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Analysis of lamotrigine and lamotrigine 2-N-glucuronide in guinea pig blood and urine by reserved-phase ion-pairing liquid chromatography

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ABSTRACT

Lamotrigine is an investigational anticonsulvant drug undergoing clinical trials. A simultaneous assay was developed to quantitate lamotrigine and its major metabolite, lamotrigine 2-N-glucuronide, from guinea pig whole blood. The extraction procedure and reversed-phase high-performance liquid chromatographic (HPLC) assay employed sodium dodecylsulfate (SDS) as an ion-pairing reagent to selectively separate lamotrigine and lamotrigine 2-N-glucuronide from endogenous blood components, other anticonvulsant drugs, and their metabolites. The mobile phase was composed of acetonitrile–50 m*M* phosphoric acid (pH 2.2) containing 10 m*M* SDS (33:67, v/v), and components were detected at 277 nm. The total coefficients of variance (C.V.) for the blood assay were ≤ 9.4% for lamotrigine (0.25–20.0 μg/ml) and ≤ 13.4% for the glucuronide metabolite (0.25–10.0 μg/ml). Separate assays for lamotrigine and its glucuronide in urine were developed. In order to quantitate low levels of lamotrigine in guinea pig urine, lamotrigine was extracted with *tert.*-butyl methyl ether–ethyl acetate (1:1). The total C.V. for lamotrigine quantitation in urine was ≤ 7.5% (0.10–10.0 μg/ml). For the determination of lamotrigine 2-N-glucuronide, urine was diluted with an SDS–phosphoric acid buffer (1:4) and injected directly onto the HPLC system, total C.V. ≤ 4.2% (0.5–50 μg/ml).

INTRODUCTION

Lamotrigine, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (LTG, Fig. 1), is a promising investigational anticonvulsant from the triazine class of chemical compounds. LTG is currently undergoing phase III clinical trials [1] and has been

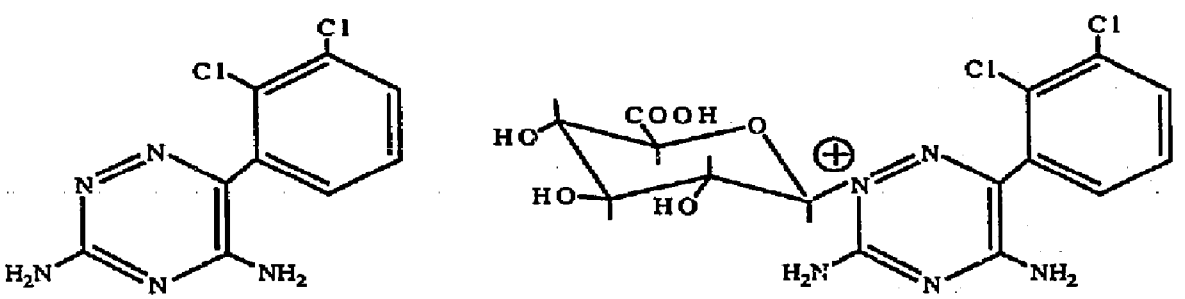

Fig. 1. Structures of lamotrigine and lamotrigine N-glucuronide.

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found to be active against minimal electroshock seizures and pentylenetetrazol-evoked hindlimb extension in mice and rats [2]. The antiepileptic profile of LTG in animals resembles that of phenytoin, a common anticonvulsant, but LTG is more potent and has a longer duration of action [2,3].

The major human metabolite of LTG is the quaternary 2-N-glucuronide (LTG N-glucuronide, Fig. 1). After a single oral dose of LTG (120 mg) in humans, approximately 70% of the dose is accounted for in the urine, and of that total 90% is the glucuronide metabolite [4]. The same glucuronide metabolite has recently been shown to be the major metabolite in guinea pigs [5], a finding that is unique because quaternary N-glucuronides are rarely excreted by animal species below monkeys and man [6–8]. After a single intravenous dose of LTG (10 mg/kg) to a guinea pig, approximately 60% of the dose was excreted in the urine as the glucuronide metabolite and 5% of the dose was recovered as intact LTG [5].

Analytical methods for the quantitation of LTG by high-performance liquid chromatography (HPLC) in human plasma have been decribed in brief terms from several clinical reports [4,9,10]. The most complete description of these normal-phase methods was by Posner *et al.* [10]. Their method employed a silica column with a mobile phase of ammonium hydroxide–ethanol–hexane (0.25:20:80, v/v). Plasma samples were extracted with a large volume of ethyl acetate (8 ml). The lower limit of detection was 0.05 μg/ml at 306 nm with a coefficient of variation of 7%. A normal-phase assay would be inappropriate for the direct quantitation of a highly polar glucuronide metabolite. In addition, the glucuronide is known to degrade at basic pH [11]. Quantitation of the glucuronide by Posner *et al.* [10] was achieved by differential hydrolysis with large amounts of β-glucuronidase.

A separation of LTG from other antiepileptic drugs by reversed-phase chromatography was described by Juergens [12] with a basic phosphate buffer (pH 9) containing triethylamine or *n*-butylamine to prevent tailing. Juergens [12] also reported the k' values for lamotrigine at different pH values on a polymeric gel column. Assay validation or extraction procedures for LTG from biological fluids were not presented. Under these conditions the glucuronide of LTG elutes with the solvent front.

Initial information collected in our laboratory indicated that the guinea pig may be a useful animal model to study the metabolism and pharmacokinetics of LTG, especially with regard to the interactions of other drugs with LTG. Therefore, a sensitive and accurate assay was developed to quantitate and evaluate these changes in pharmacokinetic parameters. This report describes a simultaneous assay for the quantitation of LTG and LTG N-glucuronide in guinea pig whole blood by means of solid-phase extraction and reversed-phase HPLC, as well as quantitation of these compounds in guinea pig urine.

EXPERIMENTAL

Chemicals

Lamotrigine and the internal standard, 3,5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine, (BW A725C), were gifts from Wellcome Research Labs. (Research Triangle Park, NC, USA). LTG-glucuronide was purified from human urine by XAD-2 column chromatography followed by semi-preparative HPLC [11]. The compound was characterized by ^{1}H NMR, fast atom bombardment, and tandem mass spectrometry [11]. The white crystalline material eluted as a single spot on two different thin-layer chromatographic (TLC) systems and as a single peak with two separate HPLC mobile phases on a reversed-phase column [11]. Stability studies at different pH values were performed, and the compound was stable in acidic solution at −20°C for at least one month [11].

Phenytoin, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, 5-(*p*-hydroxyphenyl)-5-ethylbarbituric acid (*p*-hydroxyphenobarbital), 5-ethyl-5-*p*-tolybarbituric acid (tolybarbital), and 5-(4-methylphenyl)-5-phenylhydantoin were purchased from Aldrich (Milwaukee, WI, USA). Carbamazepine, lorazepam, and phenobarbital were purchased from Sigma (St. Louis, MO, USA). Cyheptamide (a common internal standard for carbamazepine analysis) was purchased from Supelco (Bellefonte, PA, USA). Carbamazepine-*trans*-10,11-diol was a gift from Ciba-Geigy (Ardsley, NY, USA). Felbamate (2-phenyl-1,3-propanediol dicarbamate), three of its metabolites, 2-hydroxy-2-phenyl-1,3-propanediol dicarbamate (2-hydroxyfelbamate), 2-(4-hydroxyphenyl)-1,3-propanediol carbamate (*p*-hydroxyphenylfelbamate), 2-phenyl-1,3-propanediol monocarbamate (felbamate monocarbamate), and 2-phenyl-2-methyl-1,3-propanediol dicarbamate, an internal standard for the analysis of felbamate [13], were gifts from Wallace Labs. (Cranbury, NJ, USA). Ethosuximide was extracted with ethyl acetate from a Zarontin capsule (Parke-Davis). Carbamazepine-10,11-epoxide was synthesized by the method of Bellucci *et al.* [14]. Phenytoin-3,4-dihydrodiol [5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantion] was isolated from rat urine by a procedure similar to that of Maguire *et al.* [15].

Sodium dodecyl sulfate (SDS), MicroSelect grade, was purchased from Fluka BioChemika (Ronkonkama, NY, USA). HPLC-grade phosphoric acid, acetonitrile, and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

High-performance liquid chromatography apparatus and conditions

The analytical column was a 250 mm × 4.6 mm I.D., 5 μm Spherisorb C_8 cartridge column (Phase Separations, Norwalk, CT, USA). The HPLC system consisted of a Spectra-Physics SP8700 solvent delivery systems, SP 8880 autosampler, SP 4270 integrator, and a Kratos Spectroflow 754 variable-wavelength detector. The column was heated to 40°C with a variable-temperature column heater (Timberline Instrument, Boulder, CO, USA). LTG, LTG glucuronide,

and the internal standard were all detected at 277 nm with a detector sensitivity setting of 0.05 a.u.f.s. The mobile phase was composed of acetonitrile–50 m*M* phosphoric acid (pH 2.2) containing 10 m*M* SDS (33:67, v/v) at a flow-rate of 1.5 ml/min.

Sample preparation

Male Dunkin–Hartley guinea pigs (350–490 g) were obtained from Biolabs (St. Paul, MN, USA). Treatment of animals and the surgical procedure were performed as previously described [5]. Briefly, whole blood samples (300 μl) were collected from guinea pigs through a carotid artery cannula following an intravenous bolus dose of LTG (10 mg/kg). Whole blood was stored in 2.0-ml vacutainer tubes containing sodium heparin and were extracted immediately or stored in 250-μl aliquots at −20°C.

Blood samples (250 μl) were placed in 1.5-ml polyethylene microcentrifuge tubes and then 750 μl of phosphoric acid buffer, pH 1.2, containing 15 m*M* SDS, and internal standard (50 μl of a 30 μg/ml methanol–water, 1:1, v/v solution) were added. The tubes were capped, vortexed for 15 s, and centrifuged at 13 000 *g* for 7 min in a Fisher Model 235B microcentrifuge (Minneapolis, MN, USA). The supernatant was placed on a Burdick and Jackson C_{18} (100 mg) solid-phase extraction column (Baxter Healthcare, Muskegon, MI, USA) that had previously been washed with 2.0 ml of methanol, 2.0 ml of water, and 1.0 ml of 50 m*M* phosphoric acid buffer, pH 1.2. After loading of the sample, the column was washed with 300 μl of 50 m*M* phosphoric acid buffer. The compounds were then eluted with 1.0 ml of methanol. The methanol solution was evaporated to dryness with a stream of nitrogen (< 40°C). The evaporated samples were reconstituted in 250 μl of mobile phase and filtered through 0.45-μm syringe filters (Lida, Kenosha, WI, USA). A 50-μl aliquot of each sample was injected onto the HPLC system. Standards were prepared by addition of 25 μl of LTG (in methanol) and 25 μl of LTG glucuronide (in aqueous 5% acetic acid) to 250 μl of whole blood to achieve final concentrations of 0–20 and 0–10 μg/ml, respectively. The standard samples were extracted in an identical manner to animal samples as described above.

Urine was collected from each guinea pig while housed in plastic Nalgene metabolism cages. The urine was collected onto 1.0 ml of 20% acetic acid in order to prevent breakdown of the glucuronide metabolite [11]. Urine samples were combined with cage washings and stored at −20°C before analysis. Each urine sample was filtered through a 0.45-μm syringe filter before analysis or extraction. For the analysis of the glucuronide conjugate, an aliquot (100 μl) of urine was diluted with 400 μl of phosphoric acid buffer (50 m*M* , pH 1.2) containing 15 m*M* SDS. A 10–50 μl portion of this solution was injected onto the same column and under the same conditions as stated previously, except that the proportion of acetonitrile was reduced from 33 to 30% to improve the separation of the glucuronide from endogenous components in urine.

A liquid–liquid extraction procedure was employed to determine the concentration of LTG in guinea pig urine [4,14]. To 1.0 ml of filtered urine, 1.0 ml of 1.0 *M* NaH_2PO_4, pH 11.0, and 50 μl of internal standard (15 μg/ml in methanol–water, 1:1, v/v) were added. This alkaline solution was then extracted twice with 3.0 ml of ethyl acetate–*tert.*-butyl methyl ether (1:1). The organic fractions were combined and evaporated to dryness under nitrogen at 40°C. The residue was reconstituted in 250 μl of mobile phase, acetonitrile–50 m*M* phosphoric acid buffer containing 10 m*M* SDS, pH 2.2 (4:6, v/v). The column and conditions were the same as those stated earlier.

Assay validation

Peak-height ratios (LTG/internal standard and LTG N-glucuronide/internal standard) were found to give more accurate estimates of the true standard concentrations and were less susceptible to errors from interfering peaks than peak areas. Duplicate standard curves in whole blood were performed at the following concentrations of LTG and LTG-glucuronide: 0, 0.25, 0.50, 1.0, 2.5, 5.0, 10.0, 20.0 μg/ml and 0, 0.25, 0.50, 1.0, 2.5, 5.0 μg/ml, respectively. The LTG standard curves from the whole blood assay were split from 0 to 1.0 μg/ml and 1.0 to 20.0 μg/ml in order to give a more accurate determination of the LTG concentration. LTG urine standards were also extracted in duplicate at the following concentrations: 0, 0.10, 0.25, 0.50, 1.0, 5.0, and 10.0 μg/ml. The glucuronide standard curves in urine were split in the following manner: 0, 0.50, 1.0, and 5.0 μg/ml and 5.0, 10.0, 25.0, and 50.0 μg/ml.

The estimated total variability of the blood assay for a single sample measure on different days was determined by one-way analysis of variance (ANOVA), from calculated concentrations of multiple duplicate standard curves (number of replicates, $r = 2$) [16,17].

The within-day (S^2_{wd}) and between-day (S^2_b) components of variance were determined as follows:

$$S^2_{wd} = [MS_{wd}]$$

$$S^2_b = [(MS_b - MS_{wd})/r]$$

where MS_{wd} and MS_b are the within-day and between-day mean squares from the ANOVA table, respectively. The total variance of an observation (S^2_{total}) was determined from the sum of the within-day and between-day variances. The percentrage coefficient of variance (C.V.) at each concentration was determined by dividing the S_{total}, S_b, and S_{wd} by the grand mean of the calculated concentration values, multiplied by 100 [16]. Calculation of the accuracy was determined by dividing the grand mean by the true mean, and multiplied by 100.

RESULTS

Fig. 2 shows chromatograms of blank (A), standard (B), and LTG-treated (C) guinea pig blood extracts. The retention times for LTG glucuronide, internal standard, and LTG were 6.7, 11.9, and 19.9 min, respectively. After the LTG had been eluted from the column, the percentage of acetonitrile was increased to 67% for 5 min, and then adjusted to 33% for 5 min to re-equilibrate the column prior to the next sample injection. The total run time for each sample was approximately 30 min. The extraction recoveries of LTG N-glucuronide and LTG are shown in Table I and were generally 60–80%. The average extraction recovery of the internal standard was 74.9%.

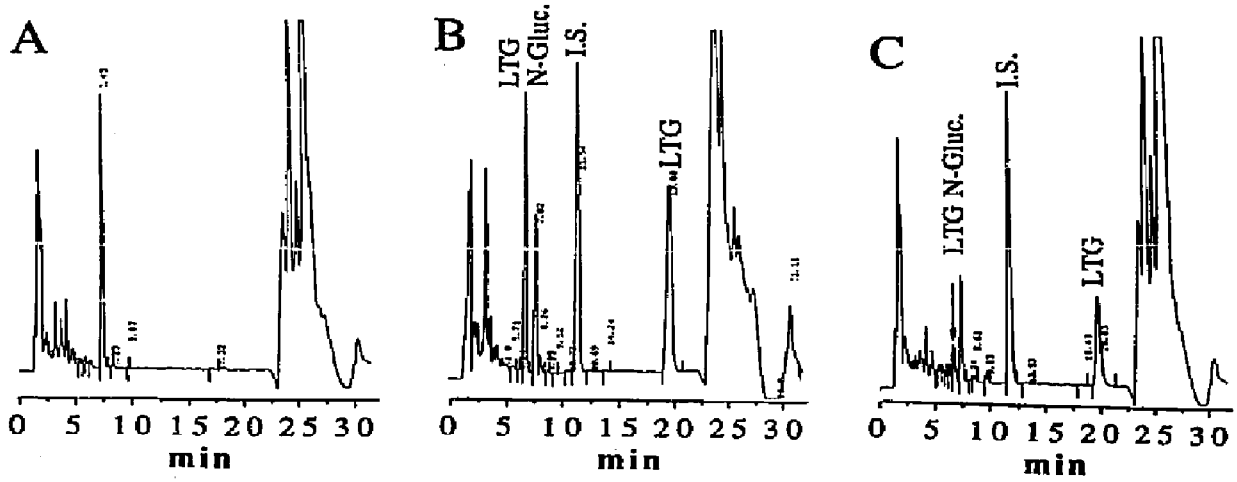

Fig. 2. HPLC of (A) extracted blank guinea pig whole blood, (B) extracted whole blood standard containing 10 μg/ml LTG, 10 μg/ml LTG N-glucuronide, and 6 μg/ml internal standard (I.S.), and (C) an extracted whole blood sample from a guinea pig 90 min after administration of LTG (10 mg/kg, intravenously). Measured LTG and LTG N-glucuronide concentrations were 5 and 1.4 μg/ml, respectively.

TABLE I

EXTRACTION RECOVERIES OF LTG AND LTG N-GLUCURONIDE FROM GUINEA PIG WHOLE BLOOD

Concentration (μg/ml)	Recovery (mean ± S.D., n = 3) (%)	
	LTG N-glucuronide	LTG
0.25	81.3 ± 15.5	71.3 ± 4.5
0.50	75.1 ± 16.2	61.6 ± 7.5
1.00	75.2 ± 7.8	65.3 ± 3.8
2.50	77.6 ± 7.1	70.2 ± 5.3
5.00	83.2 ± 6.6	74.9 ± 6.2
10.0	73.3 ± 3.3[a]	66.8 ± 3.4
20.0	—[b]	57.2 ± 4.0

[a] n = 2.
[b] Not determined.

The analytical method for the quantitation of LTG and LTG N-glucuronide in whole blood was accurate and precise. Table II shows the accuracy for LTG analysis and the within-day, between-day, and total C.V. at each concentration. Table III contains the same information for LTG N-glucuronide. All of the total C.V.s were below 10% except for the lowest concentration of LTG N-glucuronide, and within-day and between-day C.V.s were 5.0% or less. The accuracy of the LTG assay ranged from 95 to 106% of the true value, and the accuracy (relative recovery) for the measurement of the glucuronide metabolite ranged from 98 to 102%.

Table IV lists the capacity factors (k') for a variety of antiepileptic drugs, their metabolites, and internal standards which could possibly interfere with the whole blood assay. The void volume time was determined with uracil, and detection of the compounds was done at 220 nm in order to visualize all compounds (several of the compounds have poor absorbances at 277 nm). The majority of other antiepileptic drugs, metabolites, and internal standards have low k' values ($k' < 2.0$) compared to the LTG components ($k' > 2.0$). Several benzodiazepines elute with k' values between 2 and 10, although none of these compounds directly interfere with the quantitation of LTG-glucuronide or LTG.

Fig. 3A and B show chromatograms of a blank urine sample extract and an extracted urine sample from a guinea pig treated with LTG, respectively. Also shown in Fig. 3B is an unidentified metabolite at a retention time of 7.8 min. Representative chromatograms for the analysis of LTG N-glucuronide in urine are presented in Fig. 4. LTG N-glucuronide was analyzed by an external standard method and had a retention time of 9.1 min. Table V lists the total variance and C.V. values for each concentration of LTG and LTG N-glucuronide. Both assays have total C.V. values below 5% except for LTG at 1.0 μg/ml which had a C.V. value of 7.5%.

DISCUSSION

A major obstacle in the simultaneous determination of a drug and its glucuronide conjugate is the difficulty in the extraction of polar glucuronides from aqueous matrices, such as blood and urine. The parent compound (aglycone) is generally non-polar and easily extractable into organic solvents as is the case with LTG. The glucuronide conjugate of LTG contains a carboxylic acid moiety as well as a quaternary ammonium functionality and is extremely polar. To overcome this problem, glucuronide conjugates are often quantitated indirectly by differential enzymatic or chemical hydrolysis to the aglycone. Many glucuronides may be directly extracted at low pH with ethyl acetate or by solid-phase extraction on C_{18} columns [18]. Another approach is to form an ion pair with an ionizable group (usually the carboxylate anion of the glucuronic acid moiety), followed by either liquid–liquid or solid-phase extraction. To extract the quaternary N-glucuronide of LTG, we employed SDS as the ion-pairing agent for solid-phase extraction.

TABLE II

TOTAL VARIABILITY AND THE BETWEEN-DAY AND WITHIN-DAY VARIATION FOR LTG IN THE SIMULTANEOUS WHOLE BLOOD ASSAY

Concentration of LTG (μg/ml)	n	Mean measured concentration	Accuracy (%)	S_{wd}[a] (μg/ml)	$C.V._{wd}$[b] (%)	S_b[a] (μg/ml)	$C.V._b$[b] (%)	S_t[a] (μg/ml)	$C.V._t$[b] (%)
0.25	10	0.256	102.4	0.0127	5.0	0.0044	1.7	0.0135	5.3
0.50	15	0.476	95.2	0.0316	6.6	0.0316	6.6	0.0447	9.4
1.00	17	0.983	98.3	0.0447	4.6	0.0224	2.3	0.0453	4.6
2.50	6	2.64	105.8	0.0548	2.1	0.1230	4.6	0.1340	5.1
5.00	16	5.09	101.7	0.1760	3.5	0.2130	4.2	0.2770	5.4
10.0	15	10.03	100.3	0.5630	5.6	0.1160	1.2	0.5570	5.7
20.0	14	19.76	99.8	1.22	6.2	0.1110	5.6	1.23	6.2

[a] Standard deviation: S_{wd} = within-day; S_b = between-day; S_t = total.

[b] Coefficient of variation: $C.V._{wd}$ = within-day; $C.V._b$ = between-day; $C.V._t$ = total.

TABLE III

TOTAL VARIABILITY AND THE BETWEEN-DAY AND WITHIN-DAY VARIANCE FOR LTG N-GLUCURONIDE IN THE SIMULTANEOUS WHOLE BLOOD ASSAY

Concentration of LTG N-glucuronide (μg/ml)	n	Mean measured concentration (μg/ml)	Accuracy (%)	S_{wd}[a] (μg/ml)	C.V.$_{wd}$[b] (%)	S_b[a] (μg/ml)	C.V.$_b$[b] (%)	S_t[a] (μg/ml)	C.V.$_t$[b] (%)
0.25	11	0.245	98.0	0.0122	5.0	0.0304	12.4	0.0327	13.4
0.50	11	0.502	100.4	0.0161	3.2	0.0192	3.8	0.0251	5.0
1.00	12	0.987	98.7	0.0176	1.8	0.0186	1.9	0.0256	2.6
2.50	12	2.44	97.6	0.0316	1.3	0.0806	3.3	0.0866	3.6
5.00	11	5.07	101.5	0.0548	1.1	0.0242	0.44	0.0592	1.2
10.0	4	9.94	99.4	0.1100	1.1	0.0113	0.11	0.1100	1.1

[a] Standard deviation: S_{wd} = within-day; S_b = between-day; S_t = total.
[b] Coefficient of variation: C.V.$_{wd}$ = within-day; C.V.$_b$ = between-day; C.V.$_t$ = total.

TABLE IV

HPLC CAPACITY FACTORS (k') FOR LTG, LTG N-GLUCURONIDE, AND OTHER COMMONLY EMPLOYED ANTIEPILEPTIC DRUGS AND INTERNAL STANDARDS

Compound	k'	Compound	k'
Phenytoin-3,4-dihydrodiol	0.23	Tolybarbital	1.56
p-Hydroxyphenylfelbamate	0.25	Phenytoin	2.00
2-Hydroxyfelbamate	0.29	Carbamazepine	2.03
p-Hydroxyphenobarbital	0.38	LTG N-glucuronide	2.30
Carbamazepine-10,11-*trans*-diol	0.40	Cyheptamide	2.52
Felbamate monocarbamate	0.48	Oxazepam	2.84
Ethosuximide	0.50	Lorazepam	3.18
Felbamate	0.63	Temazepam	4.13
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	0.84	Internal standard for LTG	5.10
Phenobarbital	0.94	Desmethyldiazepam	8.86
5-(4-Methylphenyl)-5-phenylhydantoin	0.94	Lamotrigine	9.12
Carbamazepine-10,11-epoxide	1.22	Diazepam	11.64
Internal standard for felbamate	1.30		

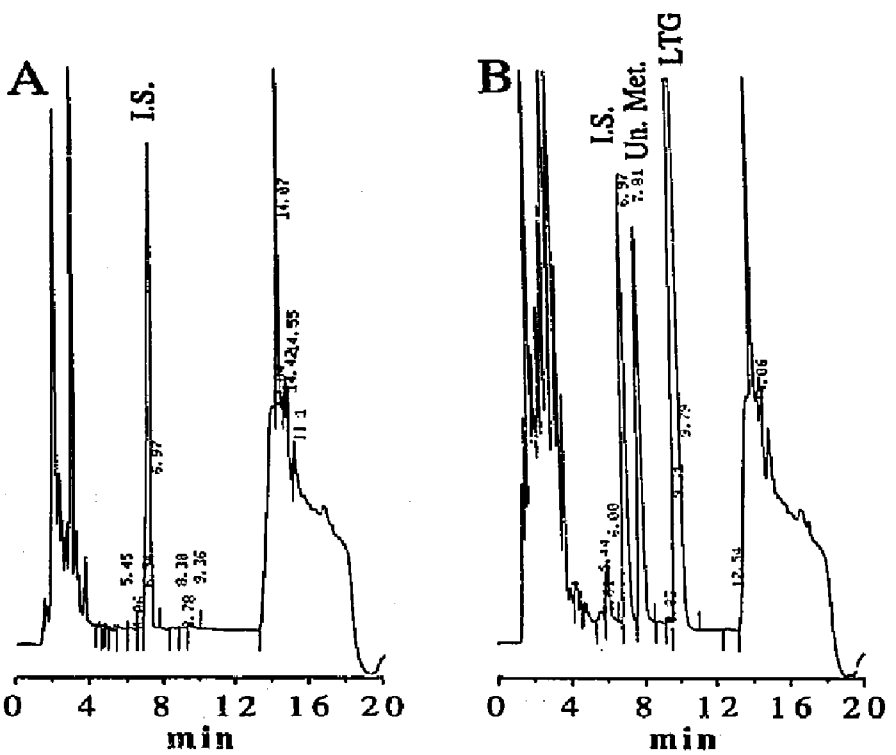

Fig. 3. HPLC from LTG urine assay. (A) Extracted blank guinea pig urine sample; (B) extracted urine sample (0–12 h) from a guinea pig that received LTG (10 mg/kg, intravenously). Measured LTG concentration was 2.1 μg/ml.

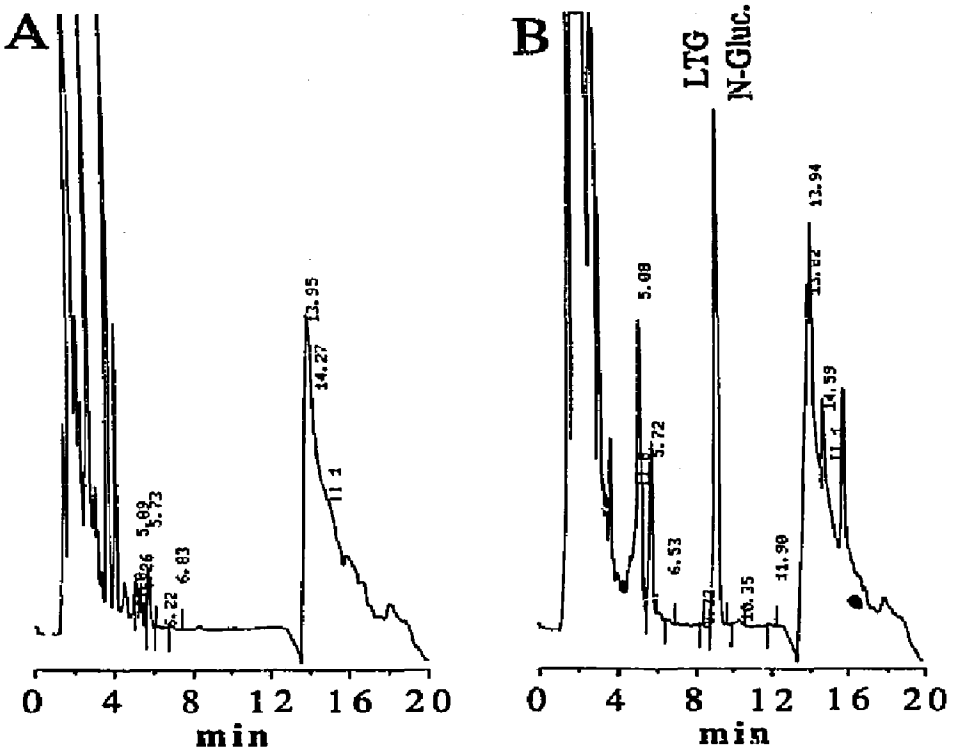

Fig. 4. HPLC from the LTG N-glucuronide urine assay. (A) Diluted (1:4, v/v) blank guinea pig urine sample; (B) diluted urine sample from a guinea pig that received LTG (10 mg/kg, intravenously, 0–12 h sample). Measured LTG N-glucuronide concentration was 211 μg/ml.

TABLE V

TOTAL VARIABILITY OF LTG AND LTG N-GLUCURONIDE IN THE URINE ASSAYS

Concentration (μg/ml)	Mean measured concentration (μg/ml)	S_t[a] (μg/ml)	C.V.$_t$[b] (%)
LTG			
0.10	0.111	0.0049	4.6
0.25	0.247	0.010[d]	4.4
0.50	0.509	0.0059	1.7
1.00	0.988	0.0590	7.5
5.00	5.065	0.1400	4.2
10.0	9.886	0.1980	0.02
LTG N-glucuronide			
0.50	0.493	0.0042	2.8
1.00	0.980	0.0359	4.2
5.00	5.01	0.0071	0.65
10.0	10.16	0.2560	2.6
25.0	25.55	0.6100	2.4
50.0	49.69	0.3300	0.71

[a] Total standard deviation.
[b] Total coefficient of variation.

At the low pH environment created by the phosphoric acid buffer the ion-pairing reagent (SDS) forms at tight ion pair with the positively charged amino groups at the 3 and/or 5 positions of the triazine ring. In the case of LTG N-glucuronide, an ion pair is formed between the quaternary nitrogen and SDS, assuming that formation of the ion pair is not sterically hindered. This allows for the retention of the quaternary N-glucuronide on the the solid-phase column eliminating the interference from polar endogenous compounds and allows for a concentration step in the procedure.

The whole blood extraction recovery of the glucuronide in spiked samples ranged from 73 to 83%, and the extraction recovery for LTG in spiked samples ranged from 57 to 75%. These extraction recoveries were high considering the initial precipitation and centrifugation of the whole blood sample (pellet contains proteins and lysed red blood cells) results in a net loss of 10–20% of the sample.

The whole blood assay was determined to be both reliable and sensitive. Fig. 5 illustrates a time course of LTG and LTG N-glucuronide blood levels after a 10 mg/kg intravenous dose of LTG in a guinea pig measured with the simultaneous blood assay. The predicted therapeutic blood concentration of LTG in humans is 1–3 μg/ml [19], a range that was bracketed by the LTG standard curve concentrations. The total C.V. values for all LTG concentrations were below 10%. The total C.V. values for LTG N-glucuronide were all below 5% except at the lowest concentration. The estimated lower limits of quantitation for LTG and LTG N-glucuronide were 0.10 and 0.05 μg/ml, respectively, with an approximate lower limit of detection of 0.05 and 0.025 μg/ml, respectively. These values are comparable to the values reported for the normal-phase assay [10]. Biddlecombe *et al.* [20] have recently validated a radioimmunoassay for the determination of LTG in human plasma. This procedure was extremely sensitive, with a lower limit of quantitation of 20 ng/ml and C.V.s ranging from 4 to 6%. Due a small linear range, extensive dilution of plasma samples was necessary. Cross-reactivity with LTG N-glucuronide was only 0.52%, so the assay would be specific for the measurement of LTG in plasma, but specificity may be a problem for measurement of LTG in urine.

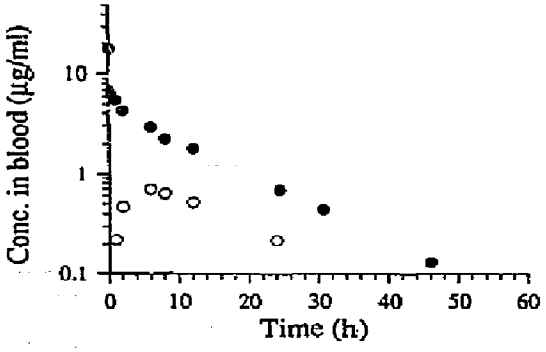

Fig. 5. Concentrations of LTG (●) and LTG N-glucuronide (○) in whole blood from a guinea pig after administration of a bolus dose of LTG (10 mg/kg, intravenously).

A simultaneous assay for LTG and LTG N-glucuronide in urine was precluded by the large difference in concentrations found in guinea pig urine. The LTG concentrations normally found in guinea pig urine after a single dose were quite low [5], and each sample required a concentration step in order to quantitate the level of LTG. On the other hand, LTG N-glucuronide concentrations in guinea pig urine were large, and dilution of these samples was required prior to analysis. Anticonvulsant drugs are generally given chronically and patients are maintained at a steady state level of the anticonvulsant. Therefore, at steady state, the concentrations of both LTG and LTG N-glucuronide excreted in the urine will be higher than the concentrations at late times after a single dose. The assay validation of both LTG and LTG N-glucuronide indicated that the higher concentrations of parent drug and metabolite in urine after administration of a constant intravenous infusion of LTG could be measured by a simultaneous assay. The C.V. values for the urine assay of both compounds were generally below 5% and were less than 1% at the highest concentrations determined for each compound.

Several reports of the direct quantitation of glucuronides by HPLC either by employing and ion-pairing reagent or low-pH buffers to protonate the carboxylic acid moiety of the glucuronic acid have been cited [21–25]. Ion-pairing reagents, such as tetramethyl ammonium chloride or tetrabutyl ammonium hydrogen sulfate have been employed to ion-pair the carboxylic functionality of the glucuronide in order to directly quantitate phenolic glucuronides [21,22]. Recently, Liu *et al.* [18] utilized a long-chain quaternary ammonium compound, cethexonium bromide for the ion-pairing chromatography of a wide variety of glucuronides. Morphine-3-O-glucuronide has been directly quantitated by forming a tertiary amine with a low-pH phosphate buffer and ion-pairing the positive charge with SDS [23]. As in the case of morphine-O-glucuronide, we also utilized a low-pH phosphate buffer and SDS as an ion-pairing agent to allow for the selective retention of the quaternary N-glucuronide of LTG on the reversed-phase column and reduce the interference from polar endogenous compounds. Also, the whole blood assay allows for the selective quantitation of LTG and its major metabolite from other common antiepileptic drugs and their metabolites. To our knowledge, this report describes the first simultaneous assay for the quantitation of a quaternary N-glucuronide and its aglycone. This same assay system has been applied to the quantitation of LTG N-glucuronide in guinea pig microsomes, guinea pig and human urine, human blood, as well as human brain tissue [5,26].

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