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Liposomal lurtotecan (NX211): determination of total drug levels in human plasma and urine by reversed-phase high-performance liquid chromatography☆

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Abstract

Lurtotecan (GI147211; LRT) is a semisynthetic and water-soluble analogue of the topoisomerase I inhibitor camptothecin. To determine whether the therapeutic efficacy of LRT in patients could be improved, the drug was encapsulated in liposomes (NX211; Gilead Sciences). In order to allow accurate description of the pharmacokinetic behavior of NX211 in cancer patients, we have developed sensitive RP-HPLC assays with fluorescence detection (λ_{ex}=378 nm; λ_{em}=420 nm) for the determination of total LRT levels in human plasma and urine. Sample pretreatment involved deproteinization with 10% (w/v) aqueous perchloric acid–acetonitrile (2:1, v/v), and chromatographic separations were achieved on an Inertsil-ODS 80A analytical column. The lower limit of quantitation (LLQ) was established at 1.00 ng/ml in plasma (200-μl sample) and at 100 ng/ml in urine (200 μl of 40-fold diluted sample). The within-run and between-run precisions were <7.5%. LRT concentrations in urine of <100 ng/ml were determined by a modified procedure comprising a single solvent extraction with *n*-butanol–diethyl ether (3:4, v/v). In this assay, the fluorescence signal of LRT was increased 14-fold prior to detection by post-column exposure to UV light (254 nm) in a photochemical reaction unit. The LLQ of this assay was 0.500 ng/ml (150-μl sample) and the within-run and between-run precisions were <10%. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Lurtotecan

1. Introduction

Lurtotecan (7-(4-methylpiperazinomethylene)-10,11 -ethylenedioxy-20(*S*)-camptothecin; also known as GI147211; LRT) (Fig. 1) is a novel semisynthetic analogue of camptothecin, a cytotoxic plant alkaloid that was first extracted from the wood and

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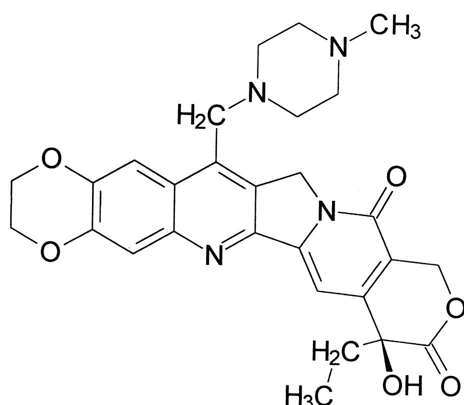

Fig. 1. Chemical structure of lurtotecan (LRT).

bark of the oriental tree, *Camptotheca acuminata* [1]. The mechanism of action of camptothecin derivatives is based on stabilization of the cleavable complex formed by the intranuclear enzyme topoisomerase I and DNA, and on induction of single-stranded DNA breaks [2]. LRT has previously been shown to have significant activity in both in vitro cytotoxicity assays and in vivo tumor model systems [3–5], and was recently introduced into clinical trials [6–8].

Clinical pharmacokinetic studies of camptothecin derivatives, including LRT, are complicated by a chemical, pH-dependent instability of the α-hydroxy-δ-lactone moiety in the core structure of the compounds, generating a ring-opened carboxylate form. This lactone functionality undergoes rapid hydrolysis in aqueous solution under physiological conditions, i.e. at pH 7 or above, and results in a virtually complete loss of biological activity [9]. In recent years, considerable effort has been put into the development of alternative formulations that would allow prolonged systemic exposure to the pharmacologically active drug form. One of these approaches is the incorporation of the lactone forms of camptothecins in liposomal particles. Indeed, recent preclinical studies indicated that liposomal encapsulation of the topoisomerase I inhibitors topotecan [10,11], camptothecin [12,13] and irinotecan [14,15] proved to be very efficient against lactone ring opening, increased antitumor activity in experimental tumor models and dramatically enhanced tissue distribution and the systemic availability. Efficacy studies performed in nude mice bearing human colon and head and neck tumor xenografts have also indicated improved therapeutic efficacy for a new liposomal formulation of LRT (NX211; NeXstar Pharmaceuticals) as compared to nonliposomal LRT [16].

Based on these favorable results, we recently started a project to study the safety profile and clinical pharmacokinetics of NX211 in patients with advanced solid tumors. In the context of this study, we have now developed sensitive RP-HPLC methods with fluorescence detection using a sample clean-up procedure that disrupts the liposomes, thus enabling determination of total drug levels in plasma and urine samples following NX211 administration. The methods have been validated in terms of sensitivity, accuracy and precision [17], and have been used in a pharmacokinetic experiment in a patient to investigate their applicability in vivo.

2. Experimental

2.1. Chemicals and reagents

LRT dihydrochloride monohydrate (lot: U2044/164/1, containing 78.11% of the free base) and NX211 (liposomal LRT, lot: 181801F, containing 0.49 mg LRT as free base/ml) were delivered by Gilead Sciences (San Dimas, CA, USA). The internal standard (I.S.) 6,7-dimethoxy-4-methylcoumarin (lot 79F3652) was obtained from Sigma (St. Louis, MO, USA). Dimethylsulfoxide (DMSO), diethyl ether, *n*-butanol, methanol and acetonitrile were purchased from Rathburn (Walkerburn, UK). Perchloric acid (70–72%, v/v, in water), neat acetic acid and sodium hydroxide were supplied by Baker (Deventer, The Netherlands). Ammonium acetate was delivered by Roth (Karlsruhe, Germany) and sodium chloride by Merck (Darmstadt, Germany). All water used in the study was filtered and deionized with a Milli-Q-UF system (Millipore, Milford, MA, USA). Drug-free human plasma for the construction of calibration curves and quality control (QC) samples originated from the Central Labora-

tory of the Blood Transfusion Service (Amsterdam, The Netherlands).

2.2. Stock solutions

Stock solutions of LRT were made in triplicate by dissolving X mg LRT in ($X \times 0.7811$) ml DMSO, resulting in a solution containing 1.00 mg/ml LRT (free base). The working stock solution of LRT, containing 0.100 mg/ml free base, was prepared by a 10-fold dilution of the stock solution in DMSO. A stock solution of the I.S. at a concentration of 1 mg/ml was prepared by dissolving 50 mg I.S. in 50 ml DMSO.

2.3. Standards for total LRT in plasma and urine

Spiked plasma samples used as calibration standards were prepared daily in duplicate by addition of 10 μl of serial dilutions in methanol–water (1:1, v/v) from the working solution of LRT to 240 μl of drug-free human plasma. This resulted in calibration standards of 1.00, 2.50, 5.00, 10.0, 25.0, 50.0 and 100 ng/ml LRT (free base) in plasma. Four pools of QC samples for LRT were prepared in human plasma at concentrations of 4.00, 20.0, 75.0 and 750 ng/ml, by addition of the appropriate volume of the LRT working solution or dilutions in 10 mM aqueous sodium hydroxide–methanol (1:1, v/v) (to shift the equilibrium to the carboxylate form) to human plasma. The QC containing 750 ng/ml LRT was used to investigate the suitability of small-volume (20 μl) injections. Lower limit of quantitation (LLQ) samples in plasma were prepared daily in separate blank plasma samples obtained from five healthy volunteers at a concentration of 1.00 ng/ml. To minimize a potential difference with clinical samples, two pools of recovery control (RC) samples containing 20.0 and 750 ng/ml NX211 were also prepared by addition of an aliquot of the NX211 stock solution (in phosphate-buffered saline) to human plasma. Two pools of QC samples were prepared in human urine at concentrations of 250 and 2500 ng/ml, which were diluted 40-fold in blank human plasma prior to extraction. The LLQ samples for urine were prepared daily at a spiked concentration of 100 ng/ml in quintuplicate using five different drug-free urine samples.

2.4. Standards of LRT in the sensitive urine assay

Calibration standards in urine were also prepared daily in duplicate by addition of 10 μl of serial dilutions of LRT in methanol–water (1:1, v/v) to 240 μl blank human urine, at final concentrations of the free base of 0.500, 1.00, 2.50, 5.00 and 10.0 ng/ml. Three pools of QC samples were prepared at concentrations of 1.25, 7.50 and 250 ng/ml by addition of appropriate volumes of a dilution of the LRT working stock solution [in 10 mM aqueous sodium hydroxide–methanol (1:1, v/v)]. The QC sample containing 250 ng/ml, identical to that used for the assay of total LRT in plasma and urine, was diluted 10-fold in blank urine before extraction, and was further used to show the applicability of low volume injections (10 μl). The LLQ samples were prepared daily at a concentration of 0.500 ng/ml, again in quintuplicate using five different drug-free urine samples.

2.5. HPLC instrumentation and conditions

The HPLC systems consisted of constaMetric 3200 and 4100 solvent delivery systems (LDC Analytical, Riviera Beach, FL, USA), Waters 717plus autosampling devices (Milford, MA, USA), a beam boost photochemical reaction unit supplied with a coil of 25 m×0.3 mm I.D. (ICT-ASS-Chem, Bad Homburg, Germany), and Jasco 821-FP and FP-920 fluorescence detectors (Jasco, Maarssen, The Netherlands). Separations were achieved on a stainless steel analytical column (150×4.6 mm I.D.) packed with Inertsil ODS-80A material (5 μm particle size), delivered by Alltech Applied Science (Breda, The Netherlands). The mobile phase was identical in both assays and was composed of 1.0 M aqueous ammonium acetate (pH 5.5)–water–acetonitrile (10:72.5:17.5, v/v/v) with the pH adjusted to 5.5 (acetic acid). The analytical columns were maintained at 60°C using a model SpH99 column oven (Spark Holland, Meppel, The Netherlands). A detailed composition of the various HPLC systems used for the two assays is provided in Table 1. Peak

Table 1
Composition of the two HPLC systems

Equipment	Sensitive urine	Total plasma/urine
Pump 4100	–	Flow rate: 1.25 ml/min
Pump 3200	Flow-rate: 0.75 ml/min	–
Autosampler 717p	Yes	Yes
Column oven SpH99	T=60°C	T=60°C
Column ODS-80A	Yes	Yes
Beam boost	Lamp: λ=254 nm Coil: 25 m× 0.3 mm I.D.	–
Detector FP-920	λ_{ex}=378 nm λ_{em}=420 nm Em band: 40 nm	–
Detector 821-FP	–	λ_{ex}=378 nm λ_{em}=420 nm Em band: 30 nm

recording and integration were performed with the CHROM-CARD data analysis system (Fisons, Milan, Italy). All calibration curves were fitted by weighted $(1/x)$ least-squares linear regression analysis using the peak height ratios of LRT and the I.S. versus the nominal concentrations of the standards.

2.6. Sample treatment for total LRT in plasma and urine

Samples were prepared by addition of 100 μl I.S. solution [100 ng/ml in 10% perchloric acid–acetonitrile (2:1, v/v)] to 200 μl human plasma, or 40-fold diluted urine in plasma, in a 1.5-ml polypropylene vial (Eppendorf, Hamburg, Germany). Samples were mixed vigorously for 30 min on a multitube vortex mixer, followed by centrifugation at 23 000 *g* (5 min) at ambient temperature. A 250-μl volume of the clear supernatant was transferred to a low volume glass insert, from which 200 μl (or 20 μl in case of QC and RC samples containing 750 ng/ml LRT and NX211, respectively) were injected into the HPLC system.

2.7. Sample treatment for LRT in the sensitive urine assay

A 100-μl volume of I.S. solution (50 ng/ml in 25 m*M* aqueous ammonium acetate, pH 3.0) was added to a 12-ml glass tube supplied with a PTFE-covered screwcap containing 150 μl urine. After incubation for 30 min at room temperature, 0.8 g solid sodium chloride was added, followed by extraction with 2 ml *n*-butanol–diethyl ether (3:4, v/v) by vigorous vortex mixing for 5 min. Subsequently, the sample was centrifuged at 4000 *g* (5 min), followed by collection of 1 ml upper organic layer, which was evaporated to dryness under a gentle stream of nitrogen at 70°C for a period of 45 min. The dried residue was redissolved in 150 μl 25 m*M* aqueous ammonium acetate (pH 3.0) and transferred to a glass insert. A volume of 50 μl (or 10 μl for the QC sample containing 250 ng/ml LRT) was injected into the HPLC system.

2.8. Validation

Validation runs of LRT in plasma and urine and of LRT in the sensitive urine assay included a set of calibration samples assayed in duplicate, and LLQ and QC samples in quintuplicate, and was performed on four separate occasions. Precisions were calculated by one-way analysis of variance (ANOVA) for each test concentration, using the run-day as the classification variable. The accuracy of at least 80% of the samples assayed at each concentration should be in the range of 80–120%. The within-run (WRP) and between-run precisions (BRP) should be $<$20% at the concentration of the LLQ and $<$15% at the concentrations of the QC samples and the average accuracy (ACC) should be within 85–115% for each concentration, including the LLQ.

The extraction recoveries of LRT and the I.S. in the assay for total LRT in plasma and urine were calculated by comparing peak heights obtained from a sample containing 25.0 ng/ml LRT in phosphate buffered saline that was extracted (as described for plasma), to those obtained in extracted QC samples prepared in the biological matrix. The extraction recoveries in the sensitive urine assay were calculated by comparing observed peak heights of the processed urine samples of the calibration curves to peak heights obtained from spiked samples containing 1.00 ng/ml LRT and 10.0 ng/ml I.S. in 25 m*M* aqueous ammonium acetate (pH 3.0).

The stability of LRT and the I.S. in plasma and urine was established (i) during three consecutive freeze–thaw cycles, in which the samples were kept at room temperature for 30 min after thawing, and (ii) during an overnight incubation at 37°C. The concentrations used were 20.0 and 75.0 ng/ml for plasma and 250 and 2500 ng/ml for urine, and were analyzed using the assay for the determination of total LRT in plasma and urine.

The selectivity of the assays was tested by the degree of separation of the compounds of interest and possible other chromatographic peaks caused by endogenous components and/or potentially coadministered drugs. The interference from endogenous material for LRT and the I.S. in human plasma and urine was determined by visual inspection of HPLC profiles of five processed blank plasma and urine samples obtained from five healthy volunteers. Interference from potentially coadministered drugs was tested at a spiked concentration of 10 μg/ml in a blank plasma extract for the assay of total LRT in plasma and urine, and at 10 μg/ml in 25 m*M* aqueous ammonium acetate (pH 3.0) for LRT in the sensitive urine assay. The tested compounds included acetaminophen, alizapride, codeine, dexamethasone, domperidon, metoclopramide, morphine, leucovorin, lorazepam, paroxetine and ranitidine.

3. Results and discussion

In approaching the present analytical procedures, we used our own previous RP-HPLC procedure for the quantitative determination of total nonliposomal LRT (lactone plus carboxylate forms) in human whole blood as a starting point [18]. Because of the pH-dependent instability of the α-hydroxy-δ-lactone moiety in the core structure of LRT, resulting in the ring-opened carboxylate form at high pH and the ring-closed lactone form at low pH, we decided to focus only on measurement of total concentrations in the present study, since disruption of liposomes while maintaining the physiologic lactone to carboxylate ratio may not be feasible. The choice of the I.S., 6,7-dimethoxy-4-methylcoumarin, and the optimal fluorescence wavelength couple of LRT (378/420 nm) was based on earlier work described for determination of LRT in human blood and dog plasma by Selinger et al. [19] and Stafford and St. Claire [20], respectively.

For the purpose of assay validation, all QC samples were prepared with the carboxylate form of LRT, to ensure a quantitative conversion to the lactone species of the total amount of LRT, prior to measurement, present in plasma and urine of clinical samples.

3.1. Assay of total LRT in plasma and urine

Initially, the assay of total LRT in plasma and urine was validated with only LLQ and QC samples of nonliposomal LRT (free base) in plasma and urine, using an extraction time of 15 min. The calibration curves were linear in the range of 1.00–100 ng/ml with Pearson's regression correlation coefficients ranging from 0.9986 to 0.9997, by using weighted $(1/x)$ linear least-squares regression analysis. The retention times of LRT and the I.S. were 11 and 15 min, respectively, with an overall run time of 20 min.

One of the tested blank plasma sample specimens showed a minor (unknown) interfering peak in the chromatogram for LRT, and was replaced by a new blank plasma obtained from a healthy volunteer to enable accurate determination of the LLQ. No interfering peaks with retention times around the I.S. were found in the tested blank plasma samples. In the five (40-fold diluted) blank human urine samples, no interfering peaks were found for LRT; however, all tested urine samples showed a small peak with the same retention time as the I.S., but this interference did not significantly alter the observed data. The tested drugs potentially coadministered with

NX211 did not interfere with the analytes of interest. Some minor peaks were found with retention times of 1–3 min, causing no problem for the determination of LRT in plasma and urine.

The LLQ was established in plasma at 1.00 ng/ml and in urine at 100 ng/ml, with 95% of the LLQ samples falling within the acceptable accuracy range of 80–120% [17]. The within- and between-run precisions at the five tested concentrations in plasma, including the LLQ, were <7.5% and <4.1%, respectively, with an accuracy range of 96–110% (Table 2). The within- and between-run precisions in urine at the three tested concentrations were <6.7% and <4.4%, respectively, with an accuracy range of 97–108% (Table 2). The extraction recoveries, estimated by comparing peak heights obtained by direct injection of standard solutions containing 25.0 ng/ml LRT and I.S. in blank plasma extracts to those obtained in extracted plasma samples of the calibration curves, were 89±8.2% (mean±standard deviation) and 67±4.8% for LRT and the I.S., respectively. No loss of LRT was estimated at the tested concentrations after three freeze–thaw cycles or during overnight incubation of the samples at 37°C (data not shown).

Using an extraction time of 15 min, we noted that the extraction recovery of plasma samples containing NX211 (liposomal LRT) was approximately 10% lower for LRT as compared to plasma samples spiked with nonliposomal LRT (free base). The extraction efficiency of the samples containing NX211 was eventually increased by extending the vortex-mixing time to 30 min, at which maximum recovery was reached (data not shown). The assays of total LRT in plasma and urine were revalidated during three analytical runs (with a 30-min mixing time during sample extraction), and the calibration curves were assayed in duplicate with the four QC samples of LRT and two RC samples containing NX211 spiked at concentrations of 20.0 and 750 ng/ml, both in plasma, in triplicate. The Pearson's regression correlation coefficients in the revalidation runs ranged from 0.9995 to 0.9998, and the ranges of the within- and between-run precisions of the QC samples containing LRT were 1.8–3.6% and 0.73–2.5%, respectively, with an overall accuracy between 99 and 104%. The within-run precisions of the RC samples were 1.9 and 2.5%, respectively, for the samples containing 20.0 and 750 ng/ml, whereas the respective between-run precisions were 2.3 and 7.3%. The extraction recoveries of LRT in the QC and RC samples containing 20.0 ng/ml LRT and NX211 were 90±2.1% and 85±3.9%, respectively, and 83±3.0% (LRT) and 82±3.0% (NX211) for the samples containing 750 ng/ml.

Table 2
Validation characteristics of total LRT in plasma and urine

Matrix	Nom. conc. (ng/ml)	Mean (ng/ml)	WRP[c] (%)	BRP[c] (%)	ACC[c] (%)
Plasma	1.00[a]	0.959	7.5	4.1	96
	4.00	3.84	7.4	[b]	96
	20.0	22.0	2.7	1.0	110
	75.0	81.4	2.3	2.1	109
	750	789	2.8	2.4	105
Urine	100[a]	96.8	6.7	4.4	97
	250	269	3.0	0.12	108
	2500	2525	4.3	2.1	101

[a] Lower limit of quantitation sample.
[b] No additional variation was observed as a result of performing the assay in different runs.
[c] Abbreviations: WRP, within-run precision; BRP, between-run precision; ACC, average accuracy.

Representative RP-HPLC chromatograms derived from a blank human plasma pool and a plasma sample spiked to contain 10.0 ng/ml LRT (free base) are shown in Fig. 2A and B. An additional chromatographic peak was found in the RC samples containing NX211 with a retention time of ≈48 min. This peak was later identified as a photochemical degradation product of NX211 by comparison of the compound's chromatographic behavior on the RP-HPLC column and spectroscopic properties with a pure reference standard. Isolation, purification and structural identification, in addition to the role of this compound in the overall drug disposition will be described separately.

3.2. Assay for the sensitive determination of LRT in urine

Previous studies have shown that renal clearance of LRT in patients treated with nonliposomal drug was low, with ≈10–14% of the delivered dose excreted as unchanged parent drug in urine [6]. In order to allow determination of low concentrations of LRT in urine, which can be anticipated following

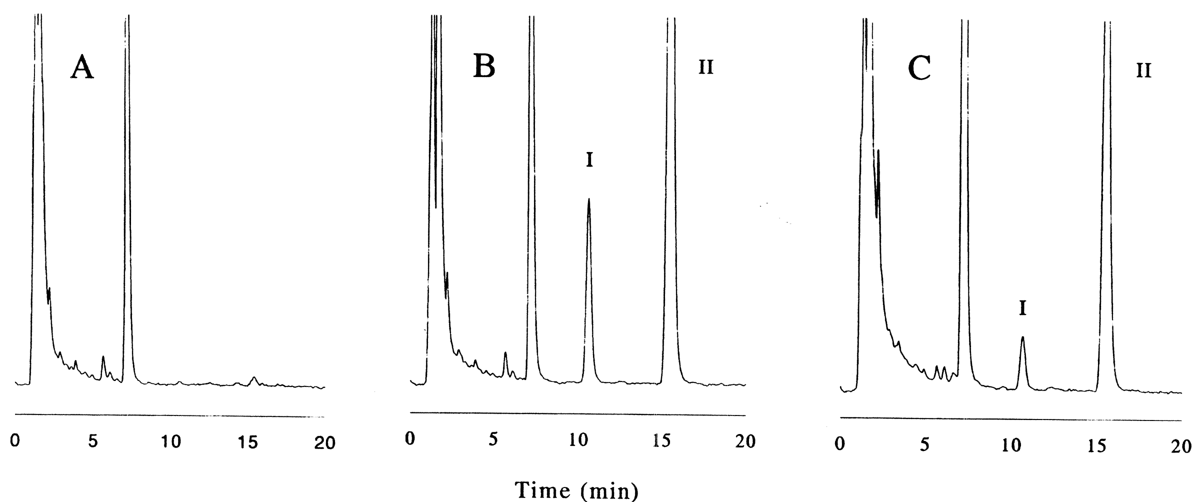

Fig. 2. Chromatograms of a blank human plasma sample (A), a plasma sample spiked with 10.0 ng/ml LRT free base (B) and a plasma sample obtained from a patient 8 h after administration of NX211 at a dose level of 0.8 mg (C). Peaks labeled I and II correspond to LRT and the I.S., respectively.

treatment with NX211 at low dosages, an assay was also required with increased sensitivity as compared to the assay described for total LRT concentrations in plasma and urine with an LLQ (for urine) of 100 ng/ml (see above). The sensitivity of this assay could theoretically be improved by decreasing the dilution factor used for urine samples prior to extraction. However, this will likely result in substantially prolonged run times in order to get sufficient separation between the peaks of interest (i.e. LRT and the I.S.) and those resulting from the presence of endogenous material, which would in turn compromise assay sensitivity.

Eventually, the assay sensitivity could be significantly improved, in part, by increasing the fluorescence intensity of LRT through a modification of the detection procedure. LRT is known to be slightly light sensitive [19] and since photochemical reactor units in combination with HPLC has been described for a wide variety of other compounds [21–25], where increased detector signal outputs have been described from 2- to 80-fold, we have evaluated the impact of post-column photodegradation on the fluorescence activity of LRT. Post-column exposure of LRT to UV light (254 nm) results in a loss of the piperazinomethylene moiety on C7 of the LRT molecule, as determined by electrospray ion-trap mass spectrometry [m/z=409 (LRT-C7 side chain)]. The influence of the photochemical reaction unit on the fluorescence of LRT was estimated by injections of 50 μl of 5 ng/ml LRT in 25 mM ammonium acetate (pH 3.0) onto the HPLC system as described for this assay. The flow-rate was varied from 0.50 to 2.00 ml/min, resulting in irradiation times of 300–75 s. At each flow-rate, two injections were performed, one with the lamp of the photochemical reaction unit on and another one with the lamp switched off. The ratios of the peak heights obtained with the lamp on and off were calculated. As displayed in Fig. 3, the fluorescence intensity of LRT increased 9 to 15-fold depending on the flow-rate used (0.50–2.00 ml/min). The use of a flow-rate set at 0.75 ml/min (i.e. an irradiation time of 200 s) resulted in a 14-fold increased fluorescence signal of LRT, and was associated with an acceptable total run time (35 min) with retention times of 19 and 24 min for LRT and the I.S., respectively.

Fig. 4 shows representative chromatograms of a blank human urine sample and a sample spiked with 2.50 ng/ml LRT (free base). The calibration curves of LRT were linear in the range of 0.500–10.0 ng/ml, with Pearson's correlation coefficients ranging from 0.9954 to 0.9994, also using weighted ($1/x$) least-squares linear regression analysis. No analytical interference was found between LRT or the I.S. and any of the tested drugs potentially coadministered

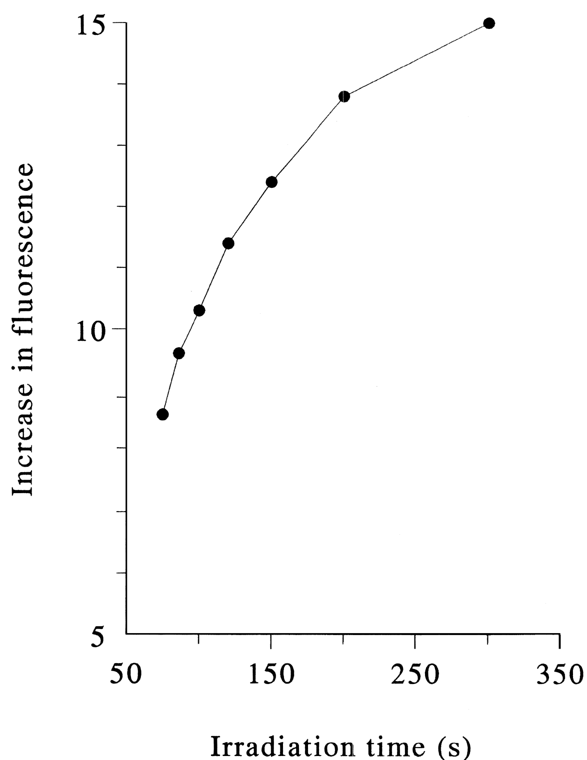

Fig. 3. Influence of the photochemical reaction unit on the fluorescence intensity of LRT.

Table 3
Validation characteristics of total LRT in urine (sensitive assay)

Nom.conc. (ng/ml)	Mean (ng/ml)	WRP[c] (%)	BRP[c] (%)	ACC[c] (%)
0.500[a]	0.539	5.5	[b]	108
1.25	1.31	3.0	3.1	105
7.50	7.27	2.8	6.7	97
250	243	4.2	10	97

[a] Lower limit of quantitation sample.
[b] No additional variation was observed as a result of performing the assay in different runs.
[c] Abbreviations: WRP, within-run precision; BRP, between-run precision; ACC, average accuracy.

with NX211. However, a number of three additional peaks were found with retention times of 6, 7 and 10 min. Since these elute in the big front of the chromatograms of urine and high concentrations of the drugs were spiked these peaks have no impact on the determination of low concentration of LRT in urine. Small peaks with the same retention time as LRT and the I.S. were found in all of the tested blank urine samples, so the LLQ could not be established below a concentration of 0.500 ng/ml, with 80% of all samples in the acceptable range of accuracy. The within- and between-run precisions at the four tested concentrations were <5.5 and $<10\%$, respectively, with the accuracy ranging from 97 to 108% (Table 3). Taking into consideration that only

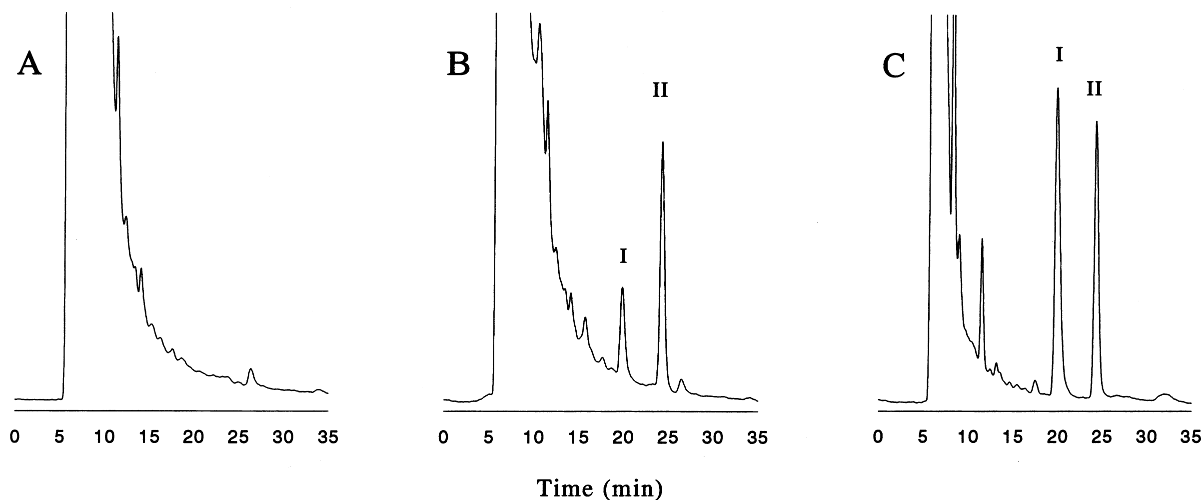

Fig. 4. Chromatograms of a blank human urine sample (A), a urine sample spiked with 2.50 ng/ml LRT free base (B) and a urine sample obtained from a patient, collected 12–24 h after administration of NX211 at a dose level of 0.8 mg (C). Peaks labeled I and II correspond to LRT and the I.S., respectively.

approximately half of the added volume of the organic layer was evaporated, the extraction recoveries were around 80 and 92% for LRT and I.S., respectively.

3.3. Assay application

The suitability of the developed methods for clinical use was demonstrated by the determination of LRT in biological specimens obtained from a patient treated with NX211 at a dose of 0.8 mg. Examples of the patient's sample trace are shown in Fig. 2C (plasma) and Fig. 4C (urine). Distinct peaks were obtained for LRT in both matrices that were well resolved from endogenous components.

4. Conclusion

In conclusion, we have developed and evaluated new liquid chromatographic methods for measuring total LRT levels in human plasma and urine. The primary elements of novelty described in this work are the use of human plasma as biological matrix containing liposomal LRT (NX211) and the utilization of a photochemical reaction unit to increase the native fluorescence intensity of LRT in order to achieve sub-nanogram per milliliter determinations. The methods were shown to meet the current requirements as to validation of bioanalytical methodologies [17], providing excellent precision and accuracy. The described methods permit the analysis of patient samples, and will be implemented in an ongoing clinical trial to investigate the disposition of LRT in cancer patients receiving NX211.

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