37 (29), it proved the accuracy of the protein binding assay. In the presence of LA, f_u remained comparable (0.41±0.02) and was not significantly different (p>0.05). This result clearly indicated that DDI was unlikely to occur at the protein binding level in our pharmacokinetic study.

Determination of VA in Rat Blood by UPLC/MS/MS

To further confirm our hypothesis and examine its significance *in vivo*, the DDI was further investigated using an animal pharmacokinetic study. The pharmacokinetic profiles following intravenous administration of VA with and without LA to Lister Hooded rats were compared. To this end, a rapid and sensitive UPLC/MS/MS method was developed for the quantification of VA in blood at various time points following administration. The method harnessed the fast and high-resolution separation of UPLC together with the sensitivity of tandem mass spectrometry. The weighted (1/x) calibration curve was linear over the blood concentration range 50–5,000 ng/ml with a coefficient of correlation (r) of 0.9987. The intra-day precision was assessed by analyzing QC samples at the low, medium and high concentration level and the coefficient of variation was found to be below 10.7%. Inter-day precision was not evaluated as all samples were processed within the same day. The overall accuracy ranged from 83.9–117.9% and the recovery from spiked blood was more than 50%. The signal-to-noise ratio at 50 ng/ml of VA in blood was approximately greater than 93. Hence the limit of detection (LOD) and lower limit of quantification (LOQ) of the assay were below 50 ng/ml. Unlike other bioanalytical assay validation, the actual LOQ of the assay was not determined in this study as the

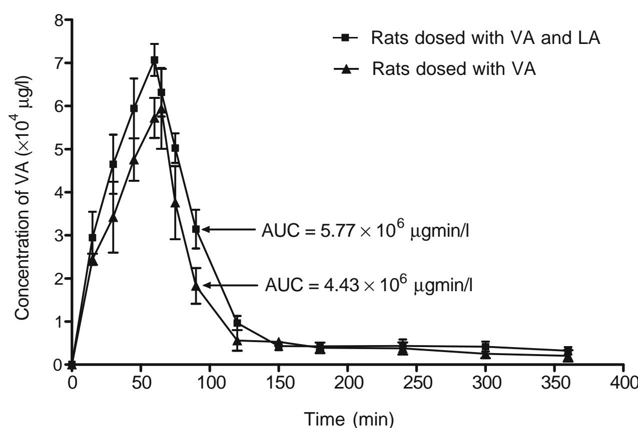

Fig. 6. Plot of mean concentration (μg/l) against time profile (min) of VA (*filled square* LA co-administered at 50 mg/kg with VA at the same dose; *filled triangle* VA was administered alone). *Each point* represents the mean±standard deviation (SD) of three rats.

concentration of VA at the last timepoint (6 h) was predicted and found to be higher than 50 ng/ml.

In the chosen chromatographic conditions, specificity was indicated by the absence of any endogenous interference at the retention time of VA as evaluated by chromatograms of blank rat blood and blood spiked with VA. As an illustration, Fig. 4B showed a representative chromatogram for rat blood spiked with VA at 50 ng/ml. Interestingly, while it is a convention to include an internal standard to help normalize any variation in extraction efficiency or volumes of LC injection, the present method was able to circumvent the use of it and yet achieved good accuracy, linearity and precision. The results of the validation test confirmed the method to be suitable for the bioanalytical study.

In the assay, the extraction of VA from blood was performed using protein precipitation by trichloroacetic acid. Liquid–liquid extraction by methyl *tert*-butyl ether was attempted but it produced a low recovery of only 30%. The low extraction efficiency may, in part, be due to the relatively low lipophilicity of VA that did not permit a good partition into the organic phase. Therefore protein–protein precipitation, which gave a better recovery of 50%, was selected as the sample extraction method.

In Vivo Inhibition of LA on VA Clearance

The validated method was used to analyze the blood samples obtained at various sampling points and a 6-h plot of mean plasma concentration *versus* time profile of VA was shown in Fig. 6. With concomitant administration of LA, the blood concentration of VA was notably higher than that of the control rats which were dosed with VA only. The area under the curve ($AUC_{0\rightarrow 6\ h}$) was increased appreciably in the presence of LA. The $AUC_{0\rightarrow 6\ h}$ values of the control (VA alone) and test (VA and LA) groups were determined to be 4.43×10^6 and 5.77×10^6 μgmin/l, respectively. Based on the $AUC_{0\rightarrow 6\ h}$ values, the absolute bioavailability of VA when given by intravenous infusion would have an absolute bioavailability of 1 (F=1) while VA given together with LA had an absolute bioavailability of 1.3. As the drugs were administered intravenously, this index indicated a DDI between LA and VA in Lister Hooded rats, a result that reinforced our data obtained with *in vitro* metabolism.

From a clinical perspective, such a DDI between VA and LA may have a huge impact given the narrow therapeutic index of VA. In particular, patients whose blood levels of VA were maintained at the upper end (100–120 μg/ml) of the therapeutic window may encounter toxicity when their exposure to VA was increased due to the DDI. However, it is important to stress that an allometric scaling of the pharmacokinetic parameters to humans was outside the scope of the present study. An animal study was only capable of predicting potential DDI between the two drugs *in vivo*, and based on the present data, such an interaction was likely. It served to highlight the need for subsequent clinical trials to investigate the DDI between LA and VA, and evaluate the need for dosage adjustments, before the two drugs are co-administered in humans.

An attempt was made to investigate the possible disposition processes, namely distribution, metabolism and excretion, at which interaction could occur between LA and VA in rats. As interaction at the oral absorption level between LA and VA may possibly occur and confound our data interpretation, LA and VA were administered via the intravenous route. A pilot *in vitro* protein binding study predicted no significant interaction at the distribution level. It had also been reported that the contribution of renal excretion to the elimination of VA was minimal (3%) (15,30). Hence attention had been correctly placed on the potential interaction at the metabolism level. As metabolic stability studies undertaken in RLM and HLM supported the supposition that CYP metabolism was not a major eliminating pathway for VA, we eliminated the possibility of interaction at the CYP450 level. As a result, the DDI observed *in vivo* was most likely attributed to inhibition of β-oxidation or glucuronidation, the two known predominant metabolic processes of VA. Remarkably, the *in vitro* work in the current study proved that significant inhibition of β-oxidation by LA was responsible for the decrease in metabolic clearance.

CONCLUSION

A sensitive HPLC/MS/MS method was developed for the determination of VA-CoA. Our data supported our hypothesis and provided the first demonstration that LA exhibited significant inhibition on the β-oxidation of VA, particularly on the formation of VA-CoA. In concordance with the *in vitro* results, our *in vivo* study confirmed that DDI occurred pharmacokinetically at the elimination level between LA and VA. While further clinical studies need to be performed to ascertain their DDI in humans, medical professionals should recognize the need to be prudent when managing patients who are co-administered with both LA and VA.

ACKNOWLEDGEMENT

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