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High-performance liquid chromatographic determination of levodropropizine in human plasma with fluorometric detection[1]

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Abstract

The present paper describes a new high-performance liquid chromatographic method with fluorescence detection for the analysis of levodropropizine [*S*-(−)-3-(4-phenylpiperazin-1-yl)-propane-1,2-diol] (Levotuss), an anti-tussive drug, in human serum and plasma. A reversed-phase separation of levodropropizine was coupled with detection of the native fluorescence of the molecule, using excitation and emission wavelengths of 240 nm and 350 nm respectively. The analytical column was packed with spherical 5 μm poly(styrene–divinylbenzene) particles and the mobile phase was 0.1 *M* NaH_2PO_4 pH 3–methanol (70:30, v/v), containing 0.5% (v/v) tetrahydrofuran. For quantitation, *p*-methoxylevodropropizine was used as the internal standard. Samples of 200 μl of either serum or plasma were mixed with 200 μl of 0.1 *M* Na_2HPO_4 pH 8.9 and extracted with 5 ml of chloroform–2-propanol (9:1, v/v). The dried residue from the organic extract was redissolved with distilled water and directly injected into the chromatograph. The limit of detection for levodropropizine, in biological matrix, was about 1–2 ng/ml, at a signal-to-noise ratio of 3. The linearity was satisfactory over a range of concentrations from 3 to 1000 ng/ml (r^2=0.99910); within-day precision tested in the range 5–100 ng/ml as well as day-to-day reproducibility proved acceptable, with relative standard deviations better than 1% in most cases. Interferences from as many as 91 therapeutic or illicit drugs were excluded.

Keywords: Levodropropizine

1. Introduction

Levodropropizine [*S*-(−)-3-(4-phenyl-piperazine-1-yl)-propane-1,2-diol] (LDP), the (−)-enantiomer of dropropizine, is an anti-cough drug with peripheral mode of action developed by Dompé S.p.A. It is marketed (Levotuss) in several European countries, including Italy, where it is the most prescribed non-narcotic antitussive.

LDP was synthesized with the aim of isolating the anti-tussive activity of dropropizine, which has been marketed as a racemate for a long time, from its side effects on the central nervous system.

Actually, in several pharmacological and clinical studies, LDP exhibits an anti-tussive activity comparable to dropropizine, with a reduced sedative effect on the central nervous system, which seems to be attributable to the (+)-enantiomer [1,2]. The main

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action mechanism of LDP is peripheral: it modulates the sensitive C fibres, whose activation causes cough and bronchial constriction [3]. This activation also induces a vicious circle, which negatively affects other pulmonary functions and, through release of neuropeptides, can lead to mucus hypersecretion and to increased capillary permeability. LDP counteracts cough mechanisms, and also seems to modulate other events associated with inflammation and bronchial constriction [3].

Until now, all the analytical determinations carried out to perform the pharmacokinetic and metabolic studies were accomplished using a gas chromatographic–mass spectrometric (GC–MS) method reported by Zaratin et al. [4]. GC–MS was found to be extremely sensitive (5 ng/ml) and specific, but requires sophisticated and delicate instrumentation and a difficult and tiresome procedure of derivatization with β-bis(trimethylsilyl)trifluoroacetamide (BSTFA). In contrast, in routine clinical studies, the analytical method should be as easy, rapid and inexpensive as possible. Because of the relatively low levels of the drug in plasma (down to 10 ng/ml, 8 h after a single dose of 60 mg of LDP administered to healthy volunteers), as reported in previous studies [4], the sensitivity appeared to be crucial.

The present paper describes a simple high-performance liquid chromatography (HPLC) method for monitoring LDP in serum and plasma, suitable for routine work using direct highly sensitive fluorometric detection (HPLC–FL).

2. Experimental

2.1. Reagents and standards

LDP and *p*-methoxylevodropropizine (*p*-MLDP) and the internal standard (I.S.) were supplied by Dompé S.p.A. (L'Aquila, Italy). Stock solutions of both standards were prepared in methanol at a concentration of 1 mg/ml and stored at −20°C. Working solutions of standards at suitable concentrations were prepared every day in water or in drug-free samples of plasma or serum, as needed.

Standards of 91 therapeutic or abusive drugs (10 μg each), dried onto glass microfibre discs impregnated with silicic acid, were from the Toxi Disc Library (Toxi-Lab, Irvine, CA, USA).

Water, all solvents and chemicals used for extraction procedures and liquid chromatography were of analytical or HPLC grade (Carlo Erba Reagenti, Milan, Italy).

2.2. HPLC equipment and analytical conditions

The HPLC system consisted of a single-piston high-pressure pump (Model 302, Gilson, Villiers-le-Bel, France), a pulse damper (Model 802 C, Gilson), a manual injector (Model 7125, Rheodyne, Cotati, CA, USA) with a 200-μl loop and a double monochromator fluorometer (Model 821 FP, Jasco, Tokyo, Japan). The excitation and the emission wavelengths were set to 240 nm (slit 18 nm) and 350 nm (slit 30 nm), respectively. Data acquisition, storage and integration were done with a computerized data system (Model 620, Gilson).

The analytical column (150×4.6 mm I.D.) was packed with spherical 5 μm poly(styrene–divinylbenzene) particles (Bio-Gel PRP 70–5, Bio-Rad Laboratories, Brussels, Belgium). Between the injector and the column, a 0.45-μm replaceable stainless steel filter (Rheodyne) was used to protect the column from clogging by particulate material present in the injected samples.

The isocratic elution was performed with a mobile phase composed of 0.1 *M* potassium monobasic phosphate buffer pH 3 (with concentrated orthophosphoric acid)–methanol (70:30, v/v), containing 0.5% of tetrahydrofuran. The separation was performed at room temperature, the usual flow-rate was 0.5 ml/min, but it was possible to increase it to 0.7 ml/min in order to reduce the time of analysis, with only a slight worsening of separation efficiency.

Usually, 100 μl of sample was injected with partial loop filling.

Quantitation was accomplished by the internal standard method (I.S.), based on the ratios of LDP peak areas of either standards or unknowns divided by the *p*-MLDP (I.S.) peak areas.

A special feature of the Jasco spectrofluorometer was used to optimise the fluorescence excitation and emission wavelengths of LDP. Fluorescence spectra of LDP and *p*-MLDP (10 mg/ml in water and in 0.1 *M* phosphate buffer pH 3) were recorded using low

resolution (slit 18 nm for excitation and emission) at stopped flow. For this purpose, the flow cell of the instrument was filled with analyte solutions or solvent blanks using an intradermal syringe.

2.3. Preparation and handling of samples

Blood bank plasma (with citrate–phosphate–dextrose as an anticoagulant) and serum samples (obtained after blood clotting at room temperature), spiked with known amounts of LDP, as well as samples from patients undergoing therapy with LDP were added with a fixed amount of I.S. solution (2 mg/ml) to obtain a final concentration of 50 ng/ml and then left to stand for about 15 min to allow equilibration with binding proteins. Plasma and serum were extracted soon after centrifugation (at +4°C) or frozen at −20°C until analysis.

Portions of 200 μl of biological sample were added with 200 μl of 0.1 *M* Na_2HPO_4 (pH 8.9) and extracted with 5 ml of chloroform–2-propanol mixture (9:1, v/v). After vortex-mixing for 2 min and centrifugation (at about 700 *g*) for 10 min, 4 ml of the organic phase was collected and evaporated under nitrogen stream. The residue was dissolved with 0.5 ml (or another suitable volume) of distilled water and a sample of 100 μl was injected.

The recovery studies were carried out by adding known amounts of LDP to blank plasma or serum, which were then processed according to the extraction procedures described above. The peak areas were compared to those from the injection of aqueous solutions of standards at corresponding concentrations.

3. Results and discussion

3.1. LDP native fluorescence

Although limited by the optic design of the instrument and by the broad slit (18 nm) available, our preliminary studies demonstrated a relevant native fluorescence of LDP and *p*-MLDP, which allowed a selective and sensitive HPLC method to be developed with direct HPLC–FL, so avoiding any derivatization. The fluorophore was identified in the benzene ring, which was linked to the nitrogen of the piperazine ring in the LDP and *p*-MLDP molecules. When dissolved in water or in phosphate buffer pH 3, the two compounds showed a broad fluorescence band with maxima at 350 nm and 240 nm for emission and excitation wavelengths, respectively. No relevant differences between the two analogues nor influences of pH on fluorescence were observed.

3.2. HPLC–FL determination

Under these conditions, LDP and *p*-MLDP eluted with baseline resolved peaks in about 20 min, with respective retention factors (k) of about 3.0 and 4.2. Using a polymeric column prevented the peak from tailing as we had observed in preliminary tests with reversed-phase silica-based columns, which could be ascribed to the interaction of silanols with the basic moieties of the molecules. Thus, peaks with asymmetry factors almost equal to 1 were obtained for both LDP and *p*-MLDP without the use of any additives. The chromatographic column was not chirally selective, thus LDP was not separated from the (+)-enantiomer of dropropizine. However, for our purposes, this limitation was not relevant because racemization of LDP was not observed in biological systems, as reported in the documentation presented for drug registration available at the Italian Ministry of Health [5].

The efficiency of the chromatographic separation was about 20 000 plates/m, which is consistent with the relatively low performance (in terms of efficiency) of polymeric columns. On the other hand, the column proved extremely stable, even after several hundred injections of biological extracts. Moreover, when the first symptoms of column fouling (peak broadening) appeared, washing in the opposite direction to the chromatographic flow, at 0.25 ml/min flow-rate, with progressively increasing percentages of acetonitrile in water (up to 80%), followed by thorough reconditioning under the original operating conditions, restored column performances completely. Using some precautions, such as in-line pre-column filter and periodical column washing, we were able to carry out the entire study with the same column. Another advantage of polymeric packing is the high selectivity of the stationary phase towards biological matrix-related interferences, which, combined with the selectivity of the fluores-

cence detection, allowed any interference in the chromatograms of real samples to be completely eliminated.

In addition to this, the use of fluorescence for LDP detection, although based on a relatively simple aromatic structure, allowed an important increase in sensitivity to take place, compared with UV detection at a wavelength of 240 nm (data not shown). The limit of detection (LOD) of the present method was about 1–2 ng/ml, with a signal-to-noise ratio of 3, in biological matrix (starting from 200 ml of sample), which is clearly better than in GC–MS (5 ng/ml starting from 1 ml of sample), as recently reported [4]. The limit of quantitation, with an R.S.D. of 6.6% and an accuracy higher than 2.5%, was 3.1 ng/ml.

However, the main advantage of the adopted detection method is, as mentioned above, the excellent selectivity towards matrix components. This makes it possible to deal with biological matrices, such as plasma and serum, without complex and tiresome sample pre-treatments. In fact, rapid but rough liquid–liquid extraction was always enough to achieve an extract suitable for injection, even at the highest sensitivity.

The linearity of LDP quantitative determination was fairly good (checked with the least squares regression analysis) over a range of concentrations from 3 to 1000 ng/ml (points at 1000, 100, 50, 25, 12.5, 6.2, 3.1 ng/ml) and is described by the equation $y=0.007388x+0.052919$, $r^2=0.99910$ (where x=LDP concentrations and y=LDP/I.S. peak-area ratios). This study was purposively conducted with the concentration of I.S. increased to 100 ng/ml, in order for it not to be too different from the highest concentrations of LDP to be measured. The linearity had to be checked over a wide range of concentrations because previous pharmacokinetic experiments carried out on healthy volunteers had shown serum LDP levels varying from 10 to 960 ng/ml [4]. However, it is well known that when the range of data is very broad, least squares regression tends to underestimate any non-linearity occurring in the lowest part of the regression plot. Therefore, we recalculated the linearity of the method over the range 3–100 ng/ml and again obtained an excellent result, described by the equation: $y=0.007796x-0.001916$, $r^2=0.99973$. The intercept on the y-axis for $x=0$ did not differ substantially from 0; when the concentration of each point on the calibration curve was back-calculated from the regression line equation, the difference from the expected value was not higher than 3.6%.

The average absolute recovery of the extraction method from both serum and plasma, tested at 25 and 50 ng/ml, was 89.0 and 86.7% for LDP and p-MLDP (n=6) with relative standard deviations (R.S.D.s) generally better than 3.5% (the recovery percentage was corrected for the loss of 1/5 of the volume of organic mixture in the extraction procedure). It is obvious that, working with an I.S., the variability of the final results is not strictly related to the absolute recoveries of the analytes, but with the reproducibility of their relative recoveries (analyte/I.S.).

Thus, the overall analytical precision was much better than that of the extraction procedure for the individual substances. As measured on 6 replicates, at the levels of 100, 50 and 5 ng/ml of LDP in biological samples (with an I.S. concentration of 50 ng/ml), intra-day precision was defined by R.S.D.s of 0.89, 0.42 and 3.9%, respectively. Day-to-day R.S.D.s were only slightly worse, at 1.2, 1.0 and 4.8%, respectively, for the same concentration levels of LDP measured on 6 subsequent days. Accuracy also proved excellent, with results varying between 92.0 and 100.2% of the expected values (mean 98.5%), when tested on the same spiked samples on the same day; analysis of the control samples (stored at −20°C), measured over 6 consecutive days, gave results varying between 89.6 and 103.3% (mean 95.6%) of the expected values.

Possible interferences were investigated by injecting mixtures of common therapeutic and/or illicit drugs contained in the Toxi Disc Library (Table 1) at an individual concentration of 20 μg/ml, with the fluorometer at a sensitivity range allowing the determination of down to 5 ng/ml of LDP. Under these conditions, none of the 91 drugs gave any significant peak-eluting at the retention time of LDP. However, since the test was carried out in vitro using parent drugs, we could not exclude possible interferences from drug metabolites originating in vivo.

As mentioned above, the injection of extracts from serum or plasma gave remarkably clean chromatograms, as shown in Fig. 1, allowing LDP to be

Table 1
Drugs investigated in order to exclude interferences in LPD determination

Opiates and antagonists			
Codeine	Dextromethorphan	Dihydrocodeine	Diphenoxilate
Ethylmorphine	Hydrocodone	Hydromorphone	Meperidine
Morphine	Methadone	Naloxone	Oxicodone
Papaverine	Propoxyphene	Terpin hydrate	
Central nervous system active drugs			
Amphetamine	Amitriptyline	Benztropine	Carbamazepine
Caffeine	Chlorprothixene	Chlorpromazine	Diazepam
Diphenylhydantoin	Doxepin	Ethinamate	Flurazepam
Imipramine	Loxapine	Meprobamate	Methamphetam.
Methaqualone	Methylphenidate	Nordiazepam	Nortriptyline
Pentobarbital	Phenmetrazine	Phentermine	Phencyclidine
Phenobarbital	Phenytoin	Phetidine	Prazepam
Protriptyline	Secobarbital	Strychnine	Thioridazine
Thiothixene	Trifluperazine	Trflupromazine	Amobarbital
Aprobarbital	Butabarbital	Barbital	Cocaine
Miscellaneous			
Acetaminophen	Atropine	Benzoylecgonine	Carisoprodol
Chlorpheniramine	Cimetidine	Diphenhydramine	Disopyramide
Doxylamine	Emetine	Erythromycin	Glutethimide
Hydrocortisone	Hydroxyzine	Lidocaine	Methapyrilene
Methocarbamol	Nicotine	Orphenadrine	Pentazocine
Phenacetin	Pyrilamine	Phenolphthalein	Phenylpropanola.
Propranolol	Procaine	Procainamide	Pseudoephedrine
Quinine	Salicylamide	Spironolactone	Triamterene
Triexyphenidyl	Trimeprazine	Trimetobenzamide	Trimethoprim

Substances injected at an individual concentration of 20 μg/ml.

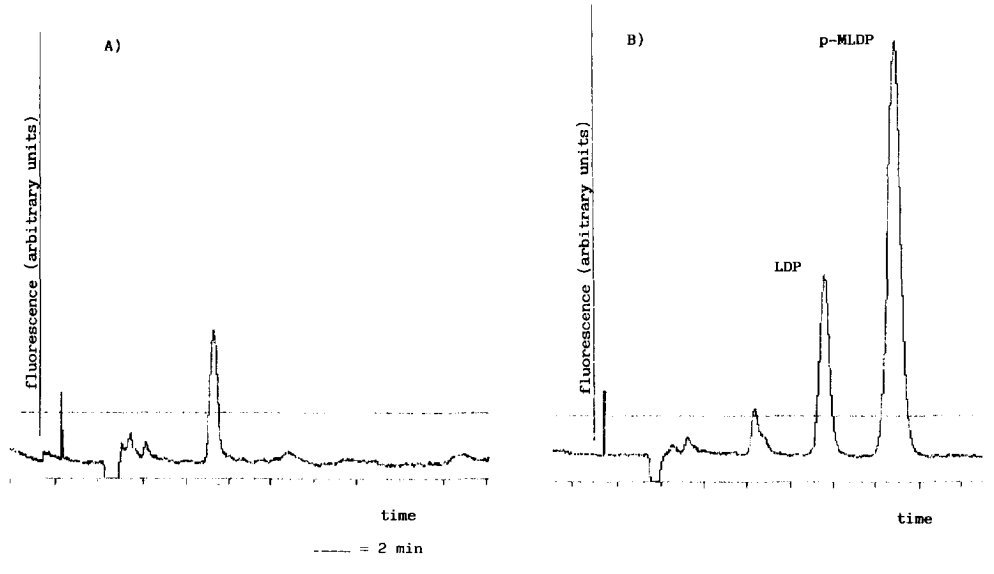

Fig. 1. (A) Chromatogram from a blank serum sample; (B) chromatogram from the serum of a patient taking an oral preparation of LDP (Levotuss, Dompé), in which 26 ng/ml LDP were measured. Flow-rate, 0.7 ml/min; paper speed, 4 mm/min; other analytical conditions are described in Section 2.2.

determined with virtually no interference from the matrix. Thus, even under real conditions, the mere electric noise of the detector was the limiting factor of sensitivity, a situation which is relatively rare when working with biological extracts.

No appreciable differences in either extraction recovery or chromatographic pattern were found between human plasma and serum, which could be processed the same way with identical results.

4. Conclusions

The HPLC–FL method reported here is, to the best of our knowledge, the first based on the direct fluorescence of LDP to be published. It proved suitable for the highly sensitive determination of this anti-tussive drug in plasma or serum with minimal sample pre-treatment. The sensitivity achieved is higher than that reported with the reference GC–MS assay [4]; accuracy and precision are comparable to the best chromatographic methods and sample pre-treatment needed is remarkably simple. In fact, the selectivity of fluorescence detection also permitted LDP to be determined where UV absorbing co-extractives were present, which the rapid but rough extraction procedure adopted could not eliminate.

Due to its simplicity, the method is rugged and can be easily automated.

Thus, the present HPLC–FL method should be a useful tool for further clinical and pharmacological studies on LDP. However, it seems also a further contribution stressing the potential usefulness of HPLC–FL for liquid chromatographic assays in biological samples. In fact, the efficiency of modern fluorometers is such that not only traditionally fluorescent molecules with polycyclic aromatic fluorophores can be detected with high sensitivity, but also simpler molecules, like many drugs containing a benzene ring, are susceptible to sensitive detection without derivatization. Moreover, the direct fluorescence of these molecules, which is limited within the UV band, has often been overlooked for analytical purposes, but in some instances (see Ref. [6], for an application for cocaine determination), modern fluorometers allow the achievement of high sensitivity in the ng/mg range with better selectivity towards matrix co-extractives than the more traditional UV detection.

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