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# Synthesis of mesocarb metabolites as reference compounds for doping analytics

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## Introduction

Mesocarb (Sydnocarb®) has been used for doping purposes due to its central nervous system stimulating effects. Presently, the metabolites of mesocarb are not commercially available, and additionally, the exact chemical structures of several mesocarb metabolites have not been solved yet. The aim of this WADA-funded project was to synthesize six potential mono-, di-, and trihydroxylated regioisomeric metabolites of mesocarb. The metabolites were compared using an LC-MS/MS-method with the *in vitro* synthesized mesocarb metabolites using human liver enzymes and with the *in vivo* formed metabolites extracted from human urine after oral administration of mesocarb.

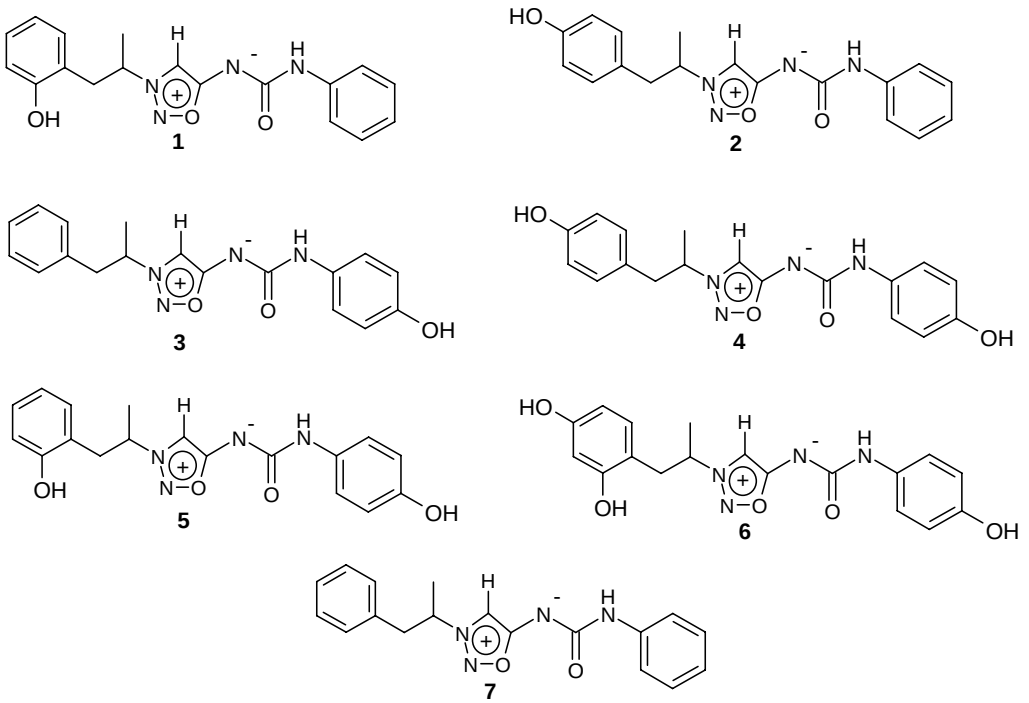
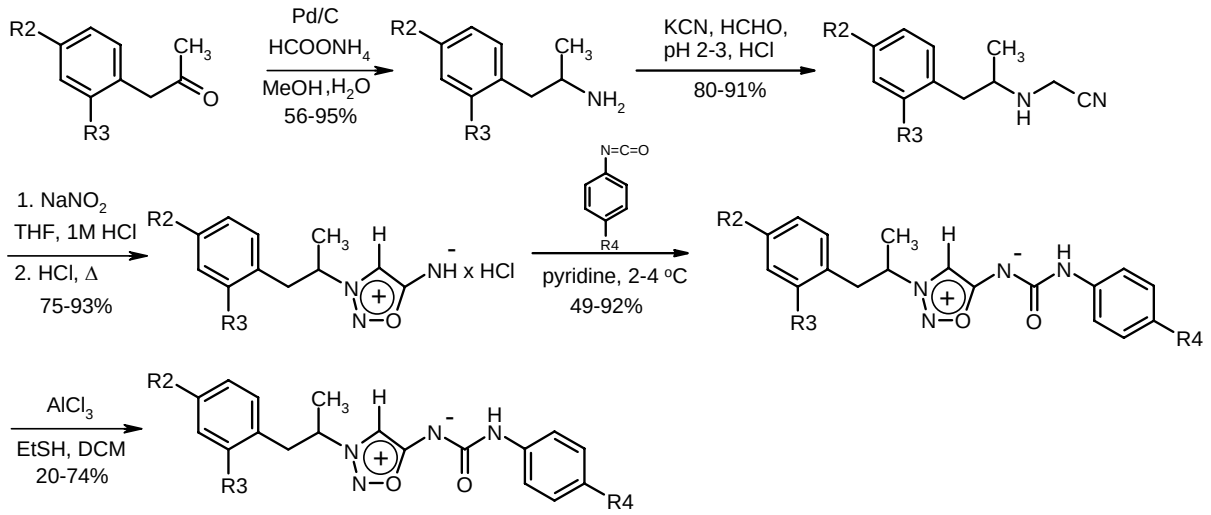



***Figure 1.*** Mesocarb (**7**) and its metabolites (**1-6**).

## Materials and methods

*Chemical synthesis.* N-exocyclic five-membered heterocyclic mesoionic compounds, i.e. N-exocarbamoylsydnone imines were synthesized according to Scheme 1. Alkyl amine (synthesized from methoxyphenylacetone) was allowed to react in the presence of potassium cyanide and formaldehyde at pH 2-3. The alkyl acetonitrile was subsequently nitrosated ($NaNO_2$, HCl) to give the corresponding N-nitrosoalkylacetonitrile. When N-nitrosoalkylacetonitrile was refluxed in concentrated hydrochloric acid, the corresponding sydnone imine was obtained. The sydnone imine was subsequently allowed to react with aryl isocyanate in the presence of pyridine to afford the N-exocarbamoyl derivatives of sydnone imine. Metabolites of mesocarb were obtained after demethylation of hydroxyl groups. The synthesized metabolites were purified either by crystallisation or chromatographic methods. Their chemical structures were characterized by means of $^{1}H$ NMR, $^{13}C$ NMR, FTIR spectrometry and liquid chromatography-mass spectrometry. The amount of synthesized metabolites was 100–500 mg. Purity of metabolites varied from 92.0 to 99.6 %.




***Scheme 1.*** Synthesis of mesocarb metabolites.

*LC/MS/MS analysis.*

| | |
|---|---|
| LC: | Surveyor (ThermoFinnigan) |
| Column: | Agilent Zorbax Eclipse 300SB-CN (2.1 × 150 mm, 5 μm) |
| Solvent A: | 2.5 mM ammonium acetate in 0.1% acetic acid, pH 4 |
| Solvent B: | Methanol |
| Gradient: | Solvent B 40%→100% (6 min, held for 2 min) |
| Flow rate: | 0.2 mL/min, at ambient temperature |
| Injection: | 10 μL |

| | |
|---|---|
| MS: | TSQuantum (ThermoFinnigan) |
| Ionization: | positive ESI (4000 V) |
| Gases: | $N_2$ as sheath and auxiliary gas, Ar as collision gas (1.5 mTorr) |
| MS-mode: | MRM, two ion transitions for each metabolite |

Ion transitions and retention time for *p*-OH-mesocarb (metabolite 2 in Figure 1) were m/z 339→196 (10 V) and m/z 339→91 (48 V) and 5.37 min, respectively.

*Urine sample pretreatment.* Methyltestosterone was used as the internal standard (500 ng/mL). A 3-mL aliquot of urine was applied to a Sep-Pak C18 cartridge (Waters), which was previously conditioned with methanol and water. The sample was first rinsed with 5 mL of water, and then eluted with 3 mL of methanol. The eluate was divided into three parts, which were evaporated to dryness and dissolved in 1 mL of a proper buffer for the isolation of free and conjugated metabolites. Enzymatic hydrolysis of the glucuronide conjugates was performed in a 0.1 M phosphate buffer (pH 7) with 25 μL of β-glucuronidase from *Escherichia coli* K12 (Boehringer Mannheim) at 50 °C for 1 hour. Sulphate conjugates were enzymatically hydrolysed in 1 mL of 0.1 M acetate buffer (pH 5) with 10 μL of arylsulfatase from *Helix Pomatia* (Sigma-Aldrich) at 37 °C for 1 hour. For isolation of the free metabolites no hydrolyzing enzyme was added. All three aliquots were finally extracted twice with 3 mL of ethyl acetate at pH 8. After centrifugation the organic phase was separated, evaporated to dryness and dissolved in 100 μL of the initial LC mobile phase. Extraction recoveries for all metabolites were higher that 80%.

## Results and Discussion

To solve the exact chemical structure of the main urinary metabolites of mesocarb, the chemically synthesized metabolites were compared both to metabolites synthesized enzymatically *in vitro,* using humane liver microsomal protein and fraction S9 of humane liver protein, and to an authentic human urine sample, which was collected during 0-48 hours after p.o. administration of 5 mg of mesocarb (a gift from Moscow Anti-Doping Laboratory).

Enzymatic synthesis yielded only *p*-OH-mesocarb (metabolite 2 in Figure 1). *p*-OH-Mesocarb was also the main metabolite found in human urine collected after oral administration of mesocarb. Most of the *p*-OH-mesocarb in urine was sulphated (76%) but also some glucuronidated (22%) and free (2%) metabolite was observed. Traces of a dihydroxylated metabolite (metabolite 4 in Figure 1) were also found in urine.

Methods to synthesize, analyze and characterize urinary hydroxylated metabolites of mesocarb were developed. *p*-Hydroxymesocarb was found to be the main metabolite in human urine. The synthesized and fully characterized *p*-hydroxymesocarb enables the reliable and legally defensible confirmation analysis of mesocarb in doping control, and could also be used in quality assurance and in development of new analytical methods. The metabolite synthesized in this project is available to all WADA-accredited anti-doping laboratories.

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