Z Rechtsmed (1984) 92:95–100

Zeitschrift für
Rechtsmedizin

Determination of *p*-Nitrophenol in Serum and Urine by Enzymatic and Non-enzymatic Conjugate Hydrolysis and HPLC. Application after Parathion Intoxication* **

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Summary. In connection with the toxicologic analysis of a number of parathion intoxications a method for determination of free and conjugated forms of *p*-nitrophenol (*p*-NP) as the main metabolite of parathion in blood and urine was established.

Quantification of conjugates is based on their hydrolysis followed by detection of *p*-NP using a sensitive HPLC method. Hydrolysis of both *p*-NP-glucuronide and *p*-NP-sulfate is performed by specific enzymes and also by mineral acid, the latter is also found to be highly selective under definite conditions. The two hydrolysis methods applied showed a good correlation. The levels of free and conjugated *p*-NP in series of blood and urine samples were established after survival from two parathion intoxications. The individual levels of *p*-NP-sulfate and *p*-NP-glucuronide in both cases are discussed in respect of results made by other authors in this field.

Key words: Parathion, enzyme hydrolysis of *p*-nitrophenol conjugates – Quantification by HPLC, parathion – Toxicology, parathion

Zusammenfassung. Zur toxikologischen Analytik von Parathion-Vergiftungen wurde eine Methode zur Bestimmung von freiem und konjugiertem *p*-Nitrophenol (*p*-NP) als Hauptmetabolit von Parathion in Blut und Urin erstellt.

Grundlage des Verfahrens bildet ein empfindlicher HPLC-Nachweis von *p*-NP nach Hydrolyse der Konjugate. Die Hydrolyse sowohl von *p*-NP-Glucuronid als auch von *p*-NP-Sulfat erfolgt enzymatisch und durch Säureeinwirkung, wobei auch das letztere Verfahren unter definierten Bedingungen sehr spezifisch ist. Beide Hydrolyseverfahren zeigten im Ergebnis gute Übereinstimmung. Die Spiegel von freiem und konjugiertem *p*-NP in einer Reihe von Blut- und Urinproben wurden nach zwei Fällen überlebter Parathion-Vergiftungen bestimmt. Die unterschiedlichen Spiegel von *p*-NP-Sulfat und *p*-NP-Glucuronid werden für beide Fälle diskutiert und Ergebnisse anderer Autoren herangezogen.

* Dedicated to Prof. H. Schweitzer, MD, on the occasion of his 65th birthday

** Presented in part at the 1st Asian-Pacific Congress on Legal Medicine and Forensic Sciences held on September 18–22, 1983, in Singapore

Schlüsselwörter: Parathion, Enzymspaltung von *p*-Nitrophenol-Konjugaten - Quantifizierung durch HPLC, Parathion - Toxikologie, Parathion

Among the phosphorus esters used as pesticides parathion is the most recognized one, especially in the form of the widely used product E 605 traded on the market.

This fact combined with the severe effects also on mammalians—as a cholinesterase blocker—is responsible for the significant importance of parathion intoxications in both clinical and forensic toxicology.

Mainly the following two methods of toxicologic analysis of parathion intoxications have been used: (1) direct gas chromatographic determination in plasma [1–4] and (2) photometric determination of the main metabolite, *p*-nitrophenol (*p*-NP), in urine resulting from hydrolysis of conjugates followed by coupling a diazo-compound [5–8]. This method—still commonly used—has a relatively low standard of sensitivity and requires rather high amounts of urine. Furthermore, *p*-NP is not suitable for the determination of blood levels.

We developed an HPLC method for the determination of *p*-NP which works with the most simple preparation of very low quantities. The high sensitivity is suitable for the analysis of 5–10 ng *p*-NP/ml urine and blood [9].

The above mentioned characteristics of this method offer the possibility of differentiation and determination of the conjugation forms of *p*-NP [10, 11].

Material and Methods

Of serum and urine 0.5 ml, respectively, was adjusted to the pH optimum of the actual enzyme by adding 0.1 *N* sulfuric acid controlled by an pH meter: pH 5.1, for β-glucuronidase/arylsulfatase; pH 6.8, for β-glucuronidase; and pH 7.1, for arylsulfatase. After addition of 0.5 ml phosphate buffer (according to Soerensen, pH 5.1, pH 6.8, pH 7.1) enzymatic incubation followed for 20 h at 37° C. The *p*-NP was then extracted with 4 ml ether, and after centrifugation 2 ml of the ether layer was aspirated and afterwards evaporated by a stream of nitrogen at ambient temperature. The residue was redissolved in 200 µl methanol, and 20 µl was submitted to HPLC. Two calibration curves were established by the same procedure after extraction of appropriate buffer solutions containing five different *p*-NP concentrations each: 10, 20, 50, 100, 500 ng and 0.5, 1.0, 5.0, 20, 100 µg, respectively.

Enzymes: β-glucuronidase/arylsulfatase (Merck, 4114), arylsulfatase (Sigma, S 1629), β-glucuronidase (Sigma, 105-2000).

HPLC: column C18 Sil-X-10, 250 × 4 mm (Perkin-Elmer), mobile phase 156 g acetonitrile + 344 g phosphate buffer (4.8 g H_3PO_4, 85%, + 6.66 g KH_2PO_4 per ml water, pH 2.30), flow 1 ml/min, isocratic mode, UV detection at 315 nm.

Experiments and Discussion of Results

The following excretion forms of *p*-NP have been mentioned by now in the literature: free *p*-NP, *p*-NP glucuronide, *p*-NP sulfate, and *p*-NP acetate [5, 7, 8].

The presence of *p*-NP acetate could be definitely excluded in the cases investigated taking into consideration the detection limit of about 10 ng/ml urine: the HPLC fraction where *p*-NP acetate was expected to be contained was collected and hydrolyzed under acidic conditions. The method proved that no *p*-NP was found there [10, 11].

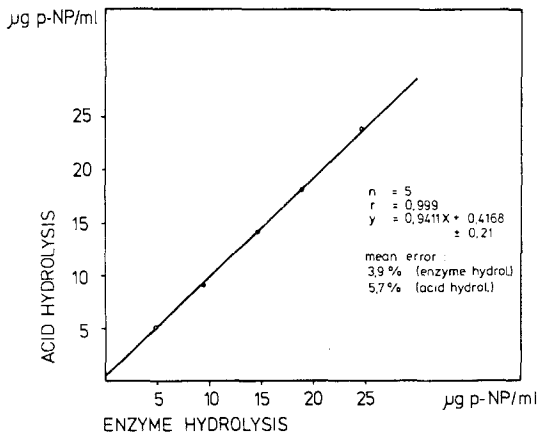

Fig. 1. Hydrolysis of *p*-NP sulfate by sulfatase and sulfuric acid. Comparison of both methods

The main interest was then paid to the quantification of the conjugates *p*-NP glucuronide and *p*-NP sulfate apart from free *p*-NP. As mentioned before, free *p*-NP can be quantified by simple extraction and by HPLC. The two conjugates, *p*-NP glucuronide and *p*-NP sulfate, are splitted together under the drastic conditions of a hydrolysis with sulfuric acid and leveled-up temperature [9]. *P*-NP in total is therefore measured after extraction, i.e., free and bound *p*-NP. The incubation of *β*-glucuronidase/arylsulfatase (from *Helix pomatia*) leads to the same result.

Furthermore, *p*-NP glucuronide can be splitted by enzyme reaction with *β*-glucuronidase (from *Escherichia coli*) selectively. As for the determination of *p*-NP sulfate, a specific hydrolysis using arylsulfatase (from *Aerobacter aerogenes*) is possible under liberation of *p*-NP.

Additionally, it was found out that *p*-NP sulfate can be splitted completely using also half-concentrated sulfuric acid at ambient temperature within 20 min. This reaction is absolutely specific: even after a period of 1 h under these conditions no *p*-NP was liberated from *p*-NP glucuronide.

The main result of the studies is that both *p*-NP glucuronide and *p*-NP sulfate in blood and urine can be quantified either enzymatically or by acid hydrolysis.

In a few separate tests both methods of hydrolysis described were compared especially in respect of *p*-NP sulfate (Fig. 1). Comparison of the two procedures, on one side the enzymatic and on the other side the non-enzymatic, presents a very slight deviation only. Using an amount of five samples, a correlation coefficient of greater than 0.99 and a deviation about the regression line of ± 0.21 are calculated.

By these two different methods blood and urine samples from two severe but survived cases of parathion intoxications were examined for a possible presence of the named conjugates. The resulting levels of *p*-NP shown by the following figures represent average values obtained after application of both methods of hydrolysis described above.

The different levels of free and bound *p*-NP in the first patient's blood are presented in Fig. 2: 30 min after ingestion (i.e., at the moment of gastric lavage

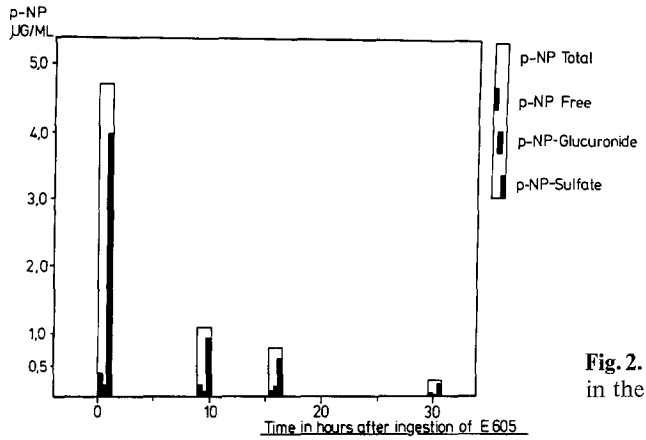

Fig. 2. Free and conjugated *p*-NP in the blood of patient 1

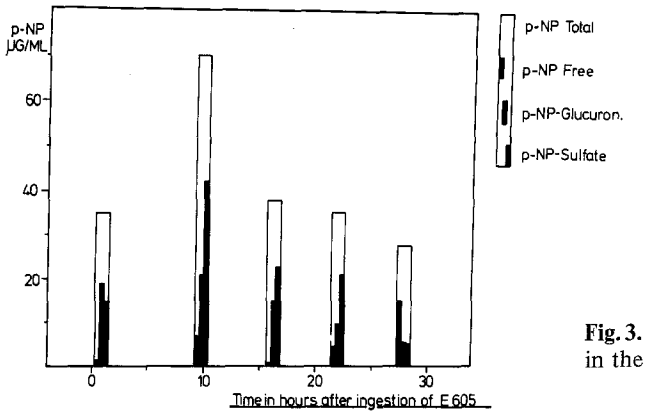

Fig. 3. Free and conjugated *p*-NP in the urine of patient 1

and the end of resorption) the level of *p*-NP in total amounts to 4.7 µg/ml plasma, and the conjugation rate is found to be 92.2%, an unexpected high standard.

During 30.5 h an elimination down to 5.3% of the original total *p*-NP value took place implying 84% of conjugated *p*-NP.

The very high levels of sulfate found in all four blood samples had been unexpected: the first blood sample contained over 85% of *p*-NP in total. Contrary to this, the values of *p*-NP glucuronide were relatively low.

The urine samples of the same patient (Fig. 3) also showed mostly high levels of sulfate. Besides, the samples contained relatively high values of *p*-NP glucuronide exceeding those in the first sample. The maximum conjugation rate is reached in the first (98%), the minimum is found in the last sample (45.1%). The correlation of the valuewise dominating sulfate levels in the blood with the relatively high glucuronide levels in the urine can be explained if presuming a short half-life of *p*-NP glucuronide in blood. This assumption would, of course, require a far better renal clearance.

The period of evaluation extended to 60 h for the second patient where only urine samples were available (Fig. 4). In the beginning, i.e., 8 h after ingestion, the total *p*-NP level of 24.55 µg/ml urine reached about half the standard of the first patient. Special attention should be paid to the overweight of *p*-NP

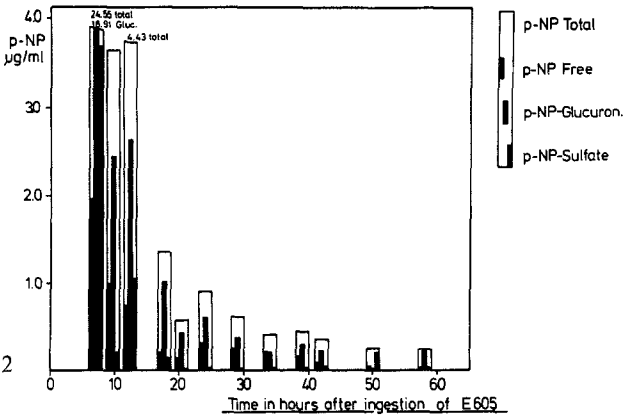

Fig. 4. Free and conjugated *p*-NP in the urine of patient 2

Table 1. Percentage of *p*-NP as glucuronide and as sulfate related to *p*-NP in total in the urine samples of both patients

Urine fractions (hours after ingestion)	*p*-NP as glucuronide (%)		*p*-NP as sulfate (%)	
	Patient 1	Patient 2	Patient 1	Patient 2
0.5	56		42	
8.0		77		15
9.5	30		60	
10.0		67		5
12.0		59		24
16.5	37		61	
18.0		75		10
20.0		74		2
22.5	27		61	
24.0		67		–
28.5	20		18	
29.0		60		–
34.0		49		–
39.0		65		2
42.0		64		12
50.0		–		86
58.0		98		–

glucuronide levels in nearly all samples contrary to the dominating sulfate levels of the first patient.

Actually, individual and different rates of metabolism as well as metabolism profiles are known in general for various drugs caused by different activity of enzymatic systems.

In this context recently published results shall be mentioned [12]: The conjugation rate for *p*-NP was examined in animal experiments after i.v. administra-

tion of *p*-NP as well as after *p*-NP incubation of homogenates of the liver, the kidneys, the lungs, and the small intestine.

The authors' main results were that sulfatation practically proceeds in the liver exclusively in contrast to glucuronidation which takes place in all the above mentioned organs.

Provided that the availability of these results is allowed to be extended to my own findings, the metabolism profile of the first patient is characterized by high hepatic sulfatation activity (except for last sample). Contrary to this, the second patient shows leveled-up hepatic or extrahepatic glucuronidation activity besides low and varying hepatic sulfatation.

A synopsis containing the percentage of *p*-NP as glucuronide and as sulfate related to total *p*-NP in urine samples of both patients is given in Table 1.

To which extent conjugates even had been splitted after elimination—presumably most likely by enzyme influence—cannot be answered definitely. The relatively high levels of free *p*-NP in some cases—to mention especially urine sample 5 of the first patient—might suggest such a process.

Acknowledgments. The author is grateful to Dr. H. J. Trampisch for his statistical advice and to Miss Petra Jahn for her review of the manuscript.

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Received October 14, 1983