



ELSEVIER

Journal of Chromatography B, 767 (2002) 277–283

JOURNAL OF  
CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

## Control of propranolol intake by direct chromatographic detection of $\alpha$ -naphthoxylactic acid in urine<sup>☆</sup>

M.J. Ruiz-Ángel<sup>a</sup>, P. Fernández-López<sup>b</sup>, J.A. Murillo-Pulgarín<sup>b</sup>,  
M.C. García-Alvarez-Coque<sup>a,\*</sup>

<sup>a</sup>Departamento de Química Analítica, Facultad de Química, Universidad de Valencia, Dr. Moliner 50, 46100 Burjassot, Valencia, Spain

<sup>b</sup>Departamento de Química Analítica y Tecnología de Alimentos, Facultad de Química, Universidad de Castilla-La Mancha, Avd. Camilo José Cela s/n, 13078 Ciudad Real, Spain

Received 2 July 2001; received in revised form 21 November 2001; accepted 22 November 2001

### Abstract

A rapid chromatographic procedure with a C<sub>18</sub> column, a mobile phase of 0.15 M sodium dodecyl sulfate (SDS)–10% (v/v) 1-propanol at pH 3 (0.01 M phosphate buffer), and fluorimetric detection, is reported for the control of propranolol (PPL) intake in urine samples, which are injected directly without any other treatment than filtration. The peak of PPL was only observed in samples taken a few hours after ingestion of the drug due to its extensive conjugation and metabolism. The detection of several unconjugated PPL metabolites was therefore considered: desisopropylpropranolol (DIP), propranolol glycol (PPG),  $\alpha$ -naphthoxylactic acid (NLT) and  $\alpha$ -naphthoxyacetic acid (NAC). NLT showed the best characteristics; it eluted at a much shorter retention time than PPL, its concentration in urine samples was greater and it did not present any interference from endogenous compounds in urine, common drugs or drugs administered in combination with PPL. The limit of quantification, measured as the concentration of analyte providing a relative standard deviation of 20%, was 24 ng/ml, and the day-to-day imprecision was below 4% for concentrations above 200 ng/ml. The procedure allows the routine control of PPL at therapeutic urine levels. Urinary excretion studies showed that the detection of NLT is possible at least up to 20–30 h after oral administration. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Micellar liquid chromatography; Propranolol;  $\alpha$ -Naphthoxylactic acid

### 1. Introduction

Propranolol (PPL, Table 1) is a  $\beta$ -adrenoceptor antagonist, which is widely used in the treatment of

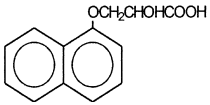
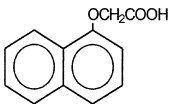
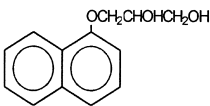
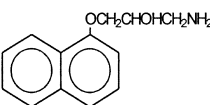
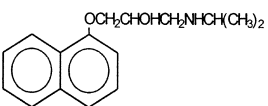
several diseases such as arrhythmias, thyrotoxicosis, angina pectoris and hypertension. It is also consumed in sport and in different stressing activities as a doping agent. Accordingly, the development of rapid and direct procedures to control PPL intake is interesting. However, PPL is rapidly metabolised after oral administration, suffering *N*-dealkylation, aromatic hydroxylation and glucuronidation of the hydroxyl group at the 2-position of the side-chain. Polar metabolites with basic (*N*-desisopropylpropranolol, DIP, and diverse hydroxypropranolols, mainly 4-hydroxypropranolol, 4-OH-PPL) and acidic ( $\alpha$ -

<sup>☆</sup>Presented at the 30th Scientific Meeting of the Spanish Group of Chromatography and Related Techniques/1st Meeting of the Spanish Society of Chromatography and Related Techniques, Valencia, 18–20 April 2001.

\*Corresponding author. Tel.: +34-963-983-003; fax: +34-963-864-436.

E-mail address: celia.garcia@uv.es (M.C. García-Alvarez-Coque).

Table 1  
Chemical structures of propranolol and several metabolites

$\alpha$ -Naphthoxyzilactic acid	
$\alpha$ -Naphthoxyzilic acid	
Propranolol glycol	
Desisopropylpropranolol	
Propranolol	

naphthoxyzilactic acid, NLT, and  $\alpha$ -naphthoxyzilic acid, NAC) functional groups are found in the physiological samples, together with neutral non-polar metabolites (propranolol glycol, PPG) (Table 1) [1,2]. PPL is also extensively conjugated, which makes its detection difficult. Consequently, several authors have proposed procedures in which PPL metabolites are analysed.

The most versatile methods in the detection of PPL and its metabolites utilise liquid or gas chromatography coupled with mass spectrometry (MS) [3,4]. Although mass-selective detection provides the necessary resolution capability, it requires access to an expensive mass spectrometer. The analyses are therefore usually accomplished using liquid chromatography with fluorimetric detection [2,5–9]. The procedures reported in the literature include however clean-up steps such as liquid–liquid or solid–liquid extraction, precipitation and hydrolysis to liberate the aglycones, and are often lengthy and tedious resulting in poor recoveries. Liquid–liquid or solid-phase extraction should be carried out at different pH

depending on the acid–base character of the metabolites. The mobile phases contain frequently several modifiers, such as acetonitrile–methanol [7,9] or acetonitrile–methanol–tetrahydrofuran [2]. A competitive amine, such as triethylamine or *n*-butylamine, is also added to reduce peak tailing by suppressing the interaction with the free silanol groups on the silica support [10].

In previous work, an RPLC procedure was developed for the analysis of several  $\beta$ -antagonists in urine samples with micellar mobile phases of sodium dodecyl sulfate (SDS), propranolol and triethylamine at pH 3 [11]. The peak of PPL was only observed in samples taken a few hours after ingestion of the drug. In this work, the chromatographic separation of the unconjugated forms of PPL and four metabolites (DIP, PPG, NLT and NAC) is studied. A rapid and sensitive procedure to control PPL intake in urine samples by detection of NLT is proposed. Micellar liquid chromatography (MLC) allowed the direct injection of urine samples without the need of previous treatments. This technique represents an attractive alternative to the conventional use of aqueous organic mobile phases for drug control [12].

## 2. Experimental

### 2.1. Reagents

Stock solutions of 10  $\mu\text{g/ml}$  of propranolol chlorhydrate (Aldrich, MI, USA), *N*-desisopropylpropranolol chlorhydrate, propranolol glycol,  $\alpha$ -naphthoxyzilic acid and  $\alpha$ -naphthoxyzilic acid (Tocris Cookson, St. Louis, MO, USA) were prepared in 0.05 *M* SDS medium (99% purity; Merck, Darmstadt, Germany). Working standard solutions were also obtained by appropriate dilution with 0.05 *M* SDS. The solutions were stored at 4 °C. The chemical structures of the compounds are shown in Table 1.

The following reagents were used for the preparation of the mobile phases: SDS, 1-propranolol, sodium monohydrogenphosphate (Scharlab, Barcelona, Spain) and HCl (Panreac, Barcelona, Spain). The pH of the mobile phase was fixed with HCl before the addition of 1-propranolol. The mobile phases were filtered through nylon membranes of 0.45  $\mu\text{m}$  and 47 mm in diameter (Micron Separations, Westboro, MA,

USA). Analyte working solutions were also filtered before injection through nylon membranes of 0.45  $\mu\text{m}$  and 25 mm in diameter (Micron). Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout to prepare all solutions.

## 2.2. Apparatus

The equipment consisted of an Agilent (Palo Alto, CA, USA) model HP 1100 chromatograph provided with an isocratic pump, an autosampler and a model HP 1046A fluorimetric detector. The excitation and emission wavelengths were 230 and 340 nm, respectively, for all compounds.

The flow-rate was 1.0 ml/min and the injection volume 20  $\mu\text{l}$ . A Spherisorb unendcapped ODS-2 analytical column (5  $\mu\text{m}$  particle size, 125 $\times$ 4.6 mm I.D.) and a guard column of similar characteristics (35 $\times$ 4.6 mm I.D.) both from Scharlab were used. The signal was acquired with a PC connected to the chromatograph through a model HP 3396A integrator using the PEAK-96 program (Hewlett-Packard, Avondale, PA, USA). Optimisation of mobile phase composition was made with MICHROM [13].

## 2.3. Procedure

The analyses were performed with 1-ml urine samples, which were diluted in a 1:25 factor with 0.05 *M* SDS. No treatment other than filtration (which was carried out directly into the autosampler vials) was made before injection into the chromatograph. The samples were chromatographed with 0.15 *M* SDS–10% (v/v) propanol, buffered with 0.01 *M* phosphate at pH 3.

## 3. Results and discussion

### 3.1. Selection of the mobile phase

The fluorescence spectra of PPL and its metabolites (DIP, PPG, NLT and NAC) were obtained in SDS solutions to select the excitation and emission wavelengths for detection, which were 230 and 340 nm, respectively, for all compounds.

The possibility of detecting the unconjugated

forms of PPL and its metabolites, in urine samples, was examined. The compounds were chromatographed with hybrid micellar mobile phases of surfactant and organic solvent at different concentrations, and variable pH. Adequate control of these factors was necessary to achieve chromatograms showing good resolution and low analysis time. A study was carried out to optimise the resolution of a mixture of the five compounds in aqueous solution, and check if baseline separation was possible with a mobile phase of SDS and propanol. For this purpose, an optimisation procedure based on the modelling of the retention behaviour at varying mobile phase composition was applied. The mobile phases of the experimental design were the following: 0.05 *M* SDS–5% (v/v) propanol, 0.15 *M* SDS–5% propanol, 0.10 *M* SDS–10% propanol, 0.05 *M* SDS–15% propanol and 0.15 *M* SDS–15% propanol.

Peak tailing was checked to decrease in mobile phases at low pH (compare Figs. 1a and 2a). The mobile phases of the experimental design were therefore buffered at pH 3 with 0.01 *M* phosphate buffer. In the literature, this pH is often used in the chromatographic determination of PPL and its metabolites with aqueous–organic mobile phases to decrease excessive peak tailing [2,7,9], which has been explained to be due to the protonation of the free silanol groups on the column [10]. However, in contrast to most reported procedures, an amine was not added to the micellar mobile phase to improve peak shape, since the surfactant layer adsorbed on the column prevents the interaction with the silanol groups, and the efficiencies are thus higher.

The peaks of NLT and PPG overlapped in the whole experimental domain, whereas NAC eluted at close retention times, but was partially or baseline resolved (Fig. 1a). DIP and PPL eluted at longer times and were usually resolved. The retention times for NLT, PPG, NAC, DIP and PPL ranged between 14.7, 15.0, 17.7, 62.2 and 84.5 min for 0.05 *M* SDS–5% propanol, and 3.8, 3.8, 4.2, 11.0 and 12.0 for 0.15 *M* SDS–15% propanol, respectively. A mobile phase of 0.15 *M* SDS–10% propanol was selected, for which the retention times of the five compounds were sufficiently low: 4.8, 4.8, 5.5, 14.3 and 16.2 min, respectively, and the resolution between the peaks of NLT and NAC was still the greatest possible with this system (Fig. 1a). The separation of NLT and NAC at pH 3 is not critical:

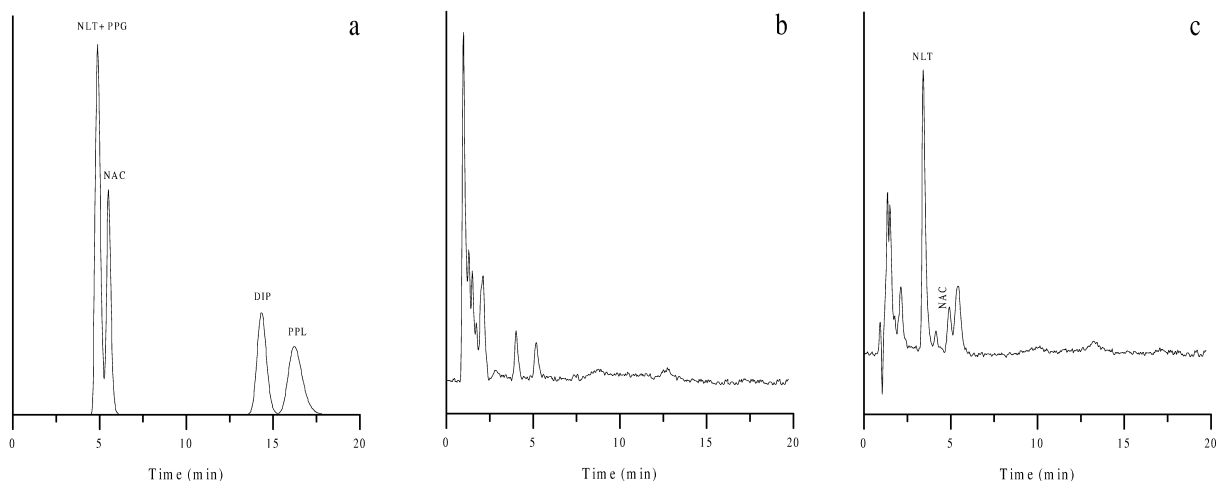


Fig. 1. Chromatograms of a standard aqueous mixture of 50 ng/ml of PPL and four metabolites (a), and diluted urine samples of a volunteer before (b) and after (c) ingestion of a pharmaceutical preparation containing PPL. Mobile phase: 0.15 M SDS–10% propanol at pH 3. The urine sample was diluted with 0.05 M SDS in a 1:25 factor. The concentration of NLT in intact urine for (c) is 250 ng/ml. Compounds: NLT= $\alpha$ -naphthoxy-lactic acid, PPG=propranolol glycol, NAC= $\alpha$ -naphthoxyacetic acid, DIP=desisopropylpropranolol, and PPL=propranolol.

mobile phase compositions (surfactant and alcohol) near the selected one gave a good separation of the two metabolites, but the retention was higher at lower concentration of SDS and propanol.

Fig. 1b and c show the chromatograms obtained with 0.15 M SDS–10% propanol at pH 3 of diluted urine samples taken from a volunteer, before and

after the ingestion of a pharmaceutical preparation that contained PPL. It should be noted that DIP and PPL are not detected, although a small peak appeared for PPL in a urine sample taken a few hours after ingestion of the drug, injected without dilution. A peak is observed at 4.0 min, a retention time slightly shorter than the peak observed for NLT and

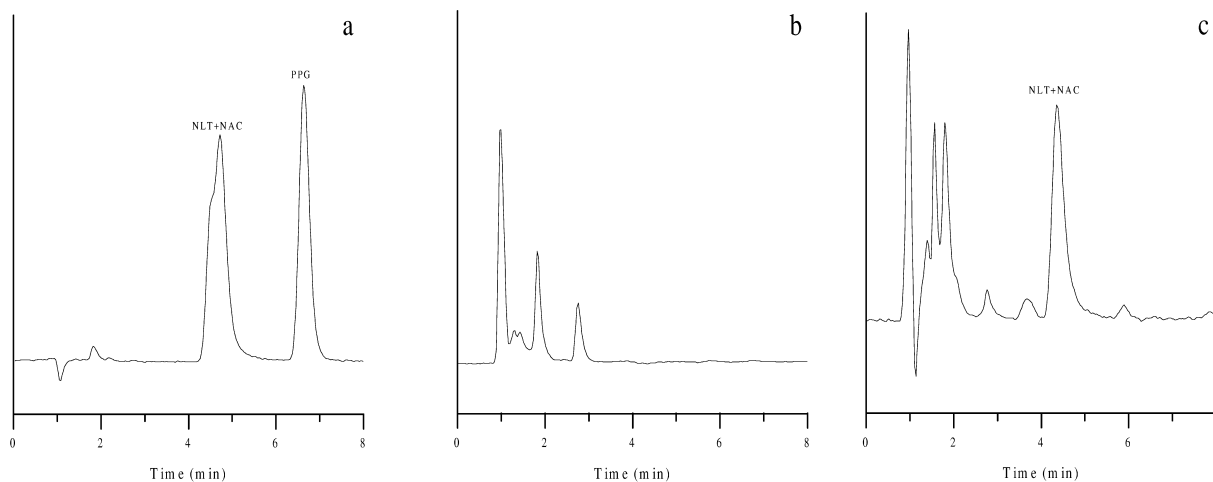


Fig. 2. Chromatograms of a standard aqueous mixture of 50 ng/ml of three metabolites of PPL (a), and urine samples of a volunteer before (b) and after (c) ingestion of a pharmaceutical preparation containing PPL. Mobile phase: 0.15 M SDS–10% propanol at pH 4. See Fig. 1 for peak identity and other details.

PPG in aqueous solution (compare Fig. 1a and c). Also, no overlapping exists with the peak of any endogenous compound. Instead, the peak of NAC is partially overlapped with an endogenous compound that eluted at 5.5 min.

The free metabolites of PPL are found in urine at different concentration levels. PPG is mostly conjugated in urine samples, while NLT is free, being considered as the primary metabolite of side-chain oxidation [2]. Therefore, the peak at 4.0 min in the urine sample should correspond only to NLT. To check this possibility, an aqueous mixture of NLT, NAC and PPG, and the urine samples of the same volunteer who was administered PPL, were eluted with mobile phases of SDS–propanol at a different pH. At  $\text{pH} \geq 4$ , PPG in the aqueous mixture was separated from NLT and NAC, which in turn were closely overlapped. Fig. 2 shows the chromatogram obtained with 0.15 M SDS–10% propanol at pH 4. Therefore, the peak at 4.0 min found in the urine samples chromatographed at pH 3 should correspond mainly or totally to NLT. Identification of the eluted compounds by direct coupling of MLC with MS was however not possible, owing to the high concentration of surfactant in the mobile phase.

The peak of NLT seems to be the most adequate for the control of PPL intake in urine and was selected. The limit of detection was lower than that found for NAC, which is derived from the oxidative metabolism of NLT. This metabolite, as commented, was partially overlapped with an endogenous compound, and although its quantification is feasible with the present assay, it showed a smaller signal than NLT.

### 3.2. Analytical figures of merit for $\alpha$ -naphthoxylic acid

The analysis of urine samples was carried out with 0.15 M SDS–10% propanol at pH 3 after dilution in a 1:25 factor. This decreased the large signal of urine matrix at the beginning of the chromatograms and permitted the injection of a larger number of urine samples without regeneration of the stationary phase. In these conditions, the retention times of different drugs in urine samples have been checked to be reproducible after at least 200 injections [14].

Calibration curves were constructed for NLT in

aqueous solution and diluted spiked urine matrix, using the measured areas of the chromatographic peaks at five increasing concentrations in the range 0.5–50 ng/ml (three replicates). Linear plots were obtained with  $r > 0.997$ . The slopes of the fitted calibration straight-lines were 0.29 and 0.23 for the aqueous solution and diluted urine, respectively. The limit of quantification (LQ) was calculated as the concentration of analyte that provided a detector response with a signal-to-noise ratio of 5:1 (relative standard deviation, RSD, of 20%). For this calculation, the standard deviation was obtained by injection of a series of ten replicates at the lower concentration of the calibration curve. The LQs were 0.37 and 0.95 ng/ml for aqueous solution and diluted urine, respectively. Taking into account the 1:25 dilution, the LQ in intact urine was 24 ng/ml. This value permits the control of PPL intake in urine samples through the detection of NLT.

Within-day repeatability was calculated by measuring the areas of the peaks obtained by injection of series of six standard solutions of NLT prepared in diluted spiked urine, at three concentrations: 1, 8 and 13 ng/ml (corresponding to 25, 200 and 325 ng/ml NLT in intact urine) (Table 2). The injections were repeated during 6 days to obtain the day-to-day repeatability, which was below 4% for 200 and 325 ng/ml.

The chromatographic behaviour of several com-

Table 2  
Within-day and day-to-day accuracy and precision for the determination of  $\alpha$ -naphthoxylic acid in urine samples<sup>a</sup>

	Added concentration (ng/ml) <sup>b</sup>		
	1	8	13
Measured concentration (ng/ml)			
	0.81±0.09	7.96±0.38	12.73±0.17
	0.70±0.12	7.79±0.22	12.65±0.40
	0.82±0.08	8.14±0.13	13.23±0.26
	0.94±0.16	7.93±0.19	13.20±0.19
	0.85±0.10	7.27±0.40	12.00±0.30
	0.80±0.16	7.72±0.15	12.59±0.23
Mean	0.82±0.07	7.80±0.23	12.73±0.45
Accuracy (%)	–18.1	–2.5	–2.0
Precision (%)	9.5	3.8	3.5

<sup>a</sup> Each data corresponds to the mean value of six replicates made inside a day. The measurements were repeated during six non-consecutive days.

<sup>b</sup> Concentration referred to diluted urine.

mon drugs and compounds administered in combination with PPL was examined to study the selectivity of the procedure: acebutolol, amiloride, aspirin, atenolol, bendroflumethiazide, diazepam, ephedrine, furosemide, hydrochlorthiazide, metoprolol, nadolol, paracetamol, piretanide, sulfadimethoxine, sulfamethoxazole, sulfisoxazole and triamterene. Solutions containing 5 µg/ml of these drugs, prepared in 0.05 M SDS, were chromatographed with the mobile phase of 0.15 M SDS–10% propanol at pH 3. No signal was observed for the compounds at the detection wavelengths, except for atenolol and nadolol, which showed peaks at 4.4 and 6.8 min. Only the peak of atenolol interfered the determination of NLT. This interference was however avoided by reducing the amount of propanol in the mobile phase to 5%, due to the different selectivity of the mobile phase towards NLT and atenolol. This mobile phase permitted also the separation of NLT and NAC, but increased the retention time of these metabolites to 6.8 and 7.4 min, respectively. Metoprolol is a hydrophobic β-antagonist which is rapidly metabolised in the liver as PPL. It has been checked however that the peaks of the main metabolites of metoprolol appear at shorter retention times than NLT, near the protein band of urine.

### 3.3. Study of the urinary excretion of α-naphthoxylactic acid

The reliability of the procedure was further checked by analysing a series of urine blank samples (diluted in a 1:25 factor with 0.05 M SDS) from 20 volunteers: ten males and ten females (including a pregnant woman) of different ages, diets and weights. In all cases, the urine matrices presented similar profiles with no endogeneous presence at the retention time of NLT.

According to this result, the possibility of detection and quantification of NLT in urine samples collected after administration of a single oral dose of a low therapeutic amount of PPL was investigated. For this study, three healthy volunteers (two males and one female) were given 10 mg of PPL chlorhydrate. Urine samples were taken during 50 h at different time intervals (during the first 6 h in 1–2-h intervals and afterwards in longer intervals), and the excreted volume was measured. The samples

were analysed immediately or frozen at –24 °C for future analysis. No preservatives were added to prevent degradation or change the pH of the samples. Fig. 3 shows the urinary excretion of NLT. As can be observed, the excreted concentration reached a maximum between 3 and 10 h from ingestion. In the urine diluted samples, the PPL metabolite was detected at least up to 20–30 h after ingestion.

Propranolol is administered as the racemic form. The pharmacodynamic and pharmacokinetic profiles of (*R*)- and (*S*)-enantiomers are different. The (*S*)-isomer is more potent than the (*R*)-isomer, and is believed to be largely responsible for the β-blocking activity of the racemic drug [15]. Thus, the mean plasma peak concentration of the (*S*)-isomer was found to be 40% higher than the (*R*)-isomer in samples taken from 12 individuals [16]. This results probably in a different proportion of the enantiomers of metabolites including NLT. Enantioselectivity bioanalysis of propranolol and 4-hydroxypropranolol has been intensively investigated [17–20]. However, the introduction of a chiral separation technique into a bioassay results in a more complex total analysis method, regarding extraction, chiral derivatisation or enantioseparation on a chiral stationary phase.

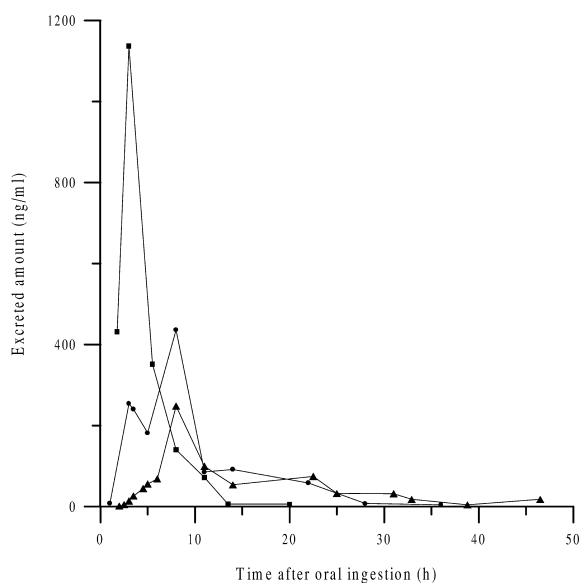


Fig. 3. Urinary excretion of NLT in urine samples of three volunteers. A single dose of 10 mg of PPL was administered. The concentrations correspond to intact urine.

This work describes a simple MLC procedure that permits the assay of PPL intake through monitoring of the total sum of the (*R*)- and (*S*)-enantiomers of NLT in urine samples. The analyses are performed in less than 10 min using a mobile phase of SDS and propanol at pH 3. In contrast to other conventional procedures with aqueous–organic mobile phases, that require time-consuming extraction steps or more complex chromatographic systems, the proposed procedure presents the advantage of the direct injection of the samples with no other treatment than sample dilution and filtration.

### Acknowledgements

The authors acknowledge the support of Project BQU2001-3047 (MCYT, Spain). M.J. Ruiz-Ángel thanks the Ministerio de Ciencia y Tecnología of Spain for the FPI Grant, and P. Fernández-López thanks the support of the Junta de Castilla–La Mancha (Spain) for a maintenance grant in the University of Valencia.

### References

- [1] T. Walle, U.K. Walle, L.S. Olanoff, *Drug Metab. Dispos.* 13 (1985) 204.
- [2] E.C. Kwong, D.D. Shen, *J. Chromatogr. B* 414 (1987) 365.
- [3] K. Hartonen, M.L. Riekkola, *J. Chromatogr. B* 676 (1996) 45.
- [4] H. Kataoka, S. Narimatsu, H.L. Lord, J. Pawliszyn, *Anal. Chem.* 19 (1999) 4237.
- [5] P.M. Harrison, A.M. Tonkin, C.M. Cahill, A.J. McLean, *J. Chromatogr.* 343 (1985) 349.
- [6] K.A. Smith, S. Wood, M. Crous, *Analyst* 112 (1987) 407.
- [7] S.A. Qureshi, H.S. Buttar, *J. Chromatogr.* 431 (1988) 465.
- [8] V.G. Belolipetskaja, V.K. Piotrovskii, V.I. Metelitsa, S.A. Pavlinov, *J. Chromatogr. B* 491 (1989) 507.
- [9] H.A. Semple, F. Xia, *J. Chromatogr. B* 655 (1994) 293.
- [10] N.E. Basci, A. Temizer, A. Bozkurt, A. Isimer, *J. Pharm. Biomed. Anal.* 18 (1998) 745.
- [11] I. Rapado-Martínez, R.M. Villanueva-Camañas, M.C. García-Alvarez-Coque, *Anal. Chem.* 71 (1999) 319.
- [12] M.C. García-Alvarez-Coque, S. Carda-Broch, *J. Chromatogr. B* 736 (1999) 1.
- [13] J.R. Torres-Lapasió, MICHROM Software, Marcel Dekker, New York, 2000.
- [14] S. Carda-Broch, J.R. Torres-Lapasió, J.S. Esteve-Romero, M.C. García-Alvarez-Coque, *J. Chromatogr. A* 893 (2000) 321.
- [15] A.M. Barrett, V.A. Cullum, *Br. J. Pharmacol.* 34 (1968) 43.
- [16] S.T. Wu, Y.P. Chang, W.L. Gee, L.Z. Benet, E.T. Lin, *J. Chromatogr. B* 692 (1997) 133.
- [17] G. Egginger, W. Lindner, C. Vandenbosch, D.L. Massart, *Biomed. Chromatogr.* 7 (1993) 277.
- [18] V.L. Herring, J.A. Johnson, *J. Chromatogr.* 612 (1993) 215.
- [19] G. Egginger, W. Lindner, G. Brunner, K. Stoschitzky, *J. Pharm. Biomed. Anal.* 12 (1994) 1537.
- [20] C. Mislanová, A. Stefanová, J. Oravcová, J. Horecký, T. Trnovec, W. Lindner, *J. Chromatogr. B* 739 (2000) 151.