

Short communication

# Determination of lauroyl-indapamide in rat whole blood by high-performance liquid chromatography

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Received 7 July 2004; accepted 31 January 2005

Available online 11 February 2005

## Abstract

A method based on a liquid–liquid extraction procedure followed by high-performance liquid chromatography (HPLC) coupled with UV–visible detection is described and validated for the determination of lauroyl-indapamide in rat whole blood. The blood sample was extracted with diethyl ether after the addition of 10% trifluoroacetic acid (aq.). The chromatographic separation was performed on a Chromasil ODS column, using methanol–acetonitrile–tetrahydrofuran–0.2% trifluoroacetic acid (170:20:15:38, v/v/v/v) as the mobile phase. The UV detection wavelength was set at 240 nm. The extraction recovery of lauroyl-indapamide was ranged from 76.5 to 82.6%, and the calibration curve had a good linearity in the range of 0.048–200 µg/ml ( $r=0.9976$ ). The method presents appropriate intra-day and inter-days repeatabilities, showing values below 7.4% in terms of the percentage of relative standard deviation (R.S.D.). The method proposed is simple, rapid and sensitive, being useful for pharmacokinetic studies in rats.

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**Keywords:** Lauroyl-indapamide; HPLC; Rat whole blood; Pharmacokinetic study

## 1. Introduction

Lauroyl-indapamide, 3-(lauroylaminosulfanyl)-4-chloro-N-(2,3-dihydro-2-methyl-1H-indol-1-yl)benzamide (Fig. 1), is a new compound derived from indapamide with an increased liposolubility to obtain a longer action and to be suitable for liposomal preparations. It belongs to a well-known pharmacological active compound series classified as an anti-hypertensive agent with diuretic activity, which can significantly reduce rat blood pressure. A number of analytical methods have been reported concerning the determination of indapamide, the parent drug of lauroyl-indapamide, in dosage form [1,2] or in biological fluids including serum, plasma, urine and whole blood [3–11]. However, to the best of our knowledge, there is little information available in the literatures about lauroyl-indapamide, even less about its assay in the biological fluids, which made it considered necessary to

develop an accurate assay to quantify it in vivo, so as to know exactly the characteristics of the substance in biological tests.

Therefore, an analytical method based on high-performance liquid chromatography (HPLC) to determine lauroyl-indapamide in biological fluids is reported here for the first time. Considering lauroyl-indapamide is preferentially bound to blood cells like indapamide [4,5], we selected rat whole blood as the biological sample. The method developed here was validated with parameters such as precision, linearity, selectivity, detection and quantitation limit, and was also successfully applied to following the pharmacokinetic behavior of lauroyl-indapamide in Wistar rats.

## 2. Experiments

### 2.1. Materials and reagents

Lauroyl-indapamide (purity  $\geq 99\%$ ) was synthesized from indapamide, isolated and purified with silica-resin chro-

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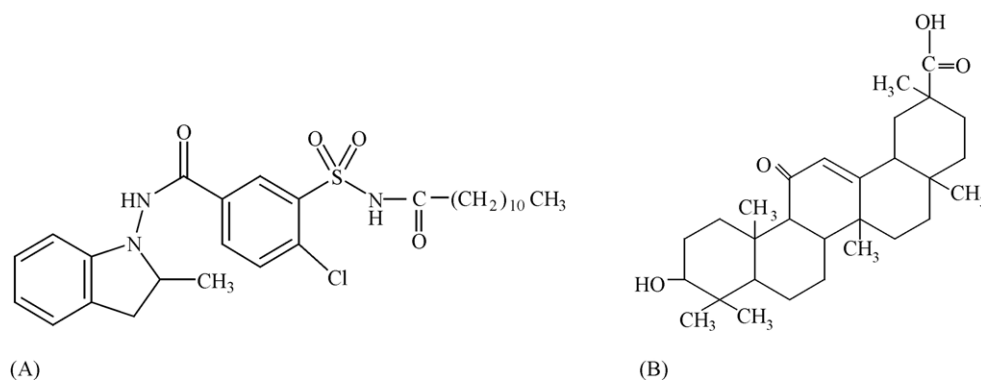


Fig. 1. The chemical structures of (A) lauroyl-indapamide and (B) glycyrrhetic acid.

matography, and its identity and purity were documented by the means of HPLC, UV, NMR and IR. Glycyrrhetic acid (internal standard, IS, see Fig. 1) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol, acetonitrile and tetrahydrofuran were of HPLC grade and purchased from Concord Technology Company (Tianjin, China), and other chemicals used were of analytical grade. Distilled water prepared from demineralized water was used throughout the study. Wistar rats were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University.

## 2.2. Instrumentations

The chromatographic system consisted of a Waters Model 510 pump (Waters Corporation, USA) and an SPD-10A UV-vis detector (Shimadzu, Japan). The signals from the detector were collected and analyzed with a computer equipped with N2000 Chromatography Data System (Zhejiang University, Zhejiang, China).

## 2.3. Chromatographic condition

Chromatographic separation was achieved on a Chromasil ODS column (250 mm  $\times$  4.6 mm, 5  $\mu$ m) (Elete, Dalian, China) with a SecurityGuard column (10 mm  $\times$  4.6 mm, 5  $\mu$ m) filled with the same materials. The temperature was maintained at ambient temperature. The mobile phase was consisted of methanol-acetonitrile-tetrahydrogen-0.2% trifluoroacetic acid (170:20:15:38; v/v/v/v), filtered with 0.45  $\mu$ m cellulosic Millipore membrane (Xinya, Shanghai, China) and degassed by ultrasonic before use, and delivered at an isocratic flow rate of 0.8 ml/min. The detection wavelength was set at 240 nm, the injection volume was 20  $\mu$ l.

## 2.4. Preparation of standard and quality control solutions

The stock solutions of lauroyl-indapamide (0.8 mg/ml) and glycyrrhetic acid (0.5 mg/ml) were prepared with methanol. A series of standard working solutions with the

concentration in the range of 0.096–400  $\mu$ g/ml for lauroyl-indapamide were obtained by a further dilution of the stock solution with methanol. The internal standard working solution (0.05 mg/ml) was prepared by the dilution of the stock internal standard solution with methanol. Quality control (QC) working solutions were prepared at the concentrations of 1.0, 10 and 100  $\mu$ g/ml with the same way as that of the standard working solutions. All the solutions were stored at 4  $^{\circ}$ C and brought to room temperature before use.

## 2.5. Sample preparation

To a 200  $\mu$ l aliquot of rat whole blood, 50  $\mu$ l of the internal standard working solution (0.05 mg/ml) and 50  $\mu$ l of 10% trichloroacetic acid were added. The mixture was extracted with 1 ml of diethyl ether by vortex for 5 min, and then centrifuged at 3000  $\times$  g for 10 min in a microcentrifuge. The upper organic phase was transferred to another tube and evaporated to dryness at 40  $^{\circ}$ C under a gentle stream of nitrogen. The residue was reconstituted in 100  $\mu$ l methanol (HPLC grade), and mixed by vortex for 30 s, a 20  $\mu$ l aliquot of the solution was injected directly into the HPLC system for analysis.

## 2.6. Validation of the analytical method

### 2.6.1. Linear range

Calibration curve data were generated by spiking a series of 200  $\mu$ l drug-free whole blood with 50  $\mu$ l of a standard working solution (1.0, 10 and 100  $\mu$ g/ml) and 50  $\mu$ l of the internal standard working solution, the following extraction procedure and HPLC analysis were performed on the samples as described above. The calibration curve was constructed by plotting the peak area ratios of the analyte to the internal standard against the concentrations of the analyte in the whole blood and analyzed by linear regression analysis.

### 2.6.2. Precision and intra-day and inter-days repeatabilities

The precision was determined by analyzing the extracted quality control samples of different concentrations more than

five times under the above HPLC conditions, and the closeness of the results at each concentration was evaluated as relative standard deviation (R.S.D.).

The intra-day repeatability was determined by analyzing three parallels of each quality control sample at the concentrations of 0.5, 5 and 50  $\mu\text{g/ml}$  at different time of the same day, and the inter-days repeatability was determined by analyzing three parallels of each quality control sample at the concentrations of 0.5, 5 and 50  $\mu\text{g/ml}$  on 3 consequent days. Both the intra-day and inter-days repeatabilities were also evaluated in terms of R.S.D.

#### 2.6.3. Limits of detection and quantitation

The limit of detection (LOD) is the lowest concentration of the analyte in a sample that can be detected, equal to  $3s$ , where “ $s$ ” is the sample standard deviation of 10 independent sample blanks fortified at lowest acceptable concentrations, measured once each. According to this rule, LOD was examined.

The limit of quantitation (LOQ) is the lowest concentration of the analyte that can be quantitatively determined with a defined precision and accuracy under a given experimental condition, which is often equal to  $10s$ , where “ $s$ ” is the standard deviation of the analyte value at each concentration. The precision of LOQ was evaluated experimentally.

#### 2.6.4. Extraction recovery

The extraction recovery at each concentration can be valued by the following equation:

$$\text{Recovery (\%)} = \frac{C_1 - C_2}{C_3} \times 100$$

where  $C_1$  is the concentration of spiked sample;  $C_2$  is the concentration of unspiked sample;  $C_3$  is the concentration of spiking. In order to calculate out the recovery,  $C_1$ ,  $C_2$  and  $C_3$  had to be determined first. To determine them, nine aliquots of 0.2 ml of the rat blank whole blood samples were used, three were spiked with 0.05 ml of the working standard solution (1.0  $\mu\text{g/ml}$ ), three were spiked with 0.05 ml of methanol, three were unspiked, the following extraction and reconstitution procedures were performed exactly the same as that of the sample preparation for all of them, except that in the reconstitution procedure of the unspiked samples, 0.05 ml of the working standard solution (1.0  $\mu\text{g/ml}$ ) and 0.05 ml of methanol were added into each of the tubes, the following mixing procedure and HPLC analysis were exactly the same as described for the samples for all of them. The peak areas were recorded for the analytes. The concentrations ( $C_1$ ,  $C_2$  and  $C_3$ ) were calculated against the calibration curve generated from a series of standard working solutions at suitable concentrations. The extraction recovery of lauroyl-indapamide at the concentration of 0.5  $\mu\text{g/ml}$  was calculated out by substituting the values of  $C_1$ ,  $C_2$  and  $C_3$  into the above equation. The recoveries of lauroyl-indapamide at the other two concentrations (5 and 50  $\mu\text{g/ml}$ ) and the internal stan-

dard at the concentration of 25  $\mu\text{g/ml}$  were determined in the same way.

#### 2.6.5. Stability

The freeze–thaw stability was evaluated by analyzing the QC samples at the concentrations of 0.5, 5 and 50  $\mu\text{g/ml}$  after undergoing three freeze ( $-20^\circ\text{C}$ )–thaw (ambient) cycles. The stability of lauroyl-indapamide in rat whole blood was assessed by placing the QC samples at room temperature for a fixed period of time before being extracted and analyzed. The stability of lauroyl-indapamide in the reconstituted methanol solutions was determined by keeping the reconstituted methanol solutions of the QC samples at room temperature for a fixed time before analysis.

### 2.7. Pharmacokinetic study

The study was conducted in accordance with the Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University. Wistar rats were used after being raised for 1 week, before the day of administration, five rats were fasted overnight but were allowed access to water ad libitum, liposomal lauroyl-indapamide at concentration of 2.93 mg/ml was administered via the tail vein. Blood samples were collected with dried heparinized tubes at 5, 15, 30, 45, 60, 90, 120, 150 and 180 min after intravenous administration respectively, as soon as possible, 0.2 ml of the whole blood was transferred accurately to another tube which was then frozen at  $-20^\circ\text{C}$  and stored until analysis.

### 2.8. Pharmacokinetic data analysis

Pharmacokinetic modeling and parameter calculations were carried out with the pharmacokinetic software Pharmacokinetics Version 1.1, one compartment model of injection was selected.

## 3. Results and discussion

### 3.1. Optimization of HPLC condition and selection of internal standard

To optimize the chromatographic conditions, the pH and organic modifiers of the mobile phase were explored, the mobile phase exceeding pH 2.5 would yield tailing peaks or even double peaks, when pH fell to around pH 2, the peaks became sharper and more symmetric. We deduced the reason might be that lauroyl-indapamide is a weak acid ( $\text{p}K_a = 3.87$ ), so it is very important to suppress its ionization by keeping the pH out of the range of  $\text{p}K_a \pm 1.5$  to eliminate the influence of pH on the peak shape obtained, thus we selected the trifluoroacetic acid as acid modifier. Tetrahydrofuran and acetonitrile in the mobile phase were acting as the organic modi-



Table 1  
Precision of the method

Concentration added ( $\mu\text{g/ml}$ )	Concentration found ( $\mu\text{g/ml}$ ) (mean $\pm$ S.D.)	Recovery (%)	R.S.D. (%)
0.5	$0.5 \pm 1.2 \times 10^{-2}$	100	2.4
5.0	$5.0 \pm 1.1 \times 10^{-1}$	100.2	2.2
50	$51.3 \pm 1.0$	102.5	1.8

$n = 5$ .

Table 2  
Intra-day repeatability of lauroyl-indapamide in the analysis of the rat whole blood

Concentration added ( $\mu\text{g/ml}$ )	Concentration found ( $\mu\text{g/ml}$ ) (mean $\pm$ S.D.)	R.S.D. (%)
0.5	$0.5 \pm 1.8 \times 10^{-2}$	3.6
5	$5.1 \pm 6.9 \times 10^{-2}$	1.4
50	$49.9 \pm 1.1$	2.2

$n = 3$ .

determination coefficient ( $r^2$ ) of the calibration curve being 0.9976 and 0.9952, respectively, the calibration curve showed an excellent linearity in the range of 0.048–200  $\mu\text{g/ml}$ . The LOQ was 0.048  $\mu\text{g/ml}$  with 98–103% recovery and relative standard deviation (R.S.D.) of 4.2%. The LOD was 0.012  $\mu\text{g/ml}$  with signal-to-standard deviation ( $s$ ) more than 3:1.

### 3.3.3. Precision and intra-day and inter-days repeatabilities

The precision of the method expressed as R.S.D. is showed in Table 1. The precision of the assay was also assessed in terms of the intra-day and inter-days repeatabilities at different concentrations. The intra-day repeatability of the assay is presented in Table 2 and the inter-days repeatability on 3 consequent days is presented in Table 3. All the R.S.D. of the intra-day and inter-days repeatabilities were less than 7.4%.

### 3.3.4. Extraction recovery

The extraction recoveries of lauroyl-indapamide and IS in the whole blood are presented in Table 4, all the recoveries were found to be more than 76.5%.

### 3.3.5. Analyte stability

The analyte was found to be stable after three freeze–thaw cycles in blood. The recoveries of the QC samples were

Table 3  
Inter-days repeatability of lauroyl-indapamide in the analysis of the rat whole blood

Concentration added ( $\mu\text{g/ml}$ )	Concentration found ( $\mu\text{g/ml}$ ) (mean $\pm$ S.D.)	R.S.D. (%)
0.5	$0.5 \pm 3.7 \times 10^{-2}$	7.4
5	$5.0 \pm 2.3 \times 10^{-1}$	4.6
50	$49.8 \pm 0.6$	1.2

$n = 3$ .

Table 4  
Extraction recoveries of lauroyl-indapamide and IS

Drug	Concentration ( $\mu\text{g/ml}$ )	Extraction recovery (%)	R.S.D. (%)
Lauroyl-indapamide	0.5	82.6	3.7
	5	76.5	1.2
	50	79.3	1.8
Internal standard	25	84.2	1.6

$n = 3$ .

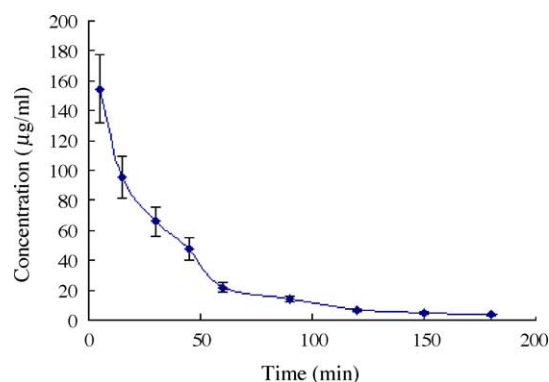


Fig. 3. Mean blood concentration-time profile of lauroyl-indapamide after intravenous administration of the liposomal lauroyl-indapamide (11.72 mg/kg) in the five rats.

ranged from 90 to 103%. The stability of the analyte in the whole blood and the reconstituted methanol at room temperature has also been investigated, lauroyl-indapamide was shown to be stable in the rat whole blood for at least 3 h (R.S.D. < 3.8%), and stable in the reconstituted methanol solution at room temperature for more than 24 h (R.S.D. < 7.1%).

### 3.4. Application of the analytical method in pharmacokinetic studies

Mean whole blood concentration-time profile of lauroyl-indapamide obtained after intravenous administration of 11.72 mg/kg in the rats is presented in Fig. 3 and the pharmacokinetic parameters obtained are shown in Table 5, from which we can see that the concentration of lauroyl-indapamide in blood decreased monotonically and rapidly after a single administration of the liposomal lauroyl-indapamide (11.72 mg/kg), and the concentration can be measurable until after 180 min with the above HPLC method. These results indicate that lauroyl-indapamide is extensively

Table 5  
Pharmacokinetic parameters obtained after intravenous administration of the liposomal lauroyl-indapamide (11.72 mg/kg) in the rats (mean value of the five rats)

$T_{1/2}$ (min)	$K_e$ ( $\text{min}^{-1}$ )	$C_0$ ( $\mu\text{g/ml}$ )	Cl (ml/min)	Vd (ml)	$\text{AUC}_{0-\infty}$ ( $\mu\text{g min/ml}$ )
71.798	0.0089	44.64	0.5842	65.64	5015.73

metabolized and degraded rapidly into its degradation products in which indapamide is the primary one. However, it should be noted that the method developed here has only determined the concentration of lauroyl-indapamide, mainly because lauroyl-indapamide shows a much great difference in the polarity from its degraded products including indapamide, which shows a much shorter retention time in our mobile phase presented above. So it is hard to follow both lauroyl-indapamide and indapamide simultaneously. To solve this problem, a gradient HPLC method is recommended, or alternatively, the blood samples are added with two suitable internal standards, and then experienced a single sample handling process, run in HPLC for two times in two different suitable mobile phases delivering at an isocratic flow rate, both lauroyl-indapamide and indapamide can be detected, the only disadvantage of this method is a little time consuming.

#### 4. Conclusion

In this study, a fast and simple HPLC method for the determination of lauroyl-indapamide in rat whole blood is presented, the linearity, precision, intra-day and inter-days repeatabilities and extraction recovery of this method were excellent. Furthermore, the method has been shown to be specific and selective. The small sample volume and good sensitivity make this method a suitable choice for a pharmacokinetic study, which has been successfully applied to the pharmacokinetic studies in rats.

#### Acknowledgements

The authors were grateful to National Natural Science Foundation of China for the grants (No. 30271548), the authors would thank Dr. Song Du and Mr. Baoqi Li for their assistants in the experiment and Dr. Yanli Hao for providing the pharmacokinetics software.

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