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# Interference in Clinical Laboratory Tests, with Special Regard to the Bilirubin Assay: Effects of a Metabolite of the New Prolyl 4-Hydroxylase Inhibitor, Lufironil

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**Summary:** During the toxicological examination of the fibrosuppressive agent, Lufironil (INN), in rats a dose-dependent positive reaction for urinary bilirubin was observed. This positive reaction was found in quantitative assays, and when using test strips.

The positive reaction for bilirubin in these assay systems was caused by a metabolite of Lufironil. It was not due to drug toxicity, and it was not caused by any endogenous substrate produced under the influence of Lufironil.

The compound responsible for this reaction was isolated by HPLC and its structure determined by spectroscopic methods. The structure was confirmed by synthesis, starting from pyridine-2,4-dicarboxylate. The synthesized compound and the compound in urine gave an identical reaction with the test reagent for bilirubin.

## Introduction

Reduction of the pathological accumulation of collagen in liver by an inhibitor of prolyl 4-hydroxylase[1]), a key enzyme of collagen biosynthesis, offers a new and promising approach in the treatment of liver fibrosis in man (1–3). Lufironil (fig. 1) is selectively activated to a competitive inhibitor of prolyl 4-hydroxylase in the liver of rats (4). Lufironil is rapidly and completely absorbed after oral administration in rats and dogs. About 75% of the compound and its metabolites are excreted in the urine (5). Studies in humans were initiated in 1990 (6).

During the toxicological examination of Lufironil, a highly positive reaction for bilirubin was observed in urine samples of rats using qualitative urinalysis tests, although the serum values remained in the normal range.

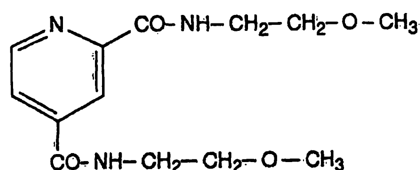


**Fig. 1** Chemical structure of Lufironil (INN), pyridine-2, 4-dicarboxylic acid bis- ((2-methoxyethyl)-amide), [Company code number: HOE 077].

Drug interference in clinical laboratory tests are well known, and a number of compounds have been published which interfere with bilirubin assay systems (7–10).

The aim of the study was to clarify whether the positive results obtained with the bilirubin assay systems in urine were caused by a toxic effect of Lufironil, or by a chemical reaction of a metabolite of Lufironil with the bilirubin test reagent.

## Materials and Methods

### Measurement of urinary bilirubin in a toxicity study in rats after repeated administrations of Lufironil

Lufironil was given to groups of 10 male and 10 female Wistar rats (110 g body weight) in doses of 100, 500 and 2500 mg/kg on 30 consecutive days. The drug was dissolved in deionized water

[1]) Enzymes: Bilirubin oxidase EC 1.3.3.5, Prolyl 4-hydroxylase EC 1.14.11.2.

and administered orally in a volume of 5 ml/kg. Two groups, n = 10 each, of age and sex-matched rats served as controls. The animals were kept under standard conditions, with a night/day rhythm from 6 p. m. to 6 a. m., at 22 °C room temperature, and fed standard pellets Altromin™ 1234 (Altromin GmbH, Lage/Lippe, Germany) and water ad libitum.

During the last week of the study the animals were placed in metabolic cages for urine collection overnight (approximately 16 hours). The concentration of bilirubin in these urine samples was measured, using qualitative and quantitative bilirubin assays (11–13).

On the day of necropsy, before the animals were sacrificed, blood was withdrawn from the conscious, non-fasted animals for analysis of the blood cell count and determination of serum values.

Investigations of serum values and macroscopical and histological examinations were performed according to *Donaubauer & Mayer* (14).

### Measurement of bilirubin in the urine of female rats after a single administration of Lufironil

Female Sprague Dawley rats (300 ± 30 g body weight) received a single dose of 1000 mg/kg Lufironil (n = 8). The compound was dissolved in saline and administered orally in a volume of 2 ml/kg. Control rats received the saline alone (n = 7). Fifteen minutes later the animals were anaesthetized by intramuscular injection of urethane (1.5 g/kg body weight). 60 minutes after the i. m. injection the animals were laparotomized and the urinary bladder drained. Saline was infused via the jugular vein with a flow rate of 6 ml/h per rat to induce diuresis, in order to obtain sufficient amounts of urine for analysis. Urine samples were collected at 30 minute intervals for a total period of 10 hours, the volume of the samples was noted and the bilirubin concentration in the urine was measured using the method of *Jendrassik & Grof* (13).

### Bilirubin test strips and tablets

Rapignost Total-Screen, Behringwerke AG, Marburg, Germany
Ictotest, Ames, Bayer Diagnostic, München, Germany

### Determination of total and conjugated bilirubin

Jendrassik-Grof, Merck, Darmstadt, Germany, cat. No. 3333
DPD-Method, BMC, Mannheim, Germany, cat. No. 123943

### Enzymatic bilirubin test

Bilirubin oxidase[1]) assay, Sigma, St. Louis, USA, cat. No. B 0390

### High-Performance Liquid Chromatography (HPLC)

HPLC: Spectra Physics, Darmstadt, Germany; Pump SP 8800, Auto sampler SP 8780, Integrator SP 4270, UV-Detector SP 8490.
Detection wavelength: 578 nm
Column: Nucleosil 120-5 C18 ET 125/8/4, Macherey + Nagel, Düren, Germany.
Gradient elution: Eluent A = 0.05 mol/l phosphoric acid + 20 ml/l
Eluent B = acetonitrile, 5 min 100% A, 20 min 90% A, 25 min 60% A, 30 min 60% A, 35 min 100% A
Flow rate: 1.2 ml/min.

### Spectroscopic measurements

UV spectra: Perkin-Elmer 554, Überlingen, Germany
NMR spectra: Bruker AM-270, Karlsruhe, Germany
Mass spectra: VG Analytical ZAB-2SEQ, Manchester, United Kingdom, equipped with a caesium ion gun operated at 30 kV; FAB matrix 3-nitro-benzylalcohol, polyethylene glycol-400 used as internal reference for high-resolution mass measurement.

### Radiochemical determination

Female Wistar rats (250–260 g body weight) received a single dose of 1000 mg/kg Lufironil (n = 3). The compound was dissolved in saline and administered orally in a volume of 4 ml/kg. Three hours later, these same animals received a second oral dose of 1000 mg/kg $^{14}$C-labelled Lufironil, (2-carbamoyl-$^{14}$C), (5). Thereafter the animals were placed in metabolic cages and urine was collected for 24 hours; the urine from all rats was pooled (U1). An aliquot (1 ml) of U1 was added to the 10 ml dichlorophenyldiazonium (DPD) reagent (15, 16). The subsequent separation by HPLC of the methanolic solution of this reaction product was performed at 578 nm, and radioactivity was detected, using the following equipment and conditions:

HPLC: series 8800, Dupont, Wilmington, United Kingdom;
Photometer with variable wavelength: SF 770, Applied Biosystems GmbH, Weiterstadt, Germany;
Absorption wavelength: 578 nm;
Radioactivity detector: LB 5026, Berthold, Wildbad, Germany;
Stationary phase: LiChrospher RP-18 (5 µm), 250 mm × 4 mm, Bischoff, Leonberg, Germany;
Mobile phase: eluent A: 0.01 mol/l phosphoric acid, eluent B 10 min 37% A–0% A, 10 min 0%–100% A;
Flow rate: 1.5 ml/min at 25 °C.

### Statistical methods

For detection of significant differences, the unpaired t-test was used. In cases of non-homogeneity of variances, the non-parametric U-test, according to *Mann-Whitney* was used. Dose response relationships were calculated by linear regression analysis (17).

## Results

### Bilirubin in urine in the toxicity study in rats after repeated administrations of Lufironil

During the toxicological examination of Lufironil in rats, a positive reaction for urinary bilirubin was observed when urine was analysed according to Rapignost and Ictotest (11, 12). To quantify the results, the urine samples were analysed using the bilirubin assay according to *Jendrassik & Grof* (13).

Bilirubin concentrations in urine from male and female rats increased dramatically, and were significantly ($r = 0.933, p < 0.001$, males and $r = 0.896, p < 0.001$ in females) correlated with the doses of Lufironil applied. The increase of bilirubin in urine was slightly more pronounced in female rats than in male rats (tab. 1).

In serum however, there was no difference in bilirubin levels between control and drug-treated rats of either sex. Identical values were obtained for control and drug-treated animals when serum was analysed with the bilirubin oxidase assay (18) or the colorimetric method of *Jendrassik & Grof* (13). In the analysis of urine, however, the enzymatic assay detected no bilirubin in urine in either group, whereas the colorimetric method gave a positive response for bilirubin in the treatment group (data not shown).

**Tab. 1** Bilirubin in urine in the toxicity study in male and female rats after repeated administrations of Lufironil

| Treatment | Dose[a] mg/kg | Sex | N | Bilirubin in urine μmol/l |
|---|---|---|---|---|
| Control | – | ♂ | 10 | 29 ± 4.2 |
| Lufironil | 100 | ♂ | 10 | 52 ± 6.4** |
| Lufironil | 500 | ♂ | 10 | 71 ± 10** |
| Lufironil | 2500 | ♂ | 10 | 159 ± 30** |
| Control | – | ♀ | 10 | 35 ± 5.9 |
| Lufironil | 100 | ♀ | 10 | 61 ± 9.5** |
| Lufironil | 500 | ♀ | 10 | 110 ± 12** |
| Lufironil | 2500 | ♀ | 10 | 179 ± 31** |

Results are means ± SD
** $p < 0.01$ vs. control
a: Total daily dose given orally

These findings indicated that the positive bilirubin reaction obtained using the colorimetric method was probably not caused by bilirubin in the urine.

## Bilirubin in the urine of rats after a single administration of Lufironil

Lufironil given as a single dose of 1000 mg/kg to rats caused a slight diuretic effect, which had also been observed in the preceding toxicity study.

As seen in the toxicity study, the urine of treated animals showed a tremendous increase of a positive bilirubin reaction, with values 26-times higher than those recorded for control animals (tab. 2).

The appearance of this positive bilirubin reaction, henceforth referred to as the *"bilirubin-like reactivity"*, started 2 hours after administration of Lufironil and rose to a maximal value of 37 nmol/0.5 h within 1 hour. The bilirubin-like reactivity plateaued for 4 hours, than declined slowly over the next 3 hours (fig. 2).

**Tab. 2** Urine secretion and bilirubin in urine after administration of a single dose of 1000 mg/kg p. o. Lufironil to female rats

| Treatment | Dose mg/kg | N | Urine[a] μl/0.5 h | Bilirubin in urine | |
|---|---|---|---|---|---|
| | | | | Total[b] nmol/10 h | Average[c] nmol/0.5 h |
| Control | – | 7 | 492 ± 276 | 19.1 | 1.03 ± 0.28 |
| Lufironil | 1000 | 8 | 745 ± 334* | 489.0 | 30.21 ± 9.08** |

Results are means ± SD
* $p < 0.05$
** $p < 0.01$ vs. control
a: Average output calculated from the whole observation period (10 h)
b: Total output calculated from the whole observation period (10 h)
c: Average output from 2.5–10 h after application of a single oral dose of 1000 mg/kg Lufironil

## Isolation of the coupling compound (IV)

To obtain the compound responsible for the bilirubin-like reactivity, a large amount of rat urine was needed. Urine samples from female Sprague Dawley rats, treated orally with a daily dose of 2500 mg/kg Lufironil over a period of 6 months, were pooled and stored frozen.

Since the diazo coupling compound of sulphanilic acid is insoluble, and therefore unsuitable for HPLC separation, the DPD-method, using 2,5-dichlorobenzene as reagent (15, 16) was chosen.

To 5 ml portions of urine, 2 ml diazonium salt (DPD-method) dissolved in 0.1 mol/l hydrochloric acid were added. The coupling product was extracted into 4 ml dichloromethane.

For the first purification step, a Bond Elut silica column (pre-treated with 2 ml dichloromethane) was loaded with the extract.

The column was washed 4 times with 2 ml dichloromethane, then the compound was eluted with 0.5 ml of a mixture of dichloromethane and methanol (7 + 1, by vol.). After evaporation to dryness, the residue was dissolved in 100 μl methanol and separated by HPLC. The isolated compound had a retention time of 24 minutes.

## Identification of the coupling compound (IV)

The isolated compound had a deep blue colour ($\lambda_{max}$ 593 nm, ε 2000 $m^2$/mol) in dichloromethane, whereas it had a red violet colour ($\lambda_{max}$ 543 nm) in methanol, indi-

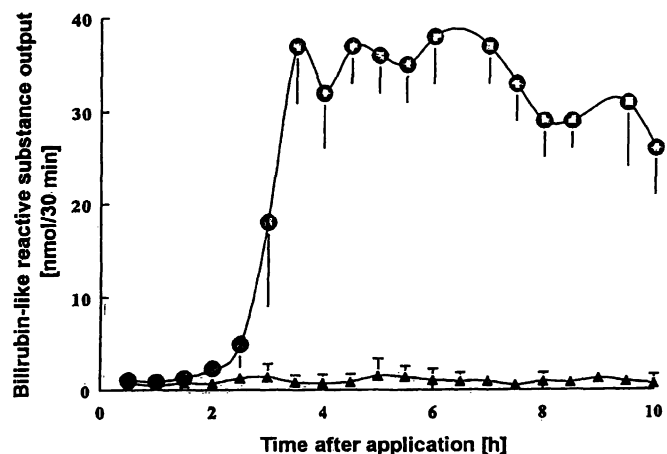


**Fig. 2** Time-dependent appearance of the bilirubin-like reactivity in the urine of female rats. Results are means ± SD. Closed circles ●–● represent the rats given a single oral dose of 1000 mg/kg Lufironil (n = 8), closed triangles ▲–▲ represent the control group given saline alone (n = 7).

Tab. 3 Proton NMR signals of the coupling compound (IV), (270 MHz, $CDCl_3$)

| Substructural unit | | (ppm), multiplicity |
|---|---|---|
| 2,5-Dichloro-phenylazo | 3-H | 7.25, d, 8 Hz |
| | 4-H | 6.9, dd, 8 + 2.5 Hz |
| | 6-H | 7.6, d, 2.5 Hz |
| Pyridine-2, 4-dicarboxamide | 3-H | 8.65, d, 2.5 Hz |
| | 5-H | 8.35, dd, 7 + 2 Hz |
| | 6-H | 9.2, d, 7 Hz |
| Methoxyethyl-amine | $N(CH_2)_2O$ | 3.65 + 3.75, m |
| | $OCH_3$ | 3.45, s |

cating the presence of a charged chromophoric system. High-resolution FAB mass spectrometry gave a molecular formula of $C_{17}H_{15}Cl_2N_5O_3$ ($M + H^+$ found m/z 408.0629, calculated 408.0630). The NMR spectrum displayed signals for one 2,5-dichloro-phenylazo group, one pyridine-2,4-dicarboxamide moiety, and one methoxyethylamine group (tab. 3). From these data the structural formula (IV) for the red dye was deduced. It was concluded that an azo-coupling product of the (possibly unstable) cyclic zwitterion (III) could have been formed under the acidic coupling conditions from the hydroxymethylene amide (II), a hitherto unknown metabolite of Lufironil (fig. 3).

## Synthesis of the metabolite (II)

The metabolite (II) was synthesized by treatment of pyridine-4-carboxy-(2-methoxyethyl)-amide-2-carboxamide (V) (synthesized according to *Baader* (19)) with 35% formaldehyde and potassium carbonate in water at room temperature for 20 hours (fig. 3).

The product was extracted with dichloromethane. The organic solvent was separated from the aqueous layer, dried with sodium carbonate and concentrated.

Pyridine-4-carboxy-(2-methoxy-ethyl)-amide-2-carboxy-(hydroxy-methyl)-amide (II), was obtained as a colourless solid (mp. 94 °C). This compound, when treated in aqueous solution with the bilirubin test reagent, gave the same colour reaction as that observed with the urine samples. The product was also spectroscopically identical with the red dye (UV, MS, NMR) after HPLC purification.

## Radiochemical analysis

That the false positive bilirubin reaction in urine was caused by a metabolite of Lufironil was further confirmed by radiochemical methods.

Investigation of the pooled urine (U1) from rats given a single dose of 1000 mg/kg unlabelled Lufironil and second a single dose of 1000 mg/kg of $^{14}C$-labelled Lufironil revealed that 63% of the administered dose was excreted in the urine.

When the diazonium salt (according to the DPD-method (15, 16)) was added to the urine, a deep blue-coloured reaction product was formed following the addition of dichloromethane.

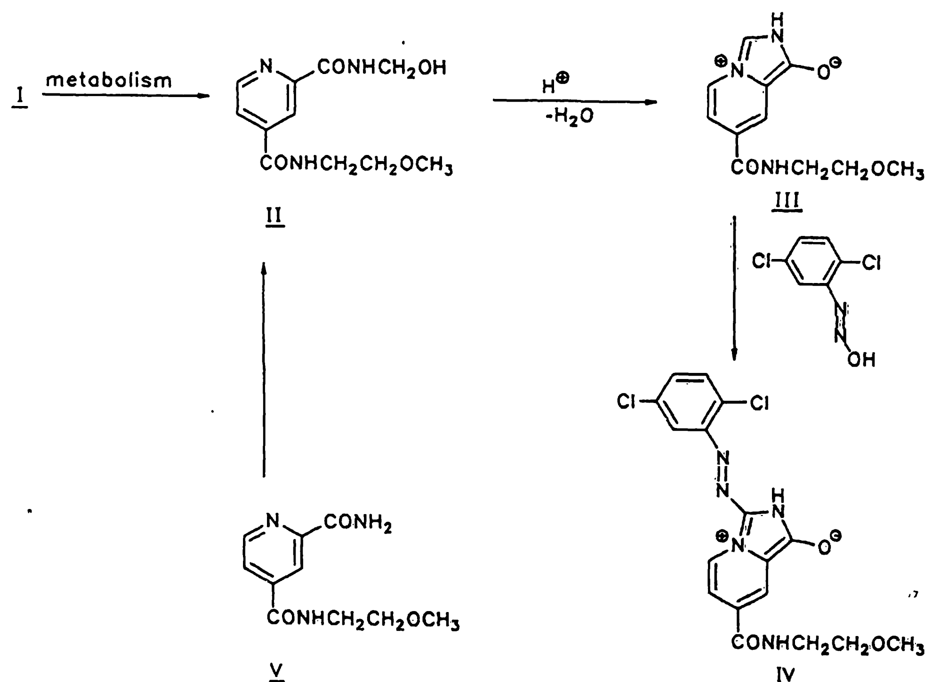


Fig. 3 Sequence of preparation of the coupling compound (IV), steps I to IV. Sequence of synthesis of the metabolite (II), steps V to II.

After extraction of the processed urine with dichloromethane, most of the radioactivity detected by HPLC was found to be present in Lufironil and its known metabolites. Only approximately 1% of the extracted radioactivity appeared on the chromatogram, with a retention time of 47.8 minutes. This peak coincided exactly with the absorption peak of the compound formed by reaction of the putative Lufironil metabolite (II) with the bilirubin reagent used in the DPD-method. From the amount of radioactivity administered and the quantities of the metabolite (II) found by HPLC, it is calculated that this metabolite accounts for less than 0.1% of the amount of Lufironil given to the animals. Thus it is unlikely that this metabolite would have been detected, if the unusual interference with the conventional bilirubin assay system had not occurred.

## Discussion

Total bilirubin in serum of healthy male and female Sprague Dawley rats ranges between 1.09–6.45 μmol/l (20). No bilirubin is present in the urine. The very small amounts of diazo-positive reaction products in urine samples (tab. 2) originate from other dipyrrole-containing structures as urobilinogen and uroporphyrin.

When bilirubin in serum exceeds concentrations of about 15 μmol/l, however, it is excreted through the kidney and appears in the urine, where it can be detected by commercially available bilirubin assay systems.

An increase of bilirubin in serum, and consequently urine, generally reflects an imbalance between bilirubin production, for which the predominant source is haemoglobin degradation (21), and bilirubin excretion by hepatocytes into the bile. Various conditions are known to generate such an imbalance: overproduction of bilirubin under haemolytic conditions, intra- or extra-hepatic obstruction of the biliary system, or a decreased capacity of hepatic bilirubin uptake combined with decreased capacity of the hepatocytes to conjugate and/or secrete the conjugated product into the bile (22). The latter condition is usually the consequence of hepatocellular damage or death, caused by infectious agents or induced by drugs, toxic to the liver.

Haematological values obtained in the toxicity study of Lufironil did not give any signs of a haemolytic process, leading to overproduction of bilirubin. Haemoglobin and red cell and reticulocyte counts were within their normal ranges.

Histological examination of the rat livers and the biliary tract of the rats in the toxicity study excluded an intra- or extra-hepatic obstructive process of the biliary draining system (24).

Competition of Lufironil and/or its metabolites with hepatic bilirubin uptake, which is known to occur with the antibiotic rifampicin (23), was unlikely, because this would have led to an elevation of serum bilirubin, which was not found.

A toxic effect of Lufironil on the hepatocytes was excluded by the histological examination of the rat livers from the toxicity study; no abnormalities of the hepatocytes were detected.

In addition, serum levels of the liver enzyme, aspartate aminotransferase, and of bilirubin were in the normal range, while alkaline phosphatase was decreased rather than increased (24).

The finding of normal bilirubin serum levels excluded a true elevation of bilirubin in urine and led us to suspect a false positive bilirubin reaction.

False positive bilirubin reactions using diazo-reagents have been reported after the administration of several drugs: sulphonamides (7), the analgesic flupirtine (8), indican (9) and methotrexate (10).

Therefore we focused our investigations on the possibility that the bilirubin-like reactivity in the urine of rats might have been caused by an unknown metabolite of Lufironil reacting with the diazo reagent of the bilirubin assay systems. We showed that the bilirubin-like reactivity found in rat urine following high doses of Lufironil was a reaction product of a very minor metabolite (II) of Lufironil (fig. 3) with the diazo-reagent.

This finding was of practical importance for ongoing human studies, in the event that bilirubin-like reactivity would be detectable in human urine.

However, elevated urinary bilirubin reactions have not yet been observed in healthy volunteers given Lufironil at doses up to 2400 mg/kg per day (6).

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