

## An on-line semi-automated solid-phase extraction procedure for high-performance liquid chromatographic determination of lonidamine in serum

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### Abstract

A semi-automated solid-phase extraction procedure on-line with gradient elution reversed-phase chromatography permits the determination of lonidamine and its metabolite in human serum. The average recovery from serum at the  $2.5 \mu\text{g ml}^{-1}$  level was  $(92.8 \pm 3.4)\%$ . The limit of quantitation for a  $100 \mu\text{l}$  sample size was  $50 \text{ ng ml}^{-1}$ . The within-day ( $n = 5$ ) and between-day ( $n = 5$ ) relative standard deviations for lonidamine determination in serum samples spiked at the  $2.5 \mu\text{g ml}^{-1}$  level were 2.7% and 4.5%, respectively.

*Keywords:* Solid-phase extraction; Reversed-phase liquid chromatography; UV detection; Lonidamine

### 1. Introduction

Lonidamine (1-[2,4-dichlorobenzyl]-1 *H*-indazole-3-carboxylic acid, LND) is a drug currently under evaluation for the treatment of human neoplasias such as advanced stage solid tumours. The primary intracellular target of this drug are mitochondria where it inhibits electron transfer at the dehydrogenase-coenzyme level when electron carriers are in a relatively oxidised state [1]. Moreover, LND seems to reduce the oxygen consumption in both normal and neoplastic cells, thereby inducing an increased aerobic glycolysis of normal cells and inhibiting that of tumour cells by acting on the mitochondrial hexokinase system, which is generally not found in normal differentiated cells. As a consequence, the growth of neoplastic cells is delayed

and the repair processes inhibited [2–4]. Further, LND seems to alter cytoplasmic membranes [5] owing to a high affinity for the inner leaflet of the lipidic bilayer [6], and to modify their protein distribution, membrane permeability, and cholesterol and phospholipid content [7–9].

The absence of haematological toxicity makes LND of particular interest especially for potential use in combination chemotherapy. In fact, several studies have shown that LND can potentiate the cytotoxic effects of radiation and hyperthermia [10], perhaps by inhibiting the repair of potentially lethal damage, as well as those of several anticancer drugs including cisplatin, cyclophosphamide, mitoxantrone and adriamycin [11–14]. Moreover, LND also shows a cytotoxic synergic action with both alkylating agents and anthracyclines without increasing myelotoxicity or cardiotoxicity, respectively [12,15].

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In order to define the optimal recommended schedule for LND administration as well as to obtain a correct evaluation of its anticancer activity and side effects, analytical methods capable of determining the drug in biological matrices are essential. In addition to a fluorimetric method [16], few chromatographic methods have been developed for LND analysis. Reversed-phase-high-performance liquid chromatography (RP-HPLC), coupled to UV [17] and spectrofluorimetric [18] detection, permits LND determination in both plasma and urine samples. A gas-liquid chromatography/electron capture detection (GLC/ECD) procedure has also been recently reported [19]. All these methods required a liquid-liquid extraction (LLE) step for sample pre-treatment that, apart from possible incomplete analyte recovery, is tedious, time-consuming and difficult to automate. On the contrary, solid-phase extraction (SPE) generally offers higher analyte recoveries and reproducibility, is less time consuming and can be automated [20]. Until now, however, no SPE method has been developed for the extraction of LND from biological samples.

This paper presents a method for LND determination based on the RP-HPLC analysis of serum samples pre-treated by a novel SPE procedure. As will be shown, the SPE described permits the co-extraction of the drug and of an unidentified metabolite. The observed LND recovery and reproducibility were quite similar to those obtained with conventional LLE procedures, but with the advantage of semi-automated multisample processing. Moreover, complete elution of analytes of concern in a few minutes and proper column conditioning were assured using the gradient elution technique.

## 2. Experimental

### 2.1. Chemicals

LND and its analogue 1-[4-chlorobenzyl]-1*H*-indazole-3 carboxylic acid (AF1312/TS), used as internal standard, were generously supplied by Istituto di Ricerca Angelini (Rome, Italy). Stock solutions were prepared in methanol and stored in the dark at 4 °C; under these conditions, analyte solutions were stable for at least 6 months [17]. More dilute solutions were prepared just before use. Methanol and acetonitrile (HPLC grade) were purchased

from Carlo Erba (Milan, Italy). Other chemicals were of analytical grade. The mobile phase was filtered through 0.45 µm filters (Whatman Limited, Maidstone, UK) and vacuum-degassed just before use.

### 2.2. Apparatus

The HPLC apparatus (Varian, Harbor City, USA) consisted of an Advanced Automated Sample Processor (AASP) PrepStation and Station modules coupled to a Model Star 9010 solvent delivery system and a Model 9050 UV-VIS detector. The analytical column was a reversed-phase Varian MicroPak SP-C18-5 (4.5 µm, 150 × 4.0 mm i.d.). A manual injection valve (Rheodyne 7125) equipped with a 10 µl loop was also used. The system was controlled by a Varian LC Star workstation running on Compaq Deskpro 286e.

### 2.3. Chromatographic and detection conditions

A binary gradient composed of acetate buffer (pH 3.5,  $I = 0.1$  M; solvent A) and acetonitrile (solvent B) was used. The gradient program was: 2.5 min isocratic at 40% B; 1 min linear to 60% B; 6.5 min linear to 70% B; 5 min linear to 40% B; equilibration time 10 min. The flow rate was 1 ml min<sup>-1</sup> and the temperature was ambient. The eluent was monitored at 300 nm.

### 2.4. Treatment protocol and sample collection

Blood samples from patients with advanced ovarian cancer under chemotherapy with LND (450 mg per day, three separate 150 mg doses every 4 h) were taken just before and every hour, for 10 h, after the start of LND therapy; a final sample was also taken after 24 h. Samples were centrifuged within 30 min from blood collection at 3000 rpm for 10 min and immediately analysed.

### 2.5. Extraction procedure

Before serum clean-up, AASP C8 microcolumns were activated on the AASP PrepStation module by fluxing 0.5 ml of methanol and 0.5 ml of 0.01 M HCl using a 15 p.s.i. pressure of helium. Serum samples (typically 10 µl, diluted to 1 ml with 0.01 M HCl containing the internal standard) were manually loaded onto microcolumns, flushed under helium pressure

and washed with 0.5 ml of 0.01 M HCl. Finally, the cassette (containing up to ten micro-columns) was loaded onto the AASP Station module where the elution–injection steps were performed on-line by passing the mobile phase through the cartridge for 1 min at 1 ml min<sup>-1</sup>.

### 2.6. Quantitation

Quantitation was performed by the internal standard (IS) method using peak area measurements (arbitrary units, a.u.). Calibration curves were obtained using standards prepared by spiking drug-free sera with 2.5 µg ml<sup>-1</sup> of IS and variable amounts of LND in order to cover the concentration range 0.25–25 µg ml<sup>-1</sup>.

### 3. Result and discussion

Fig. 1 shows a typical chromatogram of an extract of drug-free serum spiked with known amounts of LND and IS. Direct comparison with chromatograms of blank serum shows that both analytes are clearly resolved from all other endogenous serum components in a few minutes. Although analyte elution can be obtained under isocratic conditions [17,18], the technique of gradient elution was preferred because it assured complete elution of all strongly retained compounds and then proper column conditioning for the successive analyses.

Recoveries of LND and IS extracted from spiked serum samples are shown in Table 1. They were calculated by direct comparison of peak areas of analytes with those of standards dissolved in the mobile phase and directly in-

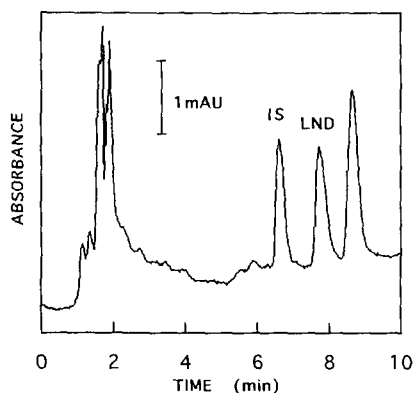


Fig. 1. Chromatogram of an extract of drug-free serum spiked with LND at the 2.5 µg ml<sup>-1</sup> level. Sample size: 10 µl.

Table 1  
Percentage recoveries of LND and IS from spiked serum samples

Analyte	Level (µg ml <sup>-1</sup> )	Mean recovery (%)	RSD (%) (n = 5)
LND	0.25	90.0	5.8
	2.5	92.8	3.4
	25	94.8	4.0
IS	2.5	99.3	3.9

jected through a conventional Rheodyne injector. In the investigated concentration range, LND recovery was independent of the analyte concentration (mean values not significantly different according to one-way analysis of variance (ANOVA) at  $p = 0.1$ ). The solid-phase extraction method developed shows recovery values similar to those already obtained by LLE methods [17,18], but with the advantage of an automated processing of at least ten serum samples, and the use of a considerably lower sample size (10–100 µl) compared to 1 ml used in other [17] procedures.

According to an unweighted regression fitting, the calibration curve, peak area (a.u.) vs. LND concentration in serum, was linear over the investigated concentration range (0.25–25.0 µg ml<sup>-1</sup>) with a correlation coefficient better than 0.999 and an intercept not significantly different from zero at the 95% confidence level. A typical calibration curve gave a slope of  $5.72 \pm 0.05$  a.u. ml µg<sup>-1</sup> and an intercept of  $0.99 \pm 0.40$  a.u.; the standard error of the fit,  $S_{y/x}$ , was 0.954. The within-day and between-day relative standard deviations (RSDs) for LND determination in serum samples spiked at several analyte levels are reported in Table 2. The limit of quantitation (signal-to-noise ratio of 10, noise calculated peak-to-peak in a blank chromatogram at the

Table 2  
Within- and between-day relative standard deviation (RSD) for LND determination in serum samples spiked at three different concentrations

Level (µg ml <sup>-1</sup> )	RDS (%)	
	Within-day (n = 5)	Between-day (n = 5)
0.25	3.2	4.6
2.5	2.7	4.5
25	3.1	4.8

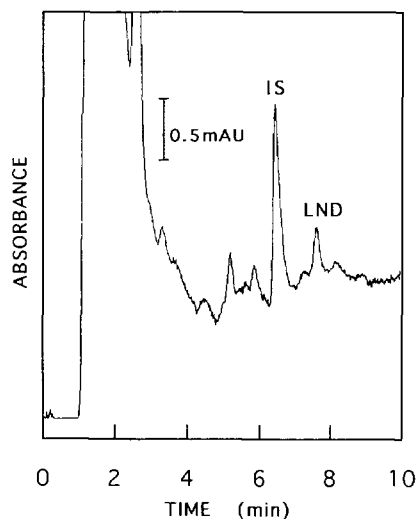


Fig. 2. Chromatogram of the extract of a drug-free serum spiked with LND ( $25 \text{ ng ml}^{-1}$ ) at a concentration near its detection limit. Sample size:  $100 \mu\text{l}$ .

elution time of LND) was  $420 \text{ ng ml}^{-1}$  for a  $10 \mu\text{l}$  sample size, which is adequate to monitor the 24 h time-course of LND concentration in the serum of patients under LND chemotherapy. However, if lower detection limits need to be achieved, a higher sample size can be employed. Using a  $100 \mu\text{l}$  sample size, a limit of detection (signal-to-noise ratio of 3) of  $15 \text{ ng ml}^{-1}$  was obtained (see Fig. 2), which is about one order of magnitude lower than those obtained by Leclaire et al. [17] using an HPLC method and  $1 \text{ ml}$  sample size or by Gatzemeier et al. [19] using a GLC/ECD method.

The analytical procedure has been applied to serum samples from cancer patients treated with LND. As an example, Fig. 3 compares chromatograms relevant to serum extracts from a patient prior to and 1 h after the start of LND therapy. As can be seen, the peak relevant to LND can easily be detected, indicating considerable drug absorption. It is worth noting in Fig. 3 the presence of an unknown peak (labelled X in the Figure) eluting at 2.6 min. Such an unknown peak was observed in all the serum samples of the investigated patients under LND treatment, but was never observed before the start of LND chemotherapy. Further, the concentration–time profile for this unknown compound had the same shape as that observed for LND (see below). The presence of an unknown metabolite in the plasma of patients following LND treatment has been already reported [18] and tentatively ascribed to a LND glucuronide. The

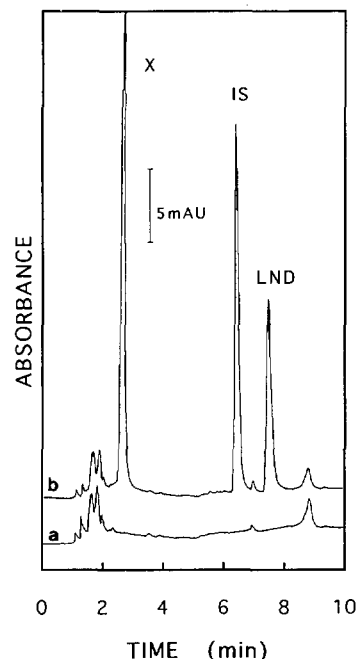


Fig. 3. Chromatograms of serum extracts taken from a patient prior to (a) and 1 h after (b) the start of LND chemotherapy (see Experimental section for the drug treatment protocol). LND calculated serum concentration was  $15.6 \mu\text{g ml}^{-1}$ . Peak labelled X denotes an unknown LND metabolite (see text). Sample size:  $10 \mu\text{l}$ .

identity of this peak is currently under investigation in our laboratory by mass spectrometry.

The time-courses of LND concentration and its metabolite in the serum of patients under LND chemotherapy are shown in Fig. 4. Unfortunately, the sampling rate was too low to permit fitting of the data to a particular pharmacokinetic model. LND concentrations measured 1 h after the first and second drug administrations were  $12.4 \pm 2.5$  and  $10.9 \pm 2.7 \mu\text{g ml}^{-1}$ , respectively, and never

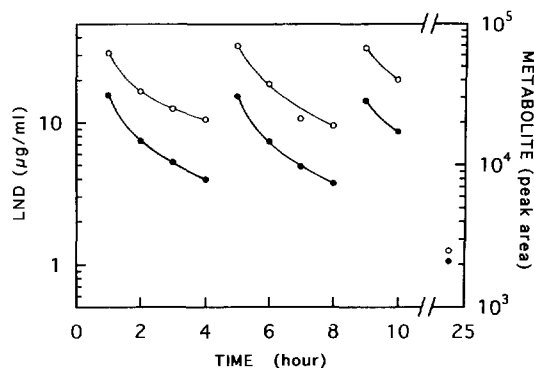


Fig. 4. Time-course of LND (●) and its metabolite (○) in the serum of a patient with advanced ovarian cancer under LND chemotherapy (see Experimental section for LND treatment protocol).

dropped below  $1 \mu\text{g ml}^{-1}$  during the time interval between successive administrations.

In conclusion, LND and its metabolite can be easily quantitated in serum by SPE coupled to gradient elution HPLC. The low sample size (10–100  $\mu\text{l}$ ) required and the automated sample processing make this method particularly promising for routine LND monitoring in patients under drug chemotherapy. Work is in progress in our laboratories to identify the LND metabolite and to study the pharmacokinetics of LND.

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