

URDIGERE R. ANIL KUMAR¹
KANAKAPURA BASAVAIHAH¹
KALSANG THARPA¹
KANAKAPURA B. VINAY²

¹Department of Chemistry, University
of Mysore, Manasagangothri,
Mysore - 570 006, India

²Advinus therapeutics, peenya 2nd
stage, Bangalore-560 058, India

SCIENTIFIC PAPER

UDC 543.544.5:615.25:61

DOI: 10.2298/CICEQ0903119K

DETERMINATION OF RALOXIFENE HYDROCHLORIDE IN HUMAN URINE BY LC-MS-MS

A sensitive and selective liquid chromatographic-tandem mass spectrometric (LC-MS-MS) method was developed to determine raloxifene hydrochloride (RLX) in human urine. After a solid-phase extraction with SPE cartridge, the urine sample was analyzed on a C₁₈ column (Symmetry 3.5 μ m; 50 mm \times 4.6 mm i.d) interfaced with a triple quadrupole tandem mass spectrometer. A positive electrospray ionization was employed as the ionization source. The mobile phase consisted of ammonium acetate (pH 4.0)-acetonitrile (60:40, v/v). The method was linear over a concentration range of 20–1000 ng mL⁻¹. The lower limit of quantitation was 20 ng mL⁻¹. The intra-day and inter-day relative standard deviation across three validation runs over the entire concentration range was <10.5%. The accuracy determined at three concentrations (50, 500 and 850 ng mL⁻¹ RLX) was within $\pm 0.84\%$ in terms of relative errors.

Key words: raloxifene; quantification; LC-MS-MS; urine.

Raloxifene hydrochloride (RLX) is a selective estrogen receptor modulator that belongs to the benzothiophene class of compounds (Fig. 1) [1]. The chemical designation is methanone[6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thien-3-yl]-[4-[2-(1-piperidinyloxy)phenyl]hydrochloride. RLX is used for the treatment and prevention of osteoporosis in postmenopausal women [2,3].

Only two HPLC methods [4,5] have been reported for the determination of RLX in body fluids. Trontelj *et al.* [6] have reported the determination of RLX in human plasma by LC-MS in the range of 0.088–60 μ g mL⁻¹.

The liquid chromatography-tandem mass spectrometry (LC-MS-MS), due to its higher sensitivity and selectivity, has been applied to the quantification of the drug in biological samples. The expected concentration of RLX is primarily excreted in feces, and less than 0.2% is excreted unchanged in urine. Less than 6% of the raloxifene dose is eliminated in urine as glucuronide conjugates [7].

The literature survey revealed that no method has been reported for the determination of RLX in human urine.

The present study was undertaken to develop a sensitive and rapid LC-MS-MS method for the determination of RLX in a urine sample using letrozole as an internal standard (Fig. 1). The sample preparation procedure was simple and a run time of each sample was 4.0 min.

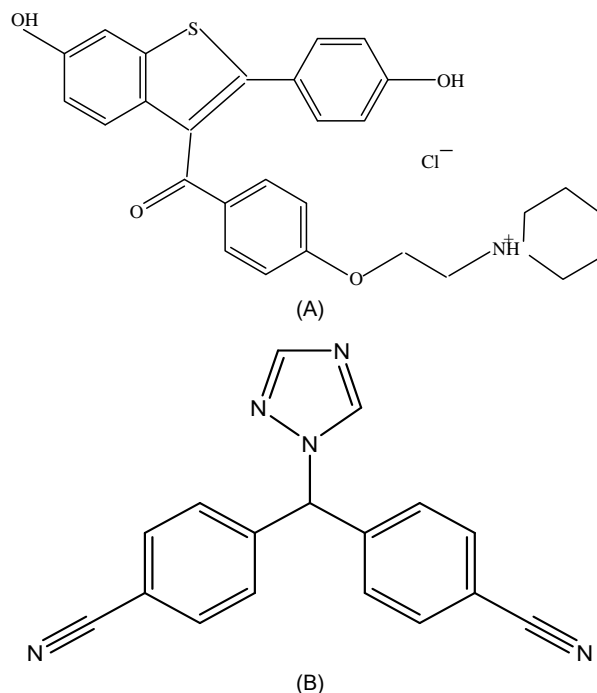


Fig. 1. Structure of raloxifene hydrochloride (A) and internal standard (B).

Corresponding author: K. Basavaiah, Department of Chemistry, University of Mysore, Manasagangothri, Mysore - 570 006, India. E-mail: basavaiahk@yahoo.co.in
Paper received: September 4, 2008.
Paper revised: March 3, 2009.
Paper accepted: March 27, 2009.

EXPERIMENTAL

Apparatus

The LC-MS-MS analysis was performed with an API 2000 mass spectrometer (Applied Biosystems) coupled to an HPLC system comprising an Agilent 1100 series low pressure quaternary gradient pump with degasser, autosampler, and the column oven.

Reagents and standards

All chemicals used were of analytical reagent grade and HPLC grade acetonitrile (Merck Ltd, Mumbai) was used. Distilled water filtered through 0.22 μm filter (Millipore) was used to prepare the solutions.

10 mM ammonium acetate (adjusted to pH 4.0 with formic acid) and acetonitrile were used as a mobile phase A and a mobile phase B, respectively. Acetonitrile was used as the diluent for the urine extract.

Pharmaceutical grade RLX, certified to be 99.8% pure was procured from Cipla India Ltd., Mumbai, India, and was used as received. A stock standard containing a 100 $\mu\text{g mL}^{-1}$ RLX solution was prepared by dissolving accurately weighed 1 mg of pure drug in a diluent and diluting it to 10 mL in a calibrated flask with the diluent. It was subsequently diluted to obtain a working concentration of RLX.

Procedures

LC-MS conditions

The chromatographic separation was achieved at ambient temperature (25 $^{\circ}\text{C}$) on the column (Symmetry, 3.5 μm ; 50 mm \times 4.6 mm i.d) using the mobile phase of 10mM ammonium acetate (pH 4.0, adjusted with formic acid) and acetonitrile in the ratio of 60:40 (v/v) at a flow rate of 0.6 mL/min. The mobile phase was degassed before use.

Quantization was performed by using the multiple reaction monitoring (MRM) of the transitions of m/z 474.6 \rightarrow m/z 269.1 for raloxifene and m/z 284.2 \rightarrow 257.5 m/z for letrozole (IS) with a scan time of 0.2 s per transition. The sensitivity and linearity range was observed for raloxifene and IS in these transition. So the same transition was used for quantization.

Figure 2 shows the product ion spectra of $[\text{M}+\text{H}]^+$ for raloxifene and letrozole.

In order to optimize all the MS parameters, a standard solution (1 $\mu\text{g mL}^{-1}$) of the analyte and IS was infused into the mass spectrometer.

The analysis was performed in a positive mode (ESI-electro spray ionization) with a turbo ion spray interface under the conditions: ion source potential, 5500 V; declustering potential, 70 V; focusing potential, 400 V, capillary temperature, 350 $^{\circ}\text{C}$; entrance

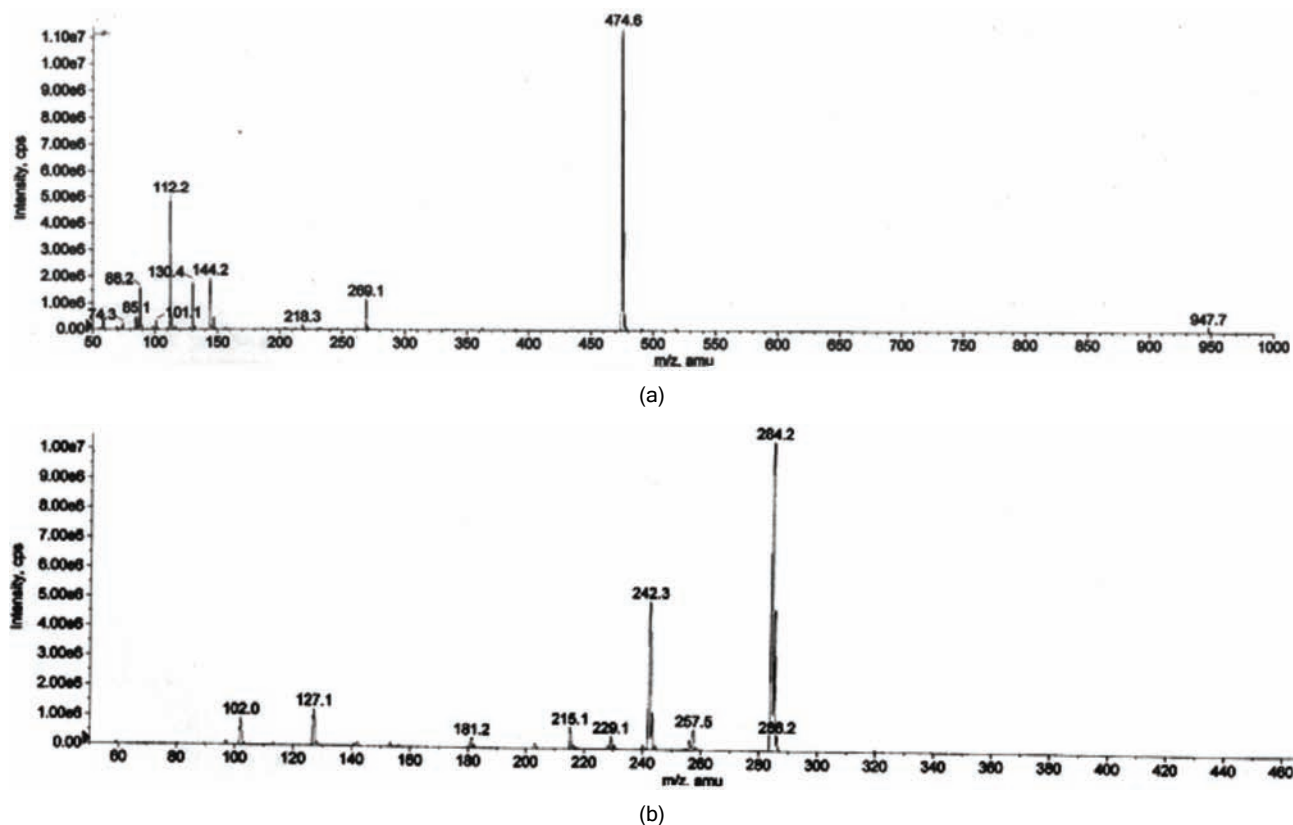


Fig. 2. Full scan product ion spectra of raloxifene (a) and letrozole (b).

potential, 10 V with nitrogen as a nebuliser gas at 25 Psi. The column eluent was introduced into the electro spray ionization chamber of the mass spectrometer with a split ratio of 3:7. Mass fragmentation studies were performed by maintaining the normalized collision energy at 30 eV.

Sample preparation

The urine samples collected from healthy volunteers were spiked with known concentration of RLX and IS and the resulting sample was diluted 1:1 with water prior to loading on SPE cartridge (Oasis HLB, 60 mg). The SPE cartridge was conditioned with 2 mL of acetonitrile and equilibrated with 1 mL of water, and then diluted 1 mL urine was loaded on. The cartridge was then washed with 1 mL of 10% acetonitrile in water and then eluted with 1 mL of 100% acetonitrile. The sample was evaporated with nitrogen and reconstituted the residue with water:acetonitrile (1:1) to 500 μL . Then 20 μL of the above solution was injected into LC-MS-MS for analysis.

Preparation of standard and quality control samples

The stock solution of RLX was prepared in acetonitrile at the concentration of 10 $\mu\text{g mL}^{-1}$. The stock solution of IS was prepared in acetonitrile at the concentration of 10 $\mu\text{g mL}^{-1}$ and diluted to 500 ng mL^{-1} with acetonitrile:water (1:1). The stock solutions were prepared in amber colored bottles and were stored in the refrigerator.

A calibration curve was prepared by diluting the stock solution with acetonitrile:water (1:1) to get appropriate concentrations. From this dilution, 20 μL of the appropriate standard solution was added to 1 mL of urine blank to get effective concentrations of 20, 80, 100, 500, 850 and 1000 ng mL^{-1} for RLX.

The quality control (QC) samples were separately prepared in the blank urine sample at the concentrations of 50, 500 and 850 ng/mL , respectively. The spiked urine samples (standards and quality controls) were then treated for SPE.

RESULTS AND DISCUSSION

Mass spectrometry

The signal intensity obtained for RLX in the positive mode was much higher than that in the negative mode. Then, the possibility of using the electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) sources under the positive ion detection mode was evaluated during the early stage of the method development. ESI spectra revealed higher signals for the molecule compared to APCI source. A further assay development was therefore limited to

ESI source. The Q1 full spectra of RLX and IS were dominated by protonated molecules $[\text{M}+\text{H}]^+$ and no significant solvent adduct ions and fragments ions were observed. The tuning of the ESI source such as capillary temperature, a flow of sheath and auxiliary gas (N_2) and the spray voltage on the transition of RLX and IS further improved the sensitivity.

Chromatography

Although in the aspect of chromatographic separation the determination of the analyte was not interfered with endogenous substances in the urine sample, yet the ionization of the analyte, especially of low concentration, was easily suppressed, which resulted in the linearity of narrow concentration range. In order to avoid the ion suppression induced by endogenous substances, the influence of the mobile phase that composed of different percentage of organic phase to the ion suppression was evaluated during the experiment. It was found that when the mobile phase consists of 10 mM ammonium acetate (pH 4.0)-acetonitrile (60:40, v/v), the spiked sample demonstrated good linearity between 20 to 1000 ng mL^{-1} for RLX. Under the present chromatographic conditions, the run time of each sample was 4.0 min. The retention times were 0.91 min and 0.90 min for RLX and IS (Fig. 3), respectively.

Method Validation

Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank urine samples with the corresponding spiked urine sample. Fig. 4 shows typical chromatograms of a blank for RLX and IS. There was no significant interference or ion suppression from endogenous substances observed at the retention times of the analytes.

Linearity of calibration curves and lower limit of quantification

Urine samples were quantified by using the ratio of the peak area of RLX to that of IS as the assay parameter. Peak area ratios were plotted against RLX concentrations and standard curves in the form of $Y = A + BX$ were calculated using weighted ($1/x^2$) least squares linear regression.

To evaluate linearity, urine calibration curves were prepared and assayed in duplicate on three separate days.

Visual inspection of the plotted duplicate calibration curves and correlation coefficients > 0.99 confirmed that the calibration curves were linear over the concentration ranges of 20-1000 ng mL^{-1} for the analyte.

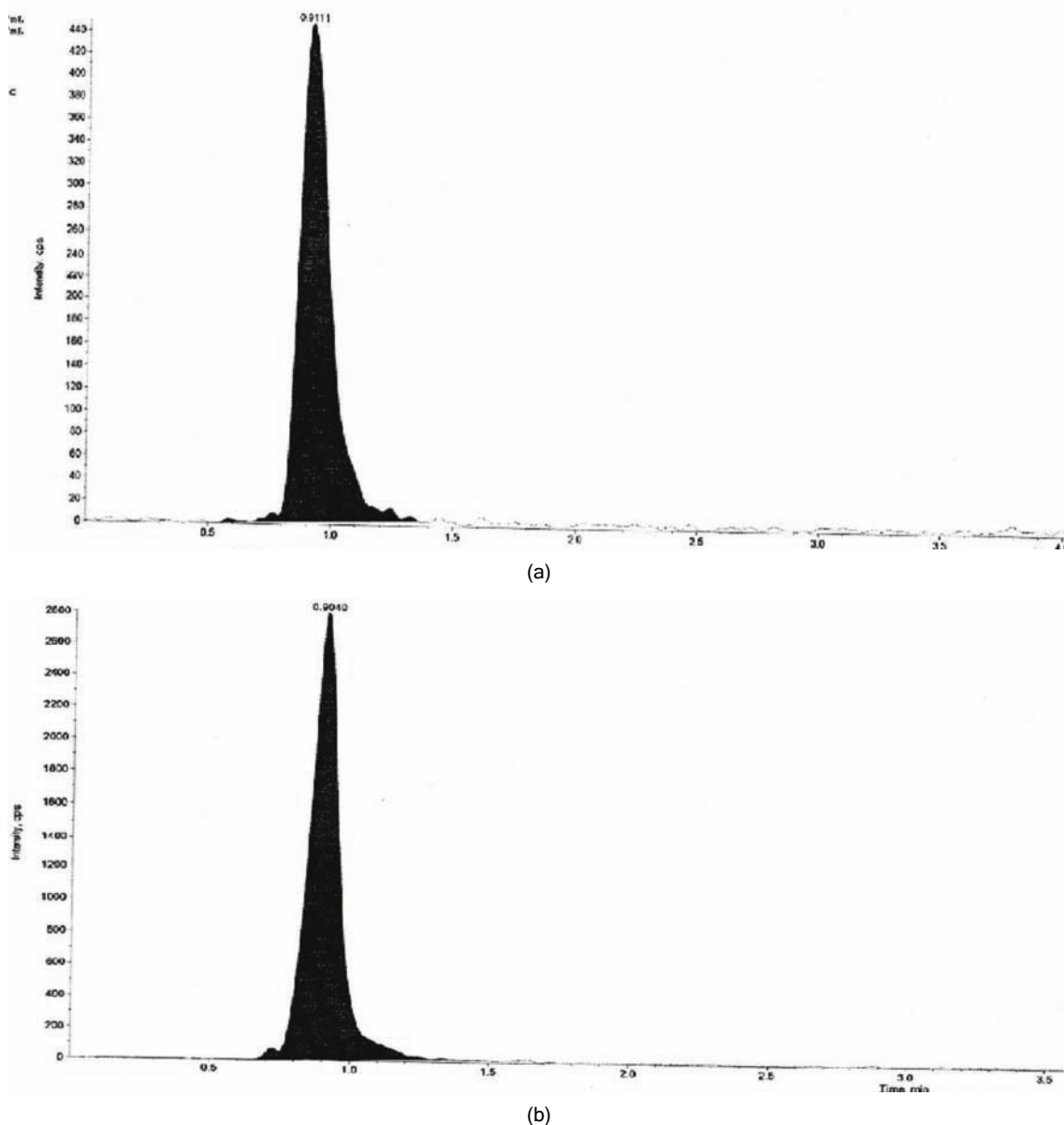


Fig. 3. Representative chromatogram of a) raloxifene (20 ng mL^{-1}) and b) IS (500 ng mL^{-1}).

The lower limit of quantification was defined as the lowest concentration on the calibration curve for which the acceptable accuracy of $\pm 15\%$ and a precision below 15% were obtained. The present LC-MS-MS method offered an *LOQ* of 20 ng mL^{-1} in 1 mL of the urine sample.

Accuracy and precision

The intra-day accuracy and precision were assessed by determining QC samples in a set of six replicates within one day. The accuracy was expressed by $(\text{mean observed concentration})/(\text{spiked concentration}) \times 100$ and the precision by relative standard deviation (*RSD*).

Table 1 summarizes the precision and accuracy for the RLX evaluated by assaying the QC samples. The inter-day precision was established by performing analyses over a period of five days on QC samples prepared afresh each day.

Recovery study

Absolute recoveries of RLX at three QC levels were determined by assaying the samples as described above and comparing the peak areas of both RLX and IS with those obtained from a direct injection of the compounds dissolved in the blank urine. The recoveries of RLX, determined at three concentrations (50 , 500 and 850 ng/mL) were $75.4 \pm 6.4\%$, $79.4 \pm 6.9\%$ and $76.4 \pm 7.4\%$ ($n = 6$) respectively.

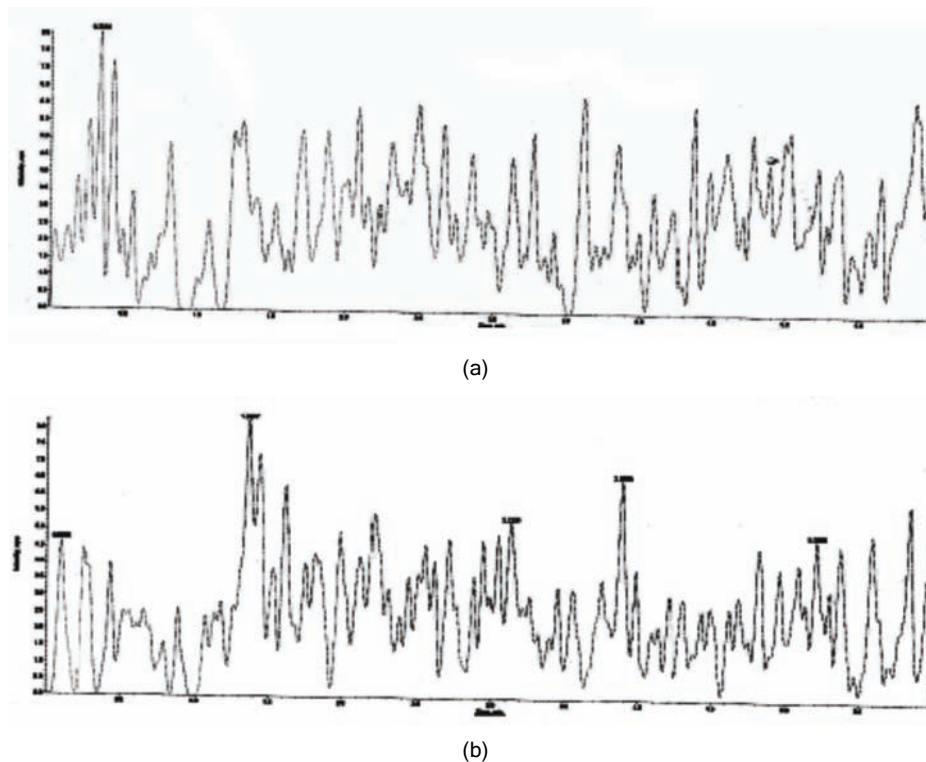


Fig. 4. Representative blank chromatogram for Raloxifene (a) and IS (b).

Table 1. Accuracy and precision

<i>RLX taken, ng mL⁻¹</i>	<i>RLX found^a, ng mL⁻¹</i>	<i>Intra-day RSD^b, %</i>	<i>Inter-day RSD, %</i>	<i>RE^c, %</i>
50	49.8	8.3	10.5	0.40
500	495.8	8.2	8.6	0.84
850	846.6	7.7	7.8	0.40

^aMean value of seven determinations; ^brelative standard deviation; ^crelative error

Stability study

It was found that the analyte in the urine sample was stable after three freeze-thaw cycles and at the 24h post-preparative stability at room temperature.

CONCLUSIONS

A sensitive LC-MS-MS method for the quantification of raloxifene in the urine sample was developed and validated. The method is rapid, sensitive and highly selective with a *LOQ* of 20 ng mL⁻¹. The determination of one urine sample needs 4 min. These results indicate that it is suitable for the routine analysis of large batches of biological samples.

REFERENCES

- [1] A. Smith, P. E. Heckelman, J. R. Obenchain, J. A. R. Galipeau, M. A. D' Arecca, S. Budavari, The Merck Index, 13th Ed., Merck Research Laboratories, Division of Merck and Co., Inc.; White house station, NJ, 2001, 1452
- [2] Y. Jin, H. G. Yu, Gongcheng Jishu **25** (2004) 56-57
- [3] J. Trontelj, V. Tomaz, M. Bogataj Marij, A. Mrhar, Pharm. Res. **52** (2005) 334-339
- [4] Zh. Y. Yang, Zh. F. Zhang, X.B. He, G.Y. Thao, Y.Q. Zhang, Chromatographia **65** (2007) 197-201
- [5] Z. Yang, X. He, Y. Zhang, Biomed. Chromatogr. **21** (2007) 229-233
- [6] J. Trontelj, M. Bogataj, J. Marc, A. Mrhar, J. Chromatogr. B **855** (2007) 220-227
- [7] K. R. Snyder, N. Sparano, J. M. Malinowski, Am. J. Health-Syst. Pharm. **57** (2000) 1669-1678.