available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/ejps

Characterization of oxalic acid derivatives as new metabolites of metamizol (dipyrone) in incubated hen's egg and human

Julia C. Wessel[a], *Magdalena Matyja*[a], *Michael Neugebauer*[b], *Heiko Kiefer*[b], *Thomas Daldrup*[c], *Fuad A. Tarbah*[c], *Horst Weber*[a,*]

[a] *Institut für Pharmazeutische und Medizinische Chemie, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany*

[b] *Pharmazeutisches Institut, Rheinische Friedrich-Wilhelms-Universität Bonn, Kreuzbergweg 26, 53115 Bonn, Germany*

[c] *Institut für Rechtsmedizin, Heinrich-Heine-Universität Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf, Germany*

ARTICLE INFO

Article history:
Received 17 August 2005
Received in revised form 24 November 2005
Accepted 28 November 2005
Published on line 18 January 2006

Keywords:
Drug metabolism
Metamizol (dipyrone)
Pyrazolinone ring opening
Oxalic acid derivatives as drug metabolites

ABSTRACT

Metamizol (dipyrone, **1**), a widely used drug with effective analgesic and antispasmodic properties, shows severe side effects like agranulocytosis and anaphylactic shock reactions, the reasons of which are not known until today. After oral administration **1** is completely metabolized. All hitherto known metabolites have an intact pyrazolinone ring structure like the parent compound and are completely extractable from urine with polar organic solvents. However, only a fractional amount of the applied dosage can be recovered by this procedure. To clarify the reason of this deficit of unknown metabolites we followed the hypothesis of oxidative rupture of the heterocyclic ring during metabolism of **1**. On the basis of former in vitro results we now were able to identify in quality three oxalic acid derivatives and one acetic acid phenylhydrazide as new metabolites of metamizol in the allantoic fluid (AF) of incubated hen's eggs as well as in human urine by means of GC–MS analysis and comparison with unequivocally synthesized authentic reference compounds. Whereas the oxamazide **7**, the phenylhydrazide **8** and *N*-methyloxamic acid **9** are only present in trace concentrations and therefore cannot account for the deficit in the balance of metabolites, the oxalic acid monohydrazide **11** seems to be excreted in higher amount. But quantitative determination of this new metabolite would be required to answer the open questions concerning the biotransformation of metamizol and thereby to detect new facts about mode of action and side effects of this drug.

1. Introduction

For 80 years, the sodium salt of metamizol (dipyrone **1**) is a widely used therapeutic drug. Due to its effective analgesic and antispasmodic properties it is indicated for severe pain and for colic of gall bladder and kidney and is commonly used in several countries in Europe, Asia and South America. In numerous developing countries it is the first line analgesic, because other analgetic drugs are not readily available (Schonhofer et al., 2003; Bensenor, 2001). In contrast to this, **1** has been banned in other regions (USA, UK, Sweden) because of its adverse drug reactions, notably agranulocytosis and ana-

* *Corresponding author*. Tel.: +49 211 8113846; fax: +49 211 204336.
E-mail address: Horst.Weber@uni-duesseldorf.de (H. Weber).

0928-0987/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejps.2005.11.010

phylactic shock reactions (Arznei-telegramm, 1974; Arellano and Sacristan, 1990). In Germany and other countries the use is restricted to special kinds of diseases.

While the International Study of Agranulocytosis and Aplastic Anemia (IAAAS) estimated a low risk of agranulocytosis after administration of **1** (1.1 cases per 1 million users, The IAAAS Group, 1986), a Swedish investigation quoted a higher rate of at least 1:1439 prescriptions (Hedenkalm and Spigset, 2002). The reasons for these severe side effects are not known and the responsible metabolites could not yet been identified.

Even the mechanism of action of metamizol is still unknown. It has been proposed that the antinocizeptive action of metamizol is mediated by its inhibition of cyclooxygenase-isoforms (Lanz et al., 1985; Weithmann and Alpermann, 1985; Campos et al., 1999; Chandrasekharan et al., 2002). On the other hand, a centrally mediated action has been suggested, including opioid receptors (Taylor et al., 1998; Beirith et al., 1998; Collares and Vinagre, 2003). In spite of the knowledge that metamizol is a prodrug and spontaneously hydrolyzed after oral intake in the gastric fluid to its effective metabolite 4-methylaminoantipyrine (**2**), most of the in vitro investigations has been carried out using **1** itself. Consequently, the results of the studies using the prodrug are not suitable to clear this problem. In addition, the metabolism of this classical drug is to date not completely clarified. The metabolites, which generate the excellent analgetic action as well as the severe side effects, are still unknown. Due to these unsolved problems the therapeutic use of **1** is still discussed controversially.

In order to clarify these open questions it would be necessary to have reliable facts with regard to the metabolic properties of this well known drug.

So far it is known that after oral administration of [^{14}C]-metamizol to man the absorption is rapid and uniform. After 48 h 96% and after 72 h 99% of the administrated radioactivity can be found in the human urine as quantity of radiation without regarding special metabolites (Christ et al., 1973). Four main metabolites **2**, **3**, **4** and **5** have been identified accounting only for 65–70% of a single dose of 480 mg of metamizol (Fig. 1). This apparent deficit of unidentified metabolites could be attributable to inadequate analytical methods and/or decomposition of metabolites (Volz and Kellner, 1980). Later on, pharmacokinetic of metamizol was studied in healthy subjects after various single doses of metamizole. The cumulative amount of main metabolites **2**–**5** excreted in urine after 72 h was determined by reversed-phase HPLC after extraction with chloroform. But only less than 50% of the corresponding dose could be recovered as defined metabolites (Vlahov et al., 1990). These results have been confirmed by a series of studies presented in an extensive review of dipyrone pharmacokinetics showing that the rate of metabolism and the composition of metabolites obviously depend on individual differences of probationers like rapid or slow acetylators (Levy et al., 1995).

Further metabolites like 4-hydroxyantipyrin (**6**) as sulfate or glucuronide conjugates and a number of other pyrazolinones derived from metamizol by chemical modification of the pyrazolinone substituents only account for minor quantities. In the literature, non-heterocyclic metabolites are mentioned only in a meeting abstract describing the biotransformation after oral and inravenous administration of ^{14}C- and ^{13}C-labeled metamizol (Volz et al., 1993). In this

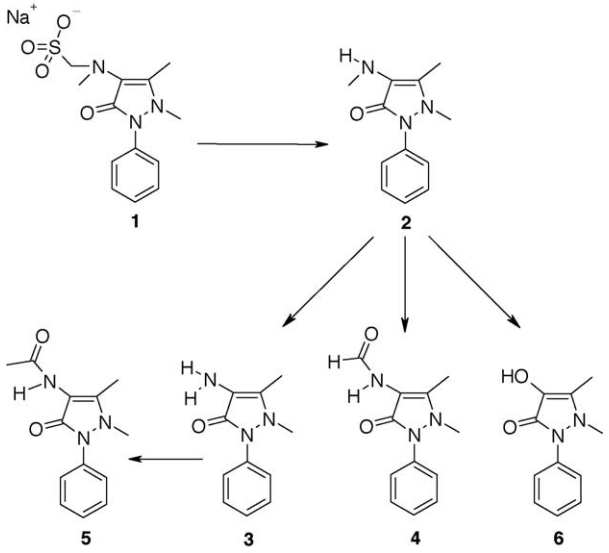

Fig. 1 – Main metabolites of 1 in human, extractable from urine with polar solvents.

short communication the authors affirmed the isolation of in total "29 mostly polar peaks". Using NMR-spectroscopy and mass spectrometry they were able to propose chemical structures for 26 of those peaks. Unfortunately, these results have never been published elsewhere in detail but it is noteworthy that one of the identified compounds was assigned to the phenylhydrazide **8** (Fig. 2) and one other as a derivative of **8** with unknown additional substituents. The total amount of elucidated metabolites in urine of man was added by these authors up to 86% of the given dose. So it still remains a deficit of unknown metabolites and pyrazolinone ring opening during metabolism of **1** seems to be possible. Previous own experiments showed that the pyrazolinone ring of **2** is easily opened by various oxidants under physiological conditions (Duchstein et al., 1988; Weber and Wollenberg, 1988; Weber and Bresser, 1996). The resulting oxamazide **7** is gently hydrolyzed at pH 7.4 and 37 °C to give 1-methyl-2-phenylacetohydrazide (**8**) and *N*-methyloxamic acid (**9**). The hydrazide **8**, however, is very unstable in the presence of oxidants, forming a variety of following products (Bresser and Weber, 1996). Therefore, the in vivo determination of **8** seems not to be an appropriate method for proving the ring opening of **2** as a real biotransformation reaction of **1**.

For that reason, we followed another strategy considering **9** as a piece of evidence for the metabolic ring opening of **1**. This acid is a fragment resulting from the oxamazide **7** and is – as far as known – no subject of further biotransformation reactions.

In order to elucidate the metabolic stability of **9** we designed a study with a small number of rats treated with an orally administrated aqueous solution of this compound. For this purpose, it was necessary to develop a reliable analytical method for the qualitative and quantitative determination of this oxamic acid in urine of rats, which was also suitable for other biological samples. Later on we applied this method to investigate the biotransformation of **1** and **7** and the recovery of **9** by using incubated hen's eggs. This is a widely used

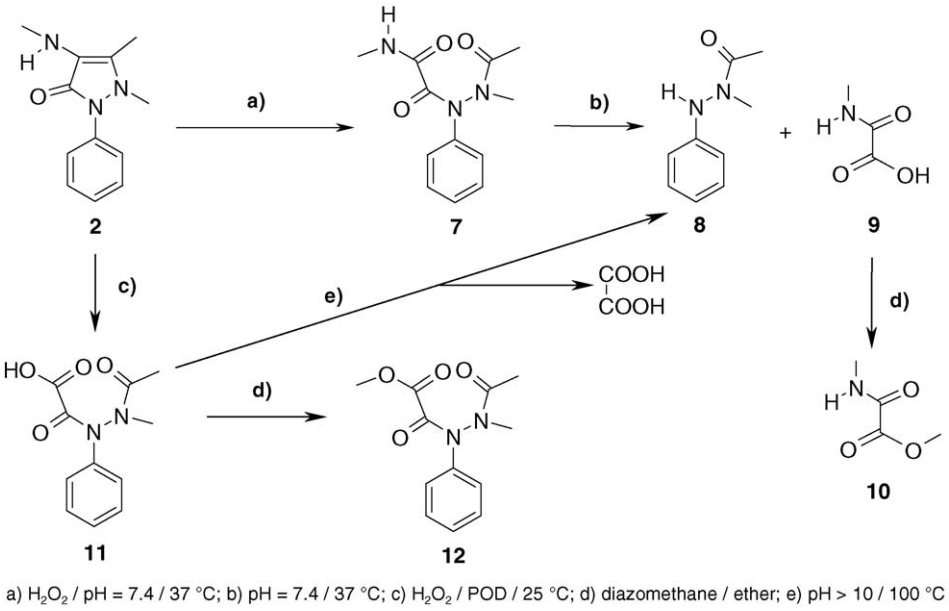

Fig. 2 – Synthesis of oxalic acid derivatives.

model of toxicological and metabolic research of xenobiotics in biology and medicine (Rosenbruch, 1994; Lüpke, 1987; Kiep and Bekemeier, 1986; Neugebauer, 1997). This method offers the possibility to apply a relatively high dosage of test compounds. Our procedure based on previous experiments (Kiep et al., 2002), who compared the biotransformation of **1** in incubated hen's eggs with the metabolism in human. The authors concluded that the incubated hen's egg is an appropriate ex vivo model for simulating the biotransformation of **1** in vivo, because they were able to recover all the well-known metabolites of **1** with an intact pyrazolinone ring. On the basis of these findings we decided to use this model to investigate the production of oxamic acids and oxalylhydrazides during biotransformation of **1**. In this connection, we also elaborated an analytical method for the detection of **7** and **8** to control the production of these compounds in the incubated hen's eggs after application of **1**.

Finally, we explored the formation of oxalic acid derivatives from **1** in human by analyzing the urine of two probationers after a single oral dose of each 1 g of metamizol. By this way, we intended to obtain the definitive evidence for the formation of oxalic acid derivatives in the biotransformation of **1** in human. To this end, it was necessary to synthesize the phenylhydrazides **7** and **8**, the oxalic acid derivatives **9** and **11** and their volatile esters **10** and **12** as authentic reference substances for GC–MS analysis (Fig. 2).

2. Materials and methods[1]

2.1. *Materials*

Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were determined by a Bruker AC-200 (200 MHz) spectrometer (Bruker Analytik GmbH, Rheinstetten, Germany) and peak positions are given in parts per million (δ) downfield from tetramethylsilane as internal standard, whereas coupling constants (J) are given in Hertz. The HMBC-spectrum was recorded on a Bruker DHX (500 MHz). Infrared spectra were measured on a Perkin-Elmer 1600 FT-IR-Spectrometer. Mass spectra of reference compounds were obtained by a Finnigan MAT 4000 (Finnigan GmbH, Puchheim/München, Germany) and by GC–MS analysis (see Section 2.3.5.3). The elemental analyses were determined by a Perkin-Elmer PE 2400 CHN Elemental Analyzer (Perkin-Elmer Instruments GmbH, Rodgau-Jügesheim, Germany). Column chromatography was performed using silica gel 60, 230–400 mesh ASTM (Merck Eurolab GmbH, Darmstadt, Germany). Solutions in organic solvents were dried over anhydrous sodium sulfate. Melting points were obtained in open capillary tubes and are uncorrected. Strongly acid cation exchanger (Amberlite® IRA-120) and strongly basic anion exchanger (Amberlite® IRA-402) were obtained from Merck.

2.2. *Synthesis of compounds*

2.2.1. *Methylaminooxoacetic acid-2-acetyl-2-methyl-1-phenylhydrazide (7)*

Synthesis and spectral data according to literature (Weber and Wollenberg, 1988).

2.2.2. *1-Methyl-2-phenylacetohydrazide (8)*

Synthesis and spectral data according to literature (Weber and Bresser, 1996).

2.2.3. *N-Methyloxamic acid (9)*

The synthesis based on a procedure described in literature (Wallach, 1877).

Diethyl oxalate 14.6 g (0.1 mol) was dissolved in 50 ml ethanol. Dimethylamine (0.1 mol, 40% aqueous solution) was added and stirred for 4 h at room temperature. This mixture

[1] More details are given in the dissertation thesis Wessel (2004).

was alkalized by adding 0.1 mol sodium hydroxide in 50 ml water and heated to reflux for 1 h. After cooling the mixture was diluted with 200 ml water and extracted with chloroform. The extracted aqueous phase was acidified by adding 10% hydrochloric acid and evaporated to dryness. The dry residue was triturated with dry magnesium sulfate and extracted several times with diethyl ether. After filtration the organic layer was evaporated in vacuum giving 4.12 g of a solid (yield 40%). The product was purified by crystallization from acetone/petroleum ether, mp = 150–151 °C. ^{1}H NMR (DMSO-d_6): δ = 2.65 (d, J = 4.8 Hz, 3H, NHC**H**$_3$), 8.8 (broad s, 1H, N**H**CH$_3$), 13.6 (broad s, 1H, COO**H**); ^{13}C NMR (DMSO-d_6): δ = 25.8 (NH**C**H$_3$), 158.8 (**C**ONH, correlation on the basis of HMBC), 162.0 (**C**OOH, correlation on the basis of HMBC). IR (KBr): ν = 1758, 1677 cm^{-1}. MS: *m*/*z* 44 (100%), 58 (84%), 59 (43%), 103 ($M^{\bullet+}$, 3%). C, H, N: $C_3H_5NO_3$ (103.08) C calcd. 34.96, found 34.67, H calcd. 4.89, found 4.88, N calcd. 13.59, found 13.32. Calculation of pk_a = 2.37 by Advanced Chemistry Development ACD, Software Solaris V 4.67, Chemical Abstracts.

2.2.4. *N-Methyloxamic acid methyl ester (**10**)*

A solution of diazomethane was prepared by a method described elsewhere (De Boer and Backer, 1963). To a solution of **9** (0.31 g, 3 mmol) in 5 ml methanol the solution of diazomethane in diethyl ether was added until the mixture retained yellow. After stirring for 20 min methanol was removed by vacuum. The residual solid weights 0.35 g (yield 100%). The product was purified by crystallization from diethyl ether/petroleum ether, mp = 78 °C. ^{1}H NMR (DMSO-d_6): δ = 2.66 (d, 3H, NHC**H**$_3$), 3.76 (s, 3H, OC**H**$_3$), 8.9 (broad, s, 1H, N**H**CH$_3$). IR (KBr): ν = 1688 cm^{-1} (C=O). MS: *m*/*z* 58 (100%), 89 (19%), 117 ($M^{\bullet+}$, 3%); GC–MS: *m*/*z* 58 (100%), 60 (45%), 117 (2%), 118 ($[M+1]^+$, 10%). C, H, N: $C_4H_7NO_3$ (117.10), C calcd. 41.03, found 40.89, H calcd. 6.03, found 5.95, N calcd. 11.96, found 11.67.

2.2.5. *Oxalic acid-(N′-acetyl-N′-methyl-N-phenyl)-hydrazide (**11**)*

A solution of 1.05 g (3 mmol) **1** in 75 ml buffer (pH 7.4) and 30 mg horseradish peroxidase in 75 ml of the same buffer were mixed. A dilution of 3.6 ml 30% hydrogen peroxide (36 mmol) in 300 ml water was added very slowly drop by drop. After stirring for 24 h at room temperature the mixture was extracted with dichloromethane and the aqueous solution acidified to pH 5 by adding 5% potassium hydrogen sulfate solution. A second extraction with dichloromethane followed. Both organic layers were dispensed. The remaining aqueous phase was brought to dryness by evaporation and the dry residue was triturated with excess potassium hydrogen sulfate and then extracted with chloroform:isopropanol 9:1. The combined extracts were dried and the solvents evaporated in vacuum yielding 0.25 g of a colorless and viscous oil (yield 35%), crystallized as ammonium salt of **11**, mp = 183–184 °C. ^{1}H NMR (DMSO-d_6): δ = 2.01 (s, 3H, NCOC**H**$_3$), 3.04 (s, 3H, NC**H**$_3$), 7.2 (m, 1H, p-ar), 7.4 (m, 4H, o-, m-ar). IR (KBr): 1681, 1624 cm^{-1} (C=O). C, H, N: $C_{11}H_{15}N_3O_4$ (253.26), C calcd. 52.17, found 51.82, H calcd. 5.97, found 5.76, N calcd. 16.59, found 16.31. The acid **11** is stable towards hydrolysis in refluxing acidic medium (pH 3, 1 h) but is readily hydrolyzed in alkaline solution to **8** and oxalic acid.

2.2.6. *Methyl-2-(N′-acetyl-N′-methyl-N-phenyl)-hydrazino-2-oxo-acetate (**12**)*

A solution of 0.25 g (1.1 mmol) **11** in 5 ml methanol was mixed with a diazomethane solution in diethyl ether (De Boer and Backer, 1963) until the mixture retained yellow. After stirring for 20 min methanol was removed by vacuum. The residual viscous oil (yield 100%) was used without further purification. ^{1}H NMR (DMSO-d_6): δ = 2.06/2.14/2.19 (s, 3 rotameric isoforms, H, NCOC**H**$_3$), 3.04/3.11/3.26/3.30 (s, 4 rotameric isoforms, 3H, NC**H**$_3$), 3.56/3.64/3.83/3.88 (s, 4 rotameric isoforms, 3H, COOC**H**$_3$), 7.3–7.6 (m, 5H, ar). IR (KBr): ν = 1751, 1704, 1677 cm^{-1} (C=O). MS: *m*/*z* 59 (70%), 77 (86%), 92 (24%), 121 (100%), 208 (17%), 250 (M^+, 0.3%). C, H, N: $C_{12}H_{14}N_2O_4$ (250.25), C calcd. 57.59, found 57.23, H calcd. 5.64, found 5.69, N calcd. 11.19, found 10.95.

2.3. *Methods*

2.3.1. *Collection of urine from rats after application of **9***

Wistar rats (female, 200 g) were divided into a *verum* group (n = 3) and a control group (n = 3). The animals were kept under controlled light/dark cycle and room temperature and fed on standard pellet diet. One milligrams of **9** in 1 ml water was administered by gavage to rats of the *verum* group. The animals of the control group received 1 ml of pure water under the same conditions. Rats were then kept in individual metabolic cages for collection of urine at 12, 24, 36 and 48 h after application. The collected samples were stored at −20 ° C. These experiments were approved by the local ethical committee.

2.3.2. *Collection of allantoic fluid (AF) from incubated hen's egg after inoculation of **1**, **7** and **9***

For this purpose, the appropriate xenobiotic was inoculated into the fertilized hen's egg before or after the beginning of incubation. After completing the incubation period the metabolites can be identified in the AF, the excretion medium of the embryo. This method depends on the description in literature (Neugebauer, 1995). Fertilized eggs (White Leghorn) were obtained from Johann Figgemeier, D-33397 Rietberg, Germany. Before starting the incubation 0.1 ml of an aqueous solution containing 5 or 10 mg of the corresponding substrate was inoculated into the albumen of 11 eggs. In a control group the same volume of pure water was inoculated. The eggs were placed horizontally in a thermostatic oven and incubated at 38.0 ± 0.5 °C and 64 ± 5% of relative humidity. They were rotated every 3 h. The experiments were stopped on the 11th or 15th day of incubation by storing the eggs for 30 min at −20 °C. The aspirated AF was pooled and stored at −20 °C.

2.3.3. *Collection of urine from human after a single dose of **1***

Metamizol (**1**) (two tablets of each 500 mg novaminsulfon ratiopharm®) was administrated to two volunteers (male, 62a, 70 kg, and female, 27a, 55 kg) as a single oral dose. The 48 h urine was collected in two fractions of each 24 h and stored in plastic bottles at −20 °C.

2.3.4. *Hydrolysis of biological samples*
Some selected samples (Section 2.3.5) were additionally treated under acidic conditions for hydrolysis of conjugates to be compared with non-hydrolyzed samples. The corresponding sample (10.0 ml) was adjusted with 1% sulfuric acid to pH 4–5 and refluxed for 20 min. After cooling the mixture was neutralized with sodium hydrogen carbonate before the isolation procedure.

2.3.5. *Isolation and determination of* **9** *from aqueous samples (e.g. urine, AF)*
2.3.5.1. *Isolation*. Because of its very high water solubility **9** is not extractable from aqueous solution with organic solvents and therefore we used the method of ion exchange chromatography based on previous experiences in the isolation of organic acids from urine samples (Spiteller and Spiteller, 1979). The undiluted sample (1.0 ml) was passed through a glass column with 3 g strongly acid cation exchanger to remove amino acids. The column was then eluted with 10 ml water and the combined eluates passed through a second column filled with 5 ml of a strongly basic anion exchanger to absorb all carboxylic acids. The anion exchanger was consecutively washed with 10 ml of water and with 10 ml of methanol and the eluats were rejected. The adsorbed acids were separated in two different fractions by using the following eluents—first fraction: 10 ml of methanol/formic acid 95:5 and 10 ml methanol successively (acids with $pk_a > 3.8$). Second fraction: 10 ml of methanol/trifluoroacetic acid 95:5 and 10 ml methanol successively (acids with $pk_a < 3.8$).

Because **9** is a strong acid ($pk_a \sim 2.4$), it was quantitatively eluted in the second fraction which was aporated to dryness. This procedure was necessary to separate the strongly acidic oxalic acid derivatives together with other native strong acids from a great variety of "normal" carboxylic acids as physiological constituents in the urine of healthy probationers (Först, 1985; Pickert, 1987).

2.3.5.2. *Esterification with diazomethane*. The residues of the eluated urine fractions were solved in 5 ml methanol and a diazomethane solution in diethyl ether was added until the mixture retained yellow. After stirring over night the solvents were removed by evaporation. Before injection in GC–MS the residues containing the volatile methyl esters were dissolved in methanol. Isolation and subsequent esterification process was improved to an efficiency of quantitative recovery by using standard **9**.

2.3.5.3. *Qualitative analysis by GC–MS*. GC–MS System Saturn 2000 Varian CP-3800, mass-detector Saturn 2200 and auto sampler Varian automatic liquid sampler 8400 (Varian GmbH, Darmstadt, Germany). Mass spectrometric measurement was performed using electron impact ionization (EI) at 70 eV. The Column: Hewlett Packard, HP-5 MS, 30 m × 0.25 mm, film thickness = 0.25 μm (Agilent Technologies Deutschland GmbH, Böblingen, Germany). Carrier gas was helium with a consistent flow of 1 ml/min, split ratio 1:80 and purge off after 0.01 min. The oven temperature was programmed to rise after an initial temperature of 50 °C for 2 min with a rate of 30 °C/min to 110 °C and afterwards with a rate of 12 °C/min to 290 °C. This temperature was hold for 15 min, injector temperature was 170 °C, detector temperature was 225 °C and transfer line temperature was 250 °C. Before injection the samples were diluted 1:10 with methanol. The injection volume was 1 μl.

2.3.5.4. *Quantitative analysis by GC–FID from rat urine*. The analysis was carried out using a Siemens Sichromat 1–4 GC, containing FID and Chromajet integrator, Spectra Physics (Spectra Physics, Darmstadt, Germany). Column: Macherey–Nagel, Permabond-OV-17, 25 m × 0.32 mm, film thickness = 0.25 μm (Macherey–Nagel GmbH, Düren, Germany). Carrier gas was nitrogen, $p = 0.5$ bar, flow rate = 17 ml/min. Temperature program: 70 °C for 3 min, 10 °C/min to 110 °C, after 3 min, 50 °C/min to 220 °C for 5 min. Injector temperature was 150 °C, detector temperature was 240 °C and injection volume was 3 μl. Five-point calibration samples containing **10** at concentrations of 25, 40, 50, 75 and 100 ng/μl were prepared. Each sample was measured three times. Calibration curve was constructed by plotting the peak area of **10** versus the concentration of the sample.

2.3.6. *Extraction and determination of* **7** *and* **8** *from aqueous samples (urine, AF)*
2.3.6.1. *Extraction*. AF or urine (0.7 ml) was adjusted to pH 8–9 by adding 0.1 ml 0.5 M di-sodium hydrogen phosphate and extracted with 1 ml dichloromethane/diethyl ether 7:3 in an eppendorf tube. The organic layer was dried with sodium sulfate and the organic solvents removed in a stream of nitrogen at room temperature. The residue was solved in 0.1 ml methanol and analyzed by GC–MS.

2.3.6.2. *Qualitative analysis by GC–MS*. Gas chromatography system consisted of Hewlett Packard 5890 MSD 5970 and auto sampler Hewlett Packard 7673 was used. Mass spectrometric measurements were performed using electron impact ionization (EI) at 70 eV. Column: Hewlett Packard, HP-5 MS, 30 m × 0.25 mm, film thickness = 0.25 μm. Carrier gas was helium ($p = 0.7$ bar), purge off after 2 min. The oven temperature was programmed to rise after an initial temperature of 50 °C for 2 min with a rate of 30 °C/min to 110 °C and afterwards with a rate of 12 °C/min to 290 °C. This temperature was held for 15 min, injector temperature was 270 °C, detector temperature was 270 °C and transfer line temperature was 280 °C. The injection volume was 1 μl. Retention times: **7**, 14.6 ± 0.2 min; **8**, 9.9 ± 0.2 min.

3. Results and discussion

In the present study, we describe the detection of non-heterocyclic metabolites **7**, **8**, **9** and **11** of metamizol (**1**) resulting from pyrazolinone ring opening reaction. For this purpose, we used two different isolation procedures from biological tissues followed by GC–MS analysis. Identification of compounds was ensured by comparing the retention time and the detected mass fragments with synthesized authentic reference compounds (Fig. 2). Only the phenylhydrazide **8** was previously mentioned as a metabolite of **1** (Volz et al., 1993; meeting abstract with no further details). Besides **8**, the oxamazide **7** and the *N*-methyloxamic acid (**9**) were now identified

as new metabolites in the allantoic fluid (AF) of incubated hen's eggs. In addition to these new metabolites we were able to detect another oxalic acid derivative **11** in human urine after oral administration of metamizol (**1**). To clarify whether *N*-methyloxamic acid (**9**) is excreted without further biotransformation, i.e. hydrolysis or conjugation, we also studied the metabolism of **9** after oral administration to female Wistar rats.

Whereas the new metabolites **7** and **8** could be detected together with the well known compounds **3**–**5** by GC–MS analysis in the organic extract of non-hydrolyzed neutral aqueous samples, the oxalic acid derivatives **9** and **11** evade such a detection because of their excellent solubility in water. Moreover, the pk_a-values of these acids are extremely lower in comparison with normal carboxylic acids. Therefore, these acids are completely ionized in neutral or weakly acidic solution and even worse to be extracted with organic solvents. This probably is the reason why such compounds have never been detected until today as metabolites of metamizol. Without application of the described ion exchange chromatography we failed to detect minor quantities of these oxalic acid derivatives in blank urine or allantoic fluid (AF). To complicate the situation there is an extreme multiplicity of organic acids in these systems. To our knowledge no work is done to analyze and identify these complex mixtures without specific enrichment procedures. The funny part of the described method is the absorption of all acids irrespective of their pk_a-values by a strongly basic anion exchanger after preceding separation of amino acids by means of a strongly acid cation exchanger. All the absorbed acids were removed from the strongly basic anion exchanger according to their pk_a-values by use of the appropriate organic acids in methanolic solution. So, with formic acid ($pk_a = 3.8$) we removed the acids with higher pk_a-values. Subsequent elution with methanolic trifluoroacetic acid ($pk_a = 0.2$) delivered the "strong acids" with a pk_a below 3.8. Before application in metabolic studies this isolation procedure was tested in various systems to separate as for instance benzoic acid ($pk_a = 4.2$) from 2,6-dihydroxybenzoic acid ($pk_a = 1.3$) as well as phenylalanine from oxamic acid ($pk_a = 1.6$). In both experiments separation was practically complete. The trifluoroacetic acid-eluate containing the strong acids was evaporated and subsequently methylated with diazomethane, which was profitable because residual amounts of trifluoroacetic acid were converted to its very volatile methylester which was removed quantitatively by a second evaporation. Solutions of these residues in methanol (methylated strong acid fraction) were analyzed by GC–MS. Finally, evaluation of this method with standard **9** was proved to give practically quantitative results in recovering.

3.1. *Metabolism of 9 in rats*

The unchanged compound following a single dose of 1 mg **9** was excreted over a period of 48 h into the urine. In spite of the specific isolation procedure the GC of the "methylated strong acid fraction" from rat urine 12 h after dosage of **9** is very complex. Fig. 3 shows the original GC and the mass spectrum at the retention time of *N*-methyloxamic acid methyl ester (**10**) as a derivative of **9**. The proposed ms-fragmentation is shown

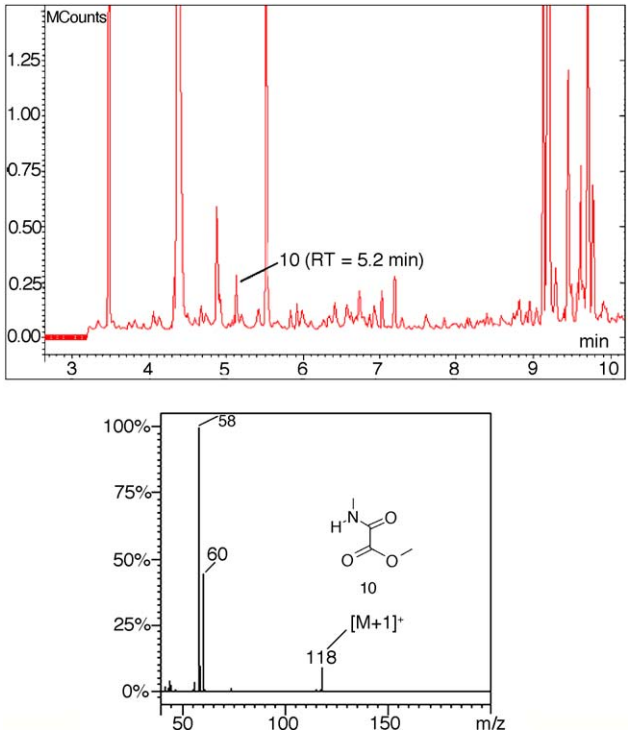

Fig. 3 – Original GC of the methylated strong acid fraction prepared from rat urine 12 h after administration of 9 (above); mass spectrum of 10 detected at RT = 5.2 min (below).

in Fig. 4. We quantified the excreted amount by GC–FID analysis. Table 1 gives the elimination of **9** in the urine of rats. Total recovery was about 90% after 48 h in rat 1 and 3. The deviating result of the second rat is difficult to explain, but individual properties of the animals or problems to gain the urine quantitatively from the cage could be responsible for the deficit. Unfortunately, the ethical committee did not agree in more animal experiments to clarify this abnormal result. Anyway, the conclusion can be drawn that **9** is no subject of further biotransformation in rats. Assuming that this is also the case in other species the detection of **9** would be an appropriate piece of evidence for the pyrazolinone ring opening in the course of metabolism of metamizol (**1**).

Table 1 – Elimination of 9 in urine of rats after oral administration of 1 mg 9 to rats

Time interval following treatment with **9** (h)	% Elimination of **9** in the urine		
	Rat 1	Rat 2	Rat 3
0–12	82.7	43.5	80.5
12–24	10.6	15.8	7.2
24–36	–	–	<1
36–48	–	–	<1
Total	93.3	59.3	87.7

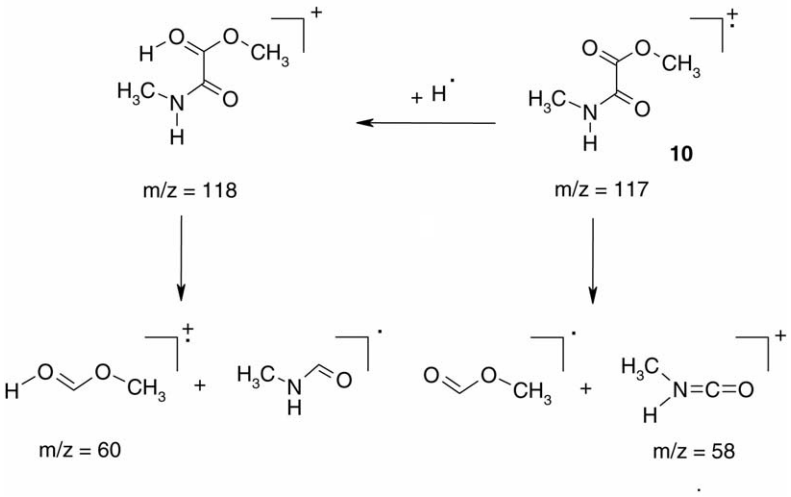

Fig. 4 – Proposed pathway for the mass fragmentation of 10.

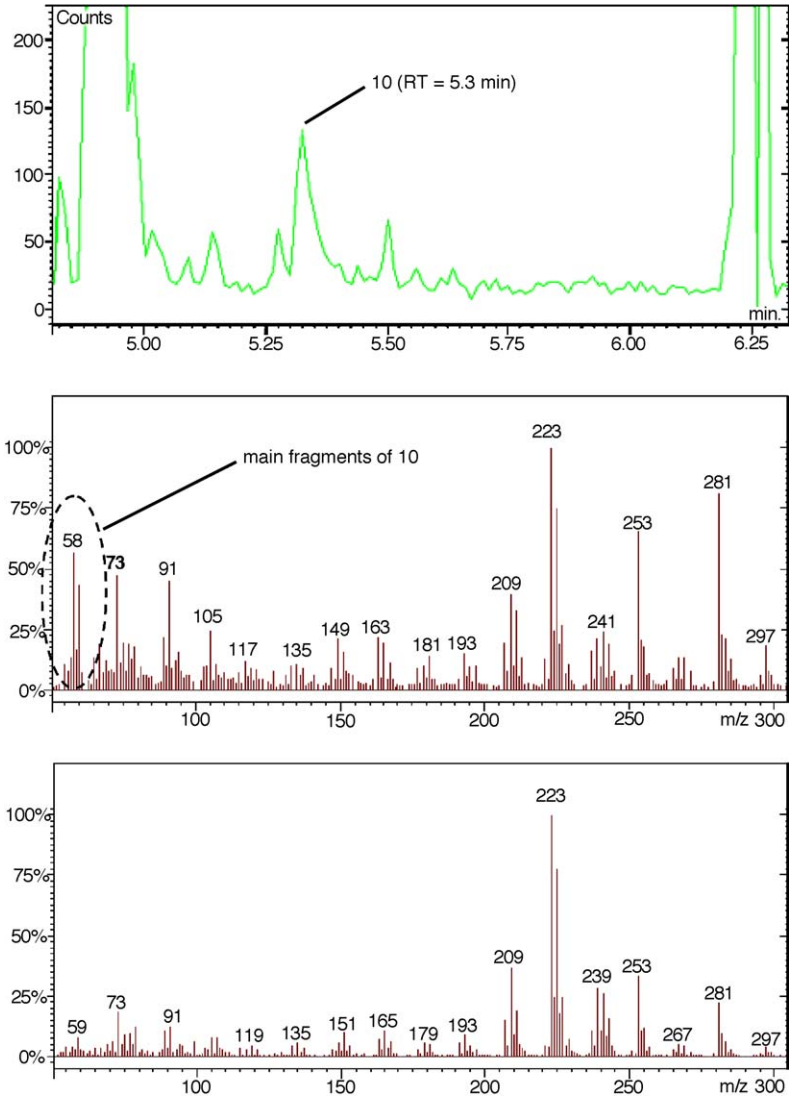

Fig. 5 – GC–MS of the methylated strong acid fraction prepared from AF after inoculation of 1; incubation time 15 days. Part of the GC with selective ion monitoring at *m/z* = 58 (above); mass spectrum at 5.3 min with main fragments of 10 (middle); in contrast the mass spectrum at RT = 5.3 min of a dry running without inoculation of 1 under same conditions (below).

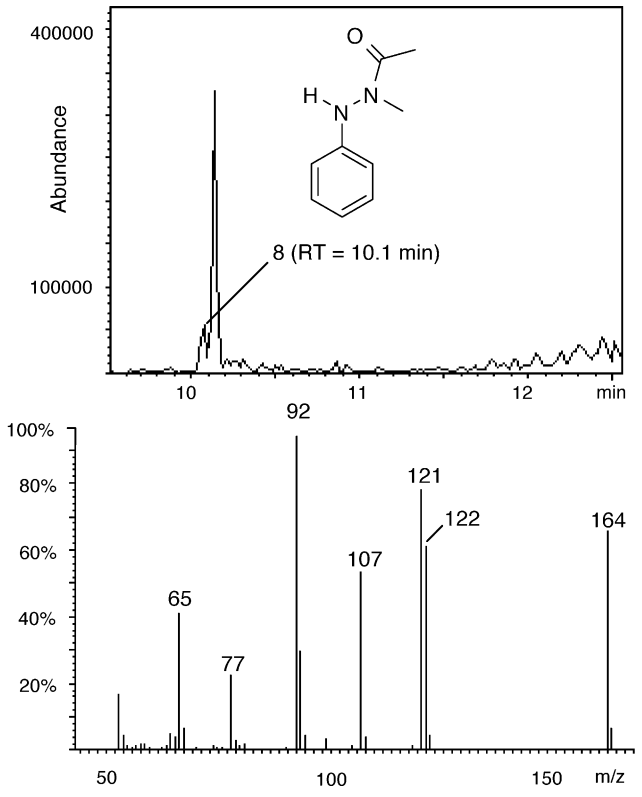

Fig. 6 – GC–MS chromatogram of the organic extract of AF after incubation of fertlized hen's eggs with 10 mg 1 (above). Identification of 8 with mass spectrum at RT = 10.1 min (below). The characteristic fragment ions are in accordance with those of the reference substance.

3.2. *Identification of oxalic acid derivatives in incubated hen's eggs*

As the metabolism of metamizol (**1**) is very similar in man and in incubated hen's egg (Kiep et al., 2002) we investigated its metabolism in this model. Besides **1** we also examined the behavior of the oxamazide **7** as a potential metabolite of **1** to find out whether this compound is detectable in the AF after biotransformation. Finally, *N*-methyloxamic acid (**9**) was also included in those investigations in order to confirm the results from rat experiments (Section 3.1).

3.2.1. *Inoculation of metamizol (1)*

After inoculation of **1**, we were able to identify **10** as the methyl ester of **9** in the "methylated strong acid fraction" of the AF by comparing its retention time and corresponding mass spectrum with authentic reference substance (Fig. 5). In addition, the phenylhydrazide **8** was identified in the organic extract of AF after inoculation of **1**. Fig. 6 shows a typical chromatogram of the corresponding AF. Retention time and characteristic fragments of this substance are in accordance with those of the reference substance. This fact shows, that **8** is not completely decomposed during the incubation in fertilized hen's eggs and should also be an appropriate metabolite to show the opening of the pyrazolinone ring during metabolism. The oxamazide **7**, however, was only present at trace concentrations after the dosage of 10 mg **1** as it was proved on the basis of the reconstructed ion chromatogram of this sample compared to the mass spectrum of the reference substance (not shown).

3.2.2. *Inoculation of oxamazide 7*

After direct injection of **7** in the fertilized eggs no parent compound could be detected, but we identified **8** and **9** as the

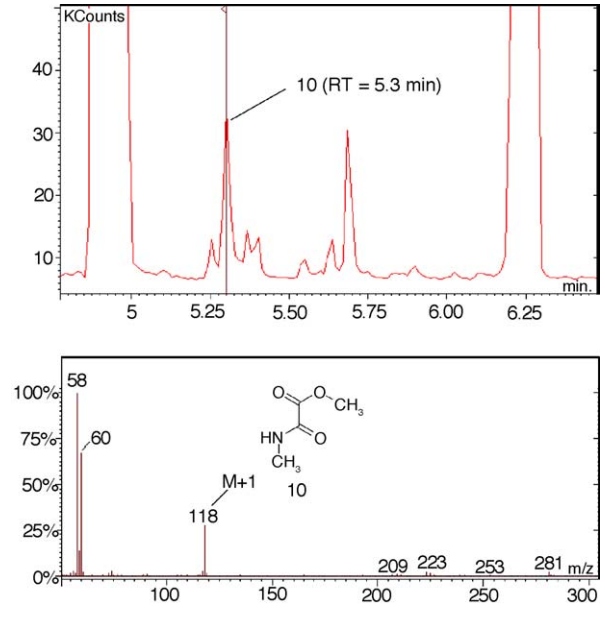

Fig. 7 – Original part of GC of the methylated strong acid fraction prepared from AF, 11 days after inoculation of 10 mg 9 (above) and the corresponding mass spectrum at RT = 5.3 min.

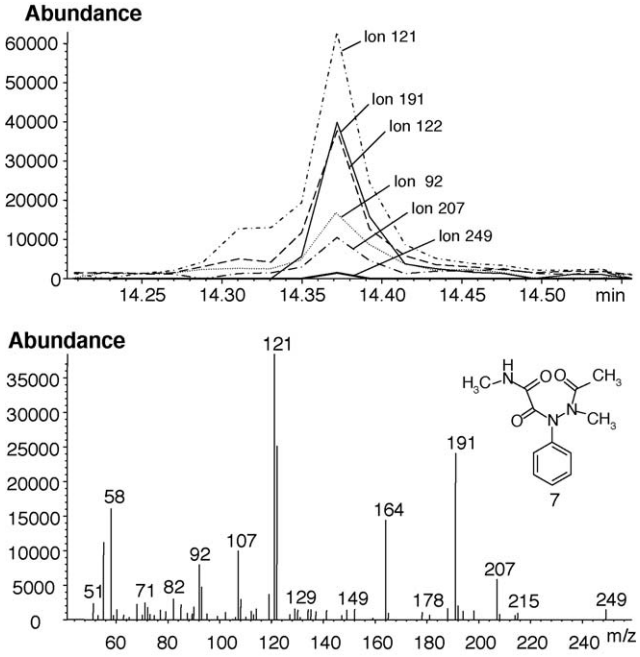

Fig. 8 – GC–MS of the organic extract of human urine after a single dose of 1 g 1 with selective ion monitoring (above) and the complete mass spectrum at RT = 14.4 min (below).

two fragments of the oxamazide structure. This surprising result possibly can be explained by regarding the sensitivity of **7** against hydrolysis ($t/2 = 22.5$ h; pH 7.4/37 °C; Weber and Bresser, 1996). Application of **7** as a single dose at the beginning of incubation time leads to a very low concentration at its end. However, if **7** is formed from **1** in a slow continuous process it is conceivable that the actual concentration is sufficient for GC–MS detection.

3.2.3. *Inoculation of N-methyloxamic acid (9)*

As was to be expected, **9** could be identified as methylester **10** in the AF as described. Fig. 7 shows a typical GC of the "methylated strong acid fraction" and the typical mass spectrum at the corresponding retention time.

3.2.4. *Toxicity of inoculated compounds*

Another observation concerning the toxicity of inoculated compounds was made during the hen's egg experiments. Whereas all eggs without any applied substance took a normal development of embryos, many of the inoculated ones showed a growth retarding or were fully devitalized. These effects were very pronounced in the case of oxamazide **7** (only 10–20% of surviving eggs), less severe with metamizol (40–60%) and could be neglected with *N*-methyloxamic acid (90–100% of surviving eggs). The reason of this remarkable toxicity of metamizol and its metabolite **7** is not clear and should be investigated in more detail.

All together, these results demonstrate clearly that the biotransformation of **1** in this model leads to hitherto unknown metabolites proceeding from opening of the pyrazolinone ring with **7** as an intermediate stage of the oxidative degradation.

3.3. *Identification of oxalic acid derivatives after administration of metamizol (1) in human*

Two volunteers were treated with a single dose of each 1 g of metamizol (Section 2.3.3). The oxamazide **7** and the phenylhydrazide **8** could be identified in the organic extract of the appropriate urine (Section 2.3.6). Detection of both compounds was achieved by means of GC–MS analysis with the ion trap technique and selective ion monitoring. Figs. 8 and 9 show the characteristic intensities according to the mass spectra of **7** and **8**, respectively. Both substances are difficult to be detected in a "normal" GC–FID because of overlapping peaks with various genuine urine components. From this we drew the conclusion that these new metabolites are present only in trace concentrations and therefore are not responsible for the remaining deficit of metabolites of **1**.

After separation of strong acids from the urine of the test persons by ion exchange chromatography (Section 2.3.5) we were also able to identify *N*-methyloxamic acid (**9**) in form of the corresponding methylester **10**. But here again only traces were present.

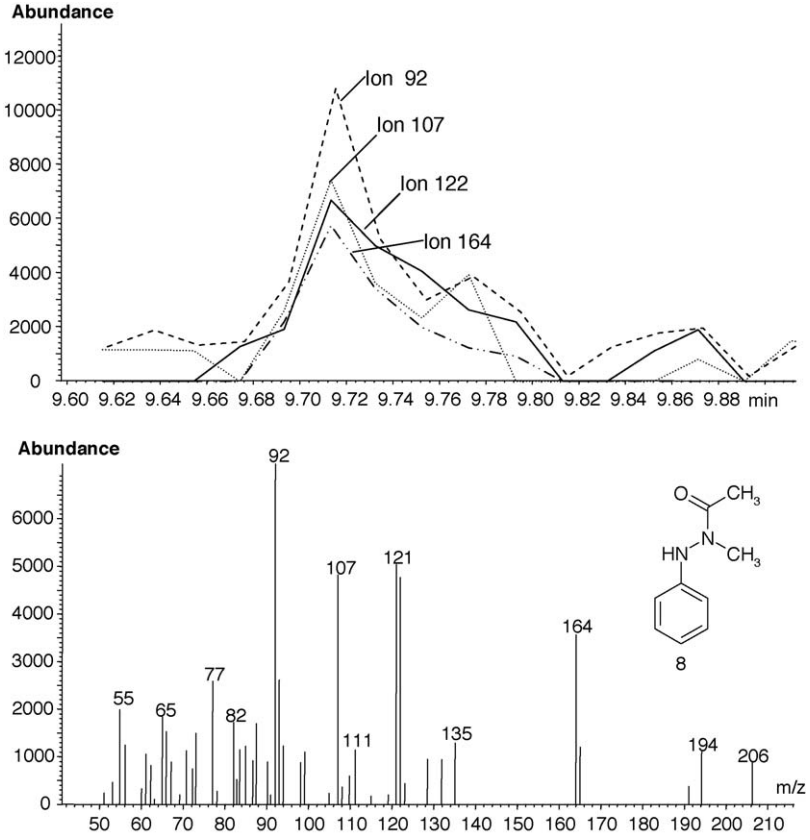

Fig. 9 – GC–MS of the organic extract of human urine after a single dose of 1 g 1 with selective ion monitoring (above) and the complete mass spectrum at RT = 9.7 min (below).

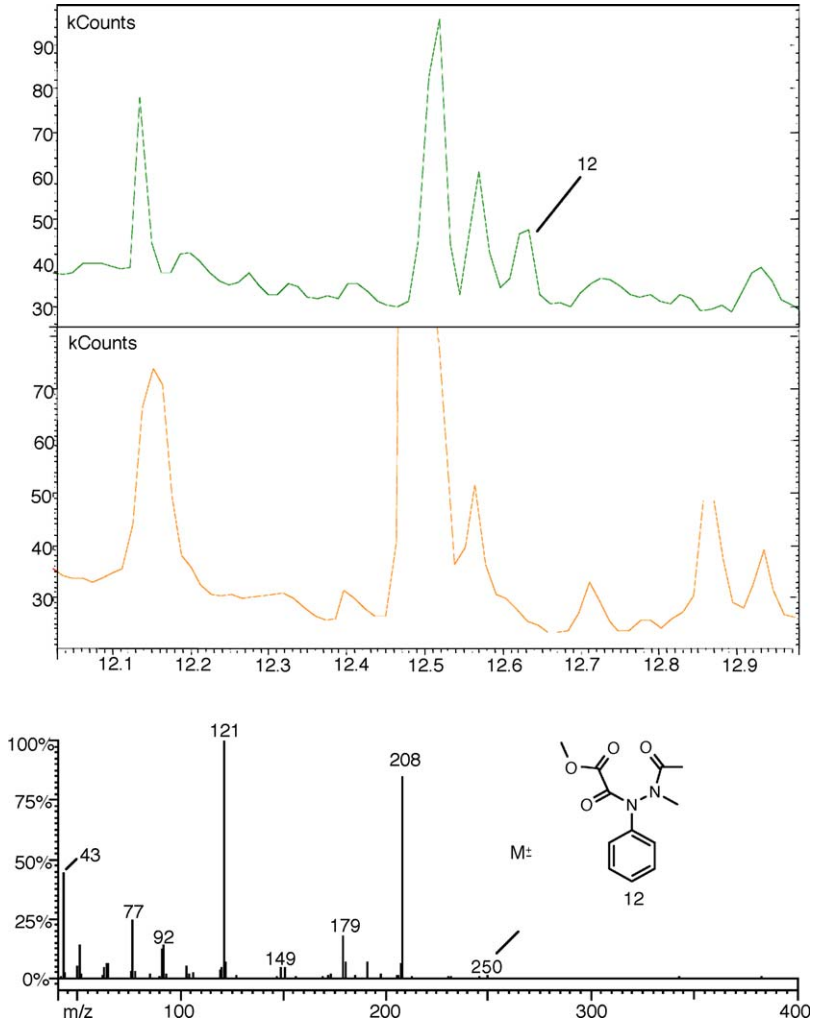

Fig. 10 – Unknown compound 12 in the methylated strong acid fraction prepared from a hydrolyzed urine sample after administration of 1 compared with a blank sample (above) and mass spectrum at RT = 12.6 min (below).

Therefore, we examined the available GC–MS data of various probes in detail to find more metabolites of **1** resulting from pyrazolinone ring rupture. Typical MS-fragments of phenylhydrazine and phenylhydrazide structures at *m/z* = 77, 92, 121 and 149 were scanned in relation to those GC-peaks that did not appear in extracts of normal urine. By this way, we were able to identify a new metabolite **11** as the corresponding methylester **12** after hydrolysis of the urine (Section 2.3.4) and preparation of the "methylated strong acid fraction" according to Section 2.3.5. Retention time of **12** was 12.6 min and the mass spectrum included typical fragments (Fig. 10). These characteristics matched with those of the authentic reference substance **12**. The original metabolite **11** was synthesized from metamizol (**1**) with hydrogen peroxide and horseradish peroxidase (POD) in neutral aqueous solution (Weber and Wollenberg, 1988; Weber and Bresser, 1996). This oxalic acid derivative **11** was never identified as a metabolite of **1** before. Its high water solubility prevented the extraction from aqueous layers by polar organic solvents even at low pH-values. Utilization of the described ion exchange matrix allowed the isolation of **11** from aqueous samples. Interestingly this procedure was only successful after preceding hydrolysis of the urine samples. So it is likely that **11** is originally eliminated in conjugated form.

4. Conclusions

In the course of our investigations we identified **7**, **8** and **9** as new metabolites of **1**. The results undoubtedly demonstrate that oxalic acid derivatives are produced in the biotransformation of **1**. By detecting these compounds the previous results (Weber and Wollenberg, 1988; Weber and Bresser, 1996) were confirmed, which describe the occurrence of oxalylhydrazides after oxidation of pyrazolinones in vitro. However, these metabolites are only present in very low concentrations in the human urine after administration of **1** and consequently they cannot be responsible for the deficit in the balance of metabolites. Additionally, the oxalic acid monohydrazide **11** could be identified as a new metabolite of **1**. We detected this new metabolite of metamizol in search of potential phenylhydrazide structures. Our results lead to the conclusion that **11** is

present in some higher amount that **7**, **8** and **9**, but it remains a quantitative determination of **11** in human urine after administration of **1**. Mode of formation of **11** is completely unclear but it is noteworthy that this oxalic acid derivative is the main product after reaction of dilute hydrogen peroxide/POD not only with **1** but also with its active form **2** and/or with hydroxyphenazone (**6**). The acid **11** is remarkably more stable against hydrolysis than the amide **7** or the ester **12**. Therefore, it would be reasonable in the future to elaborate a HPLC-method for direct determination of **11** after specific isolation as described in this paper. The results of such investigations together with studies concerning the metabolism of **11** could possibly close the gap in the biotransformation statistics of metamizol (**1**) and answer the open questions about the mechanisms of action and the observed side effects of this long known but still actual drug.

REFERENCES

Arellano, F., Sacristan, J.A., 1990. Metamizol: reassessment of its therapeutic role. Eur. J. Clin. Pharmacol. 38, 617–619.

Arznei-telegramm (Moebius, U.M., editor), issue 10, 1974, p. 76.

Beirith, A., Santos, A.R.S., Rodrigues, A.L.S., Creczynski-Pasa, T.B., Calixto, J.B., 1998. Spinal and supraspinal antinociceptive action of dipyrone in formalin, capsaicin and glutamate tests. Study of the mechanism of action. Eur. J. Pharmacol. 345, 233–245.

Bensenor, I.M., 2001. To use or not to use dipyrone? Or maybe, Central Station versus ER? That is the question. Sao Paulo Med. J. 119, 190–191.

Bresser, R., Weber, H., 1996. Untersuchungen zur stabilität von 1-methyl-2-phenylacetohydrazid. Pharmazie 51, 470–476.

Campos, C., de Gregorio, R., García-Nieto, R., Gago, F., Ortiz, P., Alemany, S., 1999. Regulation of cyclooxygenase activity by metamizol. Eur. J. Pharmacol. 378, 339–347.

Chandrasekharan, N.V., Dai, H., Roos, K.L., Evanson, N.K., Tomsik, J., Elton, T.S., Simmons, D.L., 2002. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proc. Natl. Acad. Sci. U.S.A. 99, 13926–13931.

Christ, O., Kellner, H.M., Ross, G., Rupp, W., Schwarz, A., 1973. Biopharmazeutische und pharmakokinetische untersuchungen nach gabe von metamizol-^{14}C (Novalgin®^{14}C) an ratte, hund und mensch. Arzneim. -Forsch. 23, 1760–1767.

Collares, E.F., Vinagre, A.M., 2003. Evidence of the effect of dipyrone on the central nervous system as a determinant of delayed gastric emptying observed in rats after its administration. Braz. J. Med. Biol. Res. 36, 1375–1382.

De Boer, Th.J., Backer, H.J., 1963. Diazomethane. Org. Synth. Colloid. 4, 250–253.

Duchstein, H.J., Ruch-Zaske, G., Holzmann, G., Wollenberg, E., Weber, H., 1988. Die reaktion von singulett-sauerstoff mit 4-amino-3-pyrazolin-5-onen. Arch. Pharm. (Weinheim) 321, 25–27.

Först, C., 1985. Basisprofile organischer säuren im urin—Gaschromatographisch-massenspektroskopische, analysen, vergleiche bei normaler und diabetischer stoffwechsellage. Dissertation, Heidelberg.

Hedenkalm, K., Spigset, O., 2002. Agranulocytosis and other blood dyscrasias associated with dipyrone (metamizol). Eur. J. Clin. Pharmacol. 58, 265–274.

Kiep, L., Bekemeier, H., 1986. Biotransformation und toxizität von xenobiotika im kükenembryo. Pharmazie 41, 868–872.

Kiep, L., Maderner, S., Seifert, K., 2002. Metabolism of metamizol in early stages of the incubated hen's egg. Pharmazie 57, 829–833.

Lanz, R., Peskar, A., Brune, K., 1985. Wirkung von sauren und nicht-sauren pyrazolonen auf den arachidonsäurestoffwechsel von mausperitonealmakrophagen. 100 Jahre Pyrazolone, Urban & Schwarzenberger, München, pp. 105–112.

Levy, M., Zylber-Katz, E., Rosenkranz, B., 1995. Clinical pharmacokinetics of dipyrone and its metabolites. Clin. Pharmacokinet. 28, 216–234.

Lüpke, N.P., 1987. Hühner-Ei-Test (HET). Ärztl. Kosmetolog. 17, 13–30.

Neugebauer, M., 1995. Biotransformation of (+)-methamphetamine in fertile hen's eggs. Pharmazie 50, 201–206.

Neugebauer, M., 1997. Biotransformation von arzneistoffen im bebrüteten hühnerei—Eine alternative zum tierversuch. Habilitation, Bonn.

Pickert, A., 1987. Untersuchung der organischen säuren in serum und urin bei stoffwechselgesunden und patienten mit azidosen. Dissertation, Tübingen.

Rosenbruch, M., 1994. Early stages of the incubated chicken egg as a model in experimental biology and medicine. ALTEX 11, 199–206.

Schonhofer, P., Offerhaus, L., Herxheimer, A., 2003. Dipyrone and agranulocytosis: what is the risk? Lancet 360, 1438.

Spiteller, M., Spiteller, G., 1979. Trennung und charakterisierung von sauren harnbestandteilen. J. Chromatogr. 164, 253–317.

Taylor, J., Mellström, B., Fernaud, I., Naranjo, J.R., 1998. Metamizol potentiates morphine effects on visceral pain and evoked c-Fos immunoreactivity in spinal cord. Eur. J. Pharmacol. 351, 39–47.

The International Agranulocytosis and Aplastic Anemia Study Group, 1986. Risks of agranulocytosis and aplastic anemia. A first report of their relation to drug use with special reference to analgesics. The International Agranulocytosis and Aplastic Anemia Study. JAMA 256, 1749–1757.

Vlahov, V., Badian, M., Verho, M., Bacracheva, N., 1990. Pharmacokinetics of metamizol metabolites in healthy subjects after a single oral dose of metamizol sodium. Eur. J. Clin. Pharmacol. 38, 61–65.

Volz, F., Fehlhaber, H.-W., Siebenlist, F., 1993. Biotransformation of metamizol. In: The Fifth European ISSX Meeting, Tours, p. 70 [abstract].

Volz, M., Kellner, H.M., 1980. Kinetics and metabolism of pyrazolones (propyphenazone, aminopyrine and dipyrone). Br. J. Clin. Pharmacol. 10, 299–308.

Wallach, O., 1877. Über die einwirkung von phosphorpentachlorid auf säureamide. Justus Liebigs Ann. Chem. 184, 67–68.

Weber, H., Bresser, R., 1996. Oxalsäurehydrazide als hydrolytisch labile oxidationsprodukte der pyrazolinonanalgetika. Pharmazie 51, 152–155.

Weber, H., Wollenberg, E., 1988. Oxidative ringöffnung von 3(2H)-pyrazolonen mit periodat. Arch. Pharm. (Weinheim) 321, 551–553.

Weithmann, K.U., Alpermann, H.G., 1985. Biochemical and pharmacological effects of dipyrone and its metabolites in model systems related to arachidonic acid cascade. Arzneim. -Forsch. 35, 947–952.

Wessel, J.C., 2004. Erstmaliger Nachweis von Oxalsäurederivaten bei der Biotransformation von Metamizol im bebrüteten Hühnerei und beim Menschen. Cuvillier Verlag, Göttingen, Germany.