# EVIDENCE-BASED PHARMACOTHERAPY IN PRETERM INFANTS
## Aiming at a moving target

Robert B. Flint

Cover design by Jean-Jacques Sliepen
Layout by XML 2 PUBLISH
Printed by GVO drukkers & vormgevers BV

The printing of this thesis has been financially supported by:
• Erasmus MC University Medical Center Rotterdam
• Koninklijke Maatschappij ter bevordering der Pharmacie
• Chipsoft

ISBN 978-94-6332-420-5

The work presented in this thesis was carried out at the Department of Pharmacy of the Radboudumc Nijmegen and Division of Neonatology of the ErasmusMC Rotterdam.

The research presented in this thesis was supported by the Netherlands Organisation for Health Research and Development ZonMw (Grant number: 836011022).

**Evidence-Based Pharmacotherapy in Preterm Infants**
**Aiming at a moving target**

Evidence-based farmacotherapie voor premature baby's
Mikken op een bewegend doelwit

**PROEFSCHRIFT**

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. R.C.M.E. Engels

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

14 november 2018 15:30 uur

Robert Bernard Flint
geboren te Winschoten

**Erasmus University Rotterdam**

## PROMOTIECOMMISSIE

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|---|---|
| **Promotoren** | Prof.dr. D. Tibboel<br>Prof.dr. D.M. Burger |
| **Overige leden** | Prof.dr. T. van Gelder<br>Prof.dr. S.N. de Wildt<br>Prof.dr. K. Allegaert |
| **Copromotor** | Dr. S.H.P. Simons |

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| **Paranimfen** | Jorine Roelants<br>Rob van Geest |

## CONTENTS

# 1

# General Introduction

The incidence of prematurity is still increasing worldwide and in the Netherlands, and remains the most common cause of death in children below the age of five years[1]. Yet, as a result of improvements in healthcare, to date prematurity may be considered a chronic condition that affects lifelong physiology[2,3]. Therefore, we are ethically obliged to learn from each case and therewith constantly improve future treatment of prematurely born neonates. Despite the new United States and European Union laws mandating industry to study new drugs in children, many are still used off-label–especially in the smallest newborns for whom evidence is mostly lacking. Recently we have seen a paradigm shift recognizing that the time has come to protect children and young people through research not from research.

Since preterm born infants are not small newborns, specific evidence is needed on treatment efficacy and safety of drugs, specifically obtained in the target groups rather than translated from findings in other age groups. As evidence is sparser with decreasing gestational and postnatal age, neonates can be considered therapeutic orphans, especially preterm born infants. This lack of evidence does not mean that physicians withheld neonates from (pharmacological) treatment, as this may be a higher risk of complications with possibly life-long effects and irreversible damage[4], and unethical. With decreasing gestational age, this risk increases.

Although the neonatal population by definition covers only the first 28 days after birth, taking preterm born infants into account expands the postnatal age range to 5 months. This corresponds with a more than ten-fold weight range (400g–5kg), in addition to a large range of gestational and postnatal ages, very low birth weight infants and dysmature infants. Consequently, the neonatal population is also defined by large maturational differences[5,6], before as well as after birth. Furthermore, intensive and invasive treatment and critical illness can create large physiological differences, such as hypothermia after asphyxia for neuroprotection, extracorporeal membrane oxygenation (ECMO), drug-drug interactions, and adverse drug reactions.

The large maturational changes together with the physiological differences due to treatment and disease, call for thorough investigation of (adverse) drug response in all neonatal subgroups. This was the incentive for a grant proposal approved by the Netherlands Organisation for Health Research and Development ZonMw (Grant number: 836011022). Initially, five off-label drugs–paracetamol (also known as acetaminophen), fentanyl, phenobarbital, midazolam and doxapram – were selected for a pharmacokinetics and pharmacodynamics study in preterm born infants, with the goal to achieve and implement optimized dosing regimens. Subjects were recruited from four of the ten neonatal intensive care units (NICU) in the Netherlands. The study was named the DINO-study (Drug dosage Improvements in NeOnates), and four additional drugs used in preterm born infants were added for which optimal dosage recommendations were lacking: ibuprofen, fluconazole, levetiracetam and sildenafil.

The overall aim of this thesis was to optimize pharmacological treatment for preterm born infants, using pharmacokinetic and pharmacodynamic principles.

The specific aims were:

1. To describe a) which drugs were prescribed among four NICUs, and b) the largest differences in neonatal drug use between NICUs.
2. To develop high performance analytical methods for quantification of multiple drugs and their relevant metabolites simultaneously in minimal plasma volume, which enabled neonatal research with minimal burden.
3. To describe the pharmacokinetics of phenobarbital, doxapram, paracetamol and fentanyl in preterm infants.
4. To study the pharmacodynamics of doxapram in preterm newborns, using both retrospectively and prospectively collected data. The latter enabled a continuous evaluation of pharmacotherapy which may be implemented at the bedside.

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# CURRENT NEONATAL PHARMACOTHERAPY

# 2

# Large differences in neonatal drug use between NICUs are common practice: time for consensus?

*British Journal of Clinical Pharmacology 2018 Jun;84(6):1313-1323*

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## ABSTRACT

### Introduction

Evidence for drug use in newborn is sparse, which may cause large differences in drug prescriptions. We aimed to investigate the differences between neonatal intensive care units (NICUs) in the Netherlands in currently prescribed drugs.

### Materials and Methods

This multicenter study included neonates admitted during 12 months to four different NICUs. Drugs were classified in accordance with the Anatomical Therapeutic Chemical (ATC) classification system and assessed for on/off-label status in relation to neonatal age. The treatment protocols for four common indications for drug use were compared: pain, intubation, convulsions, and hypotension.

### Results

1,491 neonates (GA-range 23-42 weeks) were included with a total of 32,182 patient days, 181 different drugs and 10,895 prescriptions of which 23% was off-label in relation to neonatal age. Overall, anti-infective drugs were most frequently used with a total of 3,161 prescriptions, of which 4% was off-label in relation to neonatal age. Nervous system drugs included 2,500 prescriptions of which 31% was off-label in relation to neonatal age. Nervous system drugs, blood and blood forming organs, and cardiovascular drugs showed the largest differences between NICUs with ranges of 919-2,278, 554-1,465, and 238-952 total prescriptions per 1,000 patients per ATC-class, respectively.

### Conclusion

We showed that drug use varies widely in neonatal clinical practice. The drug classes with the highest proportion of off-label drugs in relation to neonatal age showed the largest differences between NICUs, i.e. cardiovascular and nervous system drugs. Drug research in neonates should receive high priority to guarantee safe and appropriate medicines and optimal treatment.

## INTRODUCTION

Infants in the neonatal intensive care unit (NICU) are exposed to a large number of drugs. Most drugs are off-label for neonates and evidence for use in this population is sparse, due to a limited number of clinical trials on efficacy, dosage and safety[1, 2]. These knowledge gaps are prone to large differences in interpretation of available evidence, and will consequently be translated into different drug therapies described in local treatment protocols and neonatal practice.

Previous studies have described drug prescriptions during infancy, reporting a large proportion of off-label drug use[2-7]. The proportion of off-label prescribed drugs increases with decreasing age. Therefore the most vulnerable pediatric group- preterm infants- has the highest exposure to drugs that are insufficiently documented[2]. In neonatal care, almost all patients are exposed to al least one off-label or non-approved drug during admission. Off-label use of drugs has been associated with the risk of adverse drug reactions[8]. To improve pediatric drug therapy, new legislations were introduced more than a decade ago in the United States with the Pediatric Research Equity Act in 2003[9], the Food and Drug Administration Reauthorization Act of 2017[10], and in the European Union with the Paediatric Regulation in 2006[11] to encourage pediatric drug research in the pre- and post-marketing phase. Although these have not yet led to increased licensing[12, 13].

We aimed to investigate the differences between neonatal intensive care units (NICUs) in the Netherlands in currently prescribed drugs, and to study the off-label proportions, as well as drug-class and age related differences.

## METHODS

### Patients and setting

In this retrospective cohort, all patients with a date of admission between September 1 2014 until August 31 2015 to one of the four participating Dutch level III NICUs (Radboud University Medical Centre Nijmegen, Maastricht University Medical Centre Maastricht, Máxima Medical Centre Veldhoven and Sophia Children's Hospital Rotterdam) were eligible for inclusion. The study was conducted according to Good Clinical Practice and the Declaration of Helsinki.

### Definitions and drug classification

A prescription was defined as a patient for whom a specific drug was prescribed during admission to the NICU, regardless of how often it was prescribed and of the route of administration. Patient days were defined as the sum of treatment days of each drug during admission to the NICU, which was calculated per patient and as a total. All drugs

were classified in accordance with the Anatomical Therapeutic Chemical (ATC) classification system.

The definition by Neubert et al. for "off-label use" was applied, meaning 'all uses of a marketed drug not detailed in the summary of product characteristics (SmPC) including therapeutic indication, use in age-subsets, appropriate strength (dosage), pharmaceutical form and route of administration'[14]. Although, the on/off-label status could only be assessed of the active substance in relation to age-subsets, as information concerning dosage, route of administration, indication, drug preparation and formulation, could not be collected from all four hospitals. Therefore, on/off-label status in relation to neonatal age (<1 month after birth) was assessed according to the SmPC, which was consulted via the Dutch Medicines Evaluation Board (www.cbg-meb.org, consulted on October 12 2017). The status of a drug was considered on-label if an SmPC for that active substance describes an indication that includes infants below 1 month of age, which is also the case if the drug is indicated for children in general.

## Data collection

All four hospitals prescribed drugs using a computerized physician order entry system. Patient characteristics and drug prescriptions were retrieved from the electronical medical records of each hospital. Data were collected on date of admittance, birth date, gestational age, birthweight, gender, survival, drugs administered, and date and duration of drug administration until death or discharge from the NICU. We excluded ATC class 'Q' of veterinary drugs. We also excluded electrolytes, total parenteral nutrition, Dutch national health care system vaccines, supportive dermatological products (not containing a drug), and contrast media. We followed the guidelines in the Reporting of Studies Conducted using Observational Routinely Collected Data (RECORD) statement to report our study[15].

## Data processing and statistical analysis

Data of the four NICUs were combined for the overall analyses of neonatal prescriptions. The prescription frequency was ranked, together with an analyses of the proportion of prescriptions off-label in relation to neonatal age. For comparison of the NICU prescriptions, patients were classified into five different postmenstrual age groups at start of drug use, because gestational age groups would be confounded by drug use at a later postnatal age: <26 weeks, 26-28 weeks, 28-32 weeks, 32-37 weeks and term neonates ≥37 weeks. Exposure to drugs was defined as either the absolute number of prescriptions or expressed per 1,000 infants. Variability in prescriped drugs per ATC-class between NICUs was quantified by calculating the range of total prescriptions per ATC-class per 1,000 patients between NICUs. This range was used to select the ATC-classes for further investigation. All data were stored and analyzed in SPSS Statistics version 21 (IBM, USA),

using non-parametric Kruskal-Wallis test for continuous variables and Pearson's $\chi^2$ test for nominal variables, with a *p*-value of <0.05 for significance.



### Treatment protocol comparison

Four common indications for drug use in neonatal care were selected to compare the drugs and their suggested order as written in the treatment protocols of the four NICUs. This could give more insight in possible causes for differences in drug prescriptions. The selected indications were pain, intubation, convulsions, and hypotension.

## RESULTS

During the one-year period, 1,491 neonates were included in the study with a total of 32,182 patient days, and a median gestational age of 32 weeks (IQR: 29-37 weeks). The median birth weight of all neonates was 1,865 g (IQR: 1,253-3,000 grams), of which 14.5% had an extremely low birth weight (ELBW; < 1,000 grams). The median duration of admission to the NICU was 12 days (IQR: 5-32 days). Data on post menstrual age (PMA) at discharge was missing in six cases. Gestational age, birthweight, duration of admission to the NICU, and postmenstrual age at discharge were all significantly different between the four hospitals (Table 1).

### Overall prescription of drugs and off-label in relation to neonatal age

In total, 181 different drugs were prescribed 10,895 times, of which 23% was off-label in relation to neonatal age (see Supplementary File 1 for on-label age-range in SmPC). The proportion of prescriptions off-label in relation to neonatal age, increased with PMA at start of drug therapy: 19% for infants with PMA at start below 32 weeks, 26% for infants with PMA between 32 and 37 weeks, and 29% above 37 weeks PMA. During admission 54% of the neonates was exposed to at least one off-label drug. The median number of prescribed drugs per patient was 5 (IQR: 3-10). This was significantly different between hospitals varying from a median of 4 to 7 drugs per patient.

The ATC-class with the most frequently prescribed drugs was anti-infective drugs with a total of 3,161 prescriptions (29%), of which 4% was off-label in relation to neonatal age (Figure 1, Table 2). The second largest ATC-class was the nervous system drugs with 2,500 prescriptions (23%) of which 31% was off-label in relation to neonatal age. The drug class of blood and blood forming organs was the third largest with 1,386 prescriptions (13%). However, this result was confounded since 54% of these prescriptions concerned phytomenadione prescribed as supplementary vitamin instead of the labelled indication as an antidote to anticoagulant drugs of the coumarin type. The large proportion of 28% off-label prescriptions was caused by heparin for 86%, which was indicated for arte-

**Table 1. Baseline characteristics of hospitalized neonates in four different NICUs in the Netherlands.**

| | NICU 1 | NICU 2 | NICU 3 | NICU 4 | *p* value | Total/overall |
|---|---|---|---|---|---|---|
| **Number of beds** | 18 | 15 | 13 | 31 | | |
| **Number of patients given drugs** | 314 | 353 | 223 | 601 | | 1491 |
| **Male gender (%)** | 60 | 59 | 55 | 58 | 0.615 | 58 |
| **Gestational age (weeks$^{+days}$)** | 31 (29-35) | 33 (30-38) | 34 (30-38) | 32 (29-37) | <0.001 | 32 (29-37) |
| **<26 weeks (%)** | 17 (5.4) | 15 (4.3) | 8 (3.6) | 39 (6.5) | 0.001 | 79 (5.3) |
| **26-28 weeks (%)** | 33 (10.5) | 32 (9.1) | 15 (6.7) | 59 (9.8) | | 139 (9.3) |
| **28-32 weeks (%)** | 112 (35.7) | 96 (27.3) | 51 (22.9) | 185 (30.8) | | 444 (29.8) |
| **32-37 weeks (%)** | 82 (26.1) | 88 (25.0) | 53 (23.8) | 139 (23.2) | | 362 (24.3) |
| **≥ 37 weeks (%)** | 70 (22.3) | 121 (34.4) | 96 (43.0) | 178 (29.6) | | 465 (31.2) |
| **Birth weight (g)** | 1,695 (1,228 -2,613) | 2,012 (1,350 -3,091) | 2,100 (1,370-3,120) | 1,800 (1,200-2,970) | 0.007 | 1,865 (1,253-3,000) |
| **ELBW(%)** | 48 (15.3) | 51 (14.4) | 22 (9.9) | 95 (15.8) | 0.214 | 216 (14.5) |
| **Number of days at NICU** | 24 (8-47) | 12 (6-30) | 12 (5-25) | 7 (4-17) | <0.001 | 12 (5-32) |
| **PMA at discharge** | 37 (35-39) | 36 (33-40) | 38 (35-40) | 35 (32-39) | <0.001 | 36 (33- 40) |
| **Total patient days** | 9789 | 7769 | 4716 | 9908 | | 32182 |
| **Total prescriptions** | 2216 | 3371 | 1143 | 4165 | | 10895 |
| **Drugs per patient** | 5 (3-10) | 7 (4-14) | 4 (2-6) | 5 (3-8) | <0.001 | 5 (3-10) |
| **Patient days on drugs** | 28 (12-80) | 36 (15-98) | 18 (7-52) | 13 (6-43) | <0.001 | 21 (8-71) |
| **% OL in relation to neonatal age** | 21 | 29 | 11 | 23 | <0.001 | 23 |

Abbreviations: NICU-neonatal intensive care unit; OL -off-label; ELBW-extremely low birth weight; PMA-postmenstrual age

Data presented as median (IQR).

$\chi^2$ test

Kruskal-Wallis one-way analysis

$\chi^2$ for distributions in all strata of gestational ages in 4 NICUs

rial catherater patency. Alimenary tract and metabolism-drugs were fourth largest with 1,327 prescriptions (12%) and 17% off-label in relation to neonatal age. Cardiovascular drugs were the fifth largest class with 958 prescriptions (9%), of which 30% off-label in relation to neonatal age, for 84% due to dopamine and noradrenaline. The sixth largest ATC-class was the respiratory drugs with 36% off-label prescriptions, of which 76% was accounted for by xylometazoline and doxapram.

Table 2 provides the most prescribed drugs overall and off-label in all NICUs, which overall were phytomenadione, cholecalciferol, caffeine, amoxicillin, gentamicin, tobramycin, benzylpenicillin, paracetamol, surfactant and morphine, subsequently. Of these, none are off-label in relation to neonatal age.

## Differences in drug use between NICUs

The largest differences between NICUs were found for nervous system drugs with total prescriptions between NICUs ranging 919–2,278 per 1,000 patients followed by 554–1,465 for blood and blood forming organs, and 238-952 for cardiovascular system drugs, respectively (Figure 1). As 86% of the range of prescribed drugs from blood and blood forming organs is caused by heparin and phytomenadion, cardiovascular and nervous system drugs were considered most interesting for a more extensive comparison (Figure 2).

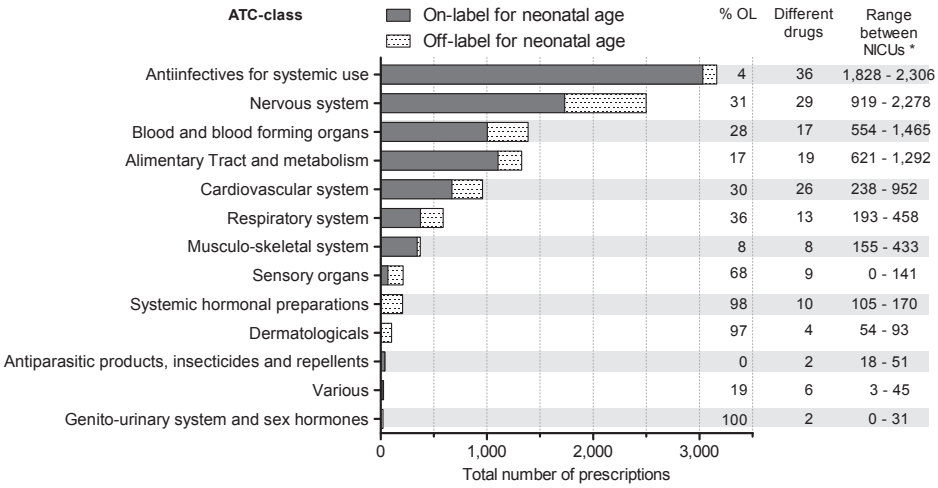


**Figure 1. Total number of prescriptions and proportion off-label in each Anatomical Therapeutic Chemical group.**

In total, 10,895 prescriptions of 181 different drugs were retrieved, of which 23% was off-label in relation to neonatal age.

* Range of total prescriptions per ATC-class per 1,000 patients between NICUs.

Abbreviations: ATC – Anatomical Therapeutic Chemical; OL – Off-label in relation to neonatal age

**Table 2. Most frequently prescribed drugs per 1,000 neonates**

| No | All drugs | Prescriptions | No | Off-label drugs in relation to neonatal age | Prescriptions |
|---|---|---|---|---|---|
| **1** | Phytomenadione | 668 | 1 | Heparin | 219 |
| **2** | Cholecalciferol | 521 | 2 | Fentanyl | 193 |
| **3** | Caffeine | 480 | 3 | Propofol | 117 |
| **4** | Amoxicillin | 375 | 4 | Dopamine | 109 |
| **5** | Gentamicin | 375 | 5 | Phenobarbital | 91 |
| **6** | Tobramycin | 302 | 6 | Hydrocortisone | 79 |
| **7** | Benzylpenicillin | 287 | 7 | Xylometazoline | 68 |
| **8** | Paracetamol | 273 | 8 | Miconazole | 66 |
| **9** | Surfactant | 251 | 9 | Phenylephrine + Tropicamide | 57 |
| **10** | Morphine | 247 | 10 | Norepinephrine | 53 |
| **11** | Heparin | 219 | 11 | Insulin | 50 |
| **12** | Fentanyl | 193 | 12 | Meropenem | 43 |
| **13** | Amoxicillin+ clavulanic acid | 165 | 13 | Dexamethasone | 42 |
| **14** | Midazolam | 148 | 14 | Doxapram | 42 |
| **15** | Atropine | 137 | 15 | Phenylephrine | 38 |
| **16** | Flucloxacillin | 133 | 16 | Chloralhydrate | 30 |
| **17** | Rocuronium | 133 | 17 | Ranitidine | 27 |
| **18** | Vancomycin | 132 | 18 | Levetiracetam | 25 |
| **19** | Furosemide | 130 | 19 | Cefazolin | 15 |
| **20** | Propofol | 117 | 20 | Cisatracurium | 15 |
| **21** | Dopamine | 109 | 21 | Ursodeoxycholic acid | 15 |
| **22** | Ceftazidime | 95 | 22 | Antitrombin | 14 |
| **23** | Phenobarbital | 91 | 23 | Esketamine | 14 |
| **24** | Ibuprofen | 91 | 24 | Tocopherol | 13 |
| **25** | Nystatin | 81 | 25 | Retinol | 11 |
| **26** | Hydrochlorothiazide | 80 | 26 | Trimethoprim | 11 |
| **27** | Spironolactone | 80 | 27 | Levomepromazine | 10 |
| **28** | Hydrocortisone | 79 | 28 | Sildenafil | 10 |
| **29** | Dobutamine | 70 | 29 | Dornase | 9 |
| **30** | Xylometazoline | 68 | 30 | Lidocaine | 9 |

**Total of 10,985 prescriptions for 1,491 patients**

Cardiovascular drug prescriptions differed between the four NICUs (Table 3), and with PMA (Figure 3A). Table 3 shows that the prescription of cardiovascular drugs varied from none to 6 different agents between the different NICUs in infants with PMA below 26 weeks. Dopamine exposure for those neonates was high in two NICUs, where another NICU showed larger variety of other haemodynamic agonists for these preterm infants, i.e. dobutamine and adrenaline. Furthermore, nervous system drugs showed large

variety (Figure 3B, Table 4). Interesting differences included the variable use of propofol, levetiracetam and diuretics between NICUs for all PMAs. Prescriptions of paracetamol, phenobarbital were particularly different in the youngest infants.



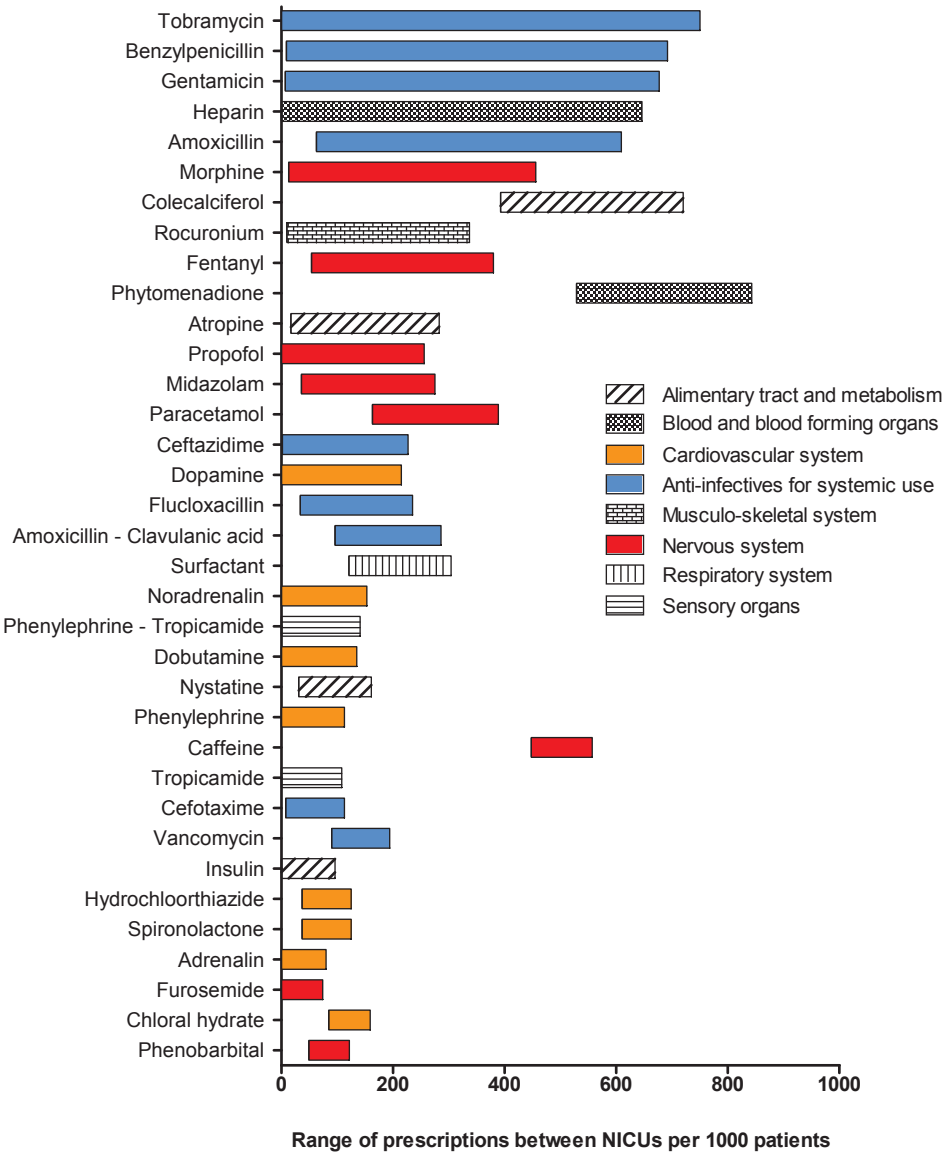


**Figure 2. Range of prescriptions per drug per 1,000 patients between NICUs in descending order.** The top 35 drugs are listed in descending order of the largest difference between minimum and maximum prescriptions.

**Table 3. Cardiovascular drug prescriptions according to PMA (per 1,000 neonates per PMA-group).**

| | NICU 1 | | NICU 2 | | NICU 3 | | NICU 4 | |
|---|---|---|---|---|---|---|---|---|
| **PMA** | **Drug** | **No** | **Drug** | **No** | **Drug** | **No** | **Drug** | **No** |
| **<26** | Dopamine | 353 | Dopamine | 467 | | | Dobutamine | 231 |
| | Norepinephrine | 59 | | | | | Dopamine | 154 |
| | | | | | | | Epinephrine | 77 |
| | | | | | | | Hydrochlorothiazide | 77 |
| | | | | | | | Spironolactone | 77 |
| | | | | | | | Furosemide | 26 |
| **26-<28** | Dopamine | 273 | Dopamine | 156 | Furosemide | 67 | Furosemide | 339 |
| | Furosemide | 242 | Furosemide | 125 | | | Dobutamine | 254 |
| | Hydrochlorothiazide | 91 | Norepinephrine | 63 | | | Hydrochlorothiazide | 237 |
| | Spironolactone | 91 | Epinephrine | 31 | | | Spironolactone | 237 |
| | Dobutamine | 61 | Dobutamine | 31 | | | Dopamine | 153 |
| | Epinephrine | 30 | Milrinone | 31 | | | Epinephrine | 85 |
| **28-<32** | Furosemide | 107 | Furosemide | 156 | Furosemide | 157 | Furosemide | 205 |
| | Dopamine | 63 | Dopamine | 146 | Hydrochlorothiazide | 78 | Hydrochlorothiazide | 184 |
| | Epinephrine | 27 | Norepinephrine | 94 | Spironolactone | 78 | Spironolactone | 184 |
| | Norepinephrine | 27 | Hydrochlorothiazide | 42 | | | Dobutamine | 114 |
| | Hydrochlorothiazide | 18 | Spironolactone | 42 | | | Dopamine | 97 |
| | Spironolactone | 18 | Dobutamine | 31 | | | Epinephrine | 92 |

**Table 3. Cardiovascular drug prescriptions according to PMA (per 1,000 neonates per PMA-group). (continued)**

| | NICU 1 | | NICU 2 | | NICU 3 | | NICU 4 | |
|---|---|---|---|---|---|---|---|---|
| **PMA** | **Drug** | **No** | **Drug** | **No** | **Drug** | **No** | **Drug** | **No** |
| **32-<37** | Furosemide | 98 | Dopamine | 170 | Hydrochlorothiazide | 113 | Furosemide | 151 |
| | Dopamine | 85 | Norepinephrine | 148 | Spironolactone | 113 | Hydrochlorothiazide | 122 |
| | Hydrochlorothiazide | 85 | Furosemide | 125 | Furosemide | 75 | Spironolactone | 122 |
| | Spironolactone | 85 | Hydrochlorothiazide | 34 | Metoprolol | 19 | Dobutamine | 94 |
| | Dobutamine | 24 | Spironolactone | 34 | | | Epinephrine | 58 |
| | Norepinephrine | 24 | Epinephrine | 23 | | | Dopamine | 58 |
| **≥37** | Dopamine | 100 | Dopamine | 289 | Furosemide | 63 | Dobutamine | 129 |
| | Hydrochlorothiazide | 71 | Norepinephrine | 248 | Hydrochlorothiazide | 52 | Epinephrine | 84 |
| | Spironolactone | 71 | Furosemide | 215 | Spironolactone | 52 | Dopamine | 56 |
| | Norepinephrine | 57 | Milrinone | 149 | Digoxine | 21 | Furosemide | 51 |
| | Epinephrine | 43 | Alprostadil | 116 | Propranolol | 10 | Alprostadil | 45 |
| | Alprostadil | 29 | Dobutamine | 99 | | | Hydrochlorothiazide | 39 |

The top 5 prescribed cardiovascular drugs has been shown for every PMA-group.
Abbreviations: PMA-postmenstrual age; NICU – neonatal intensive care unit

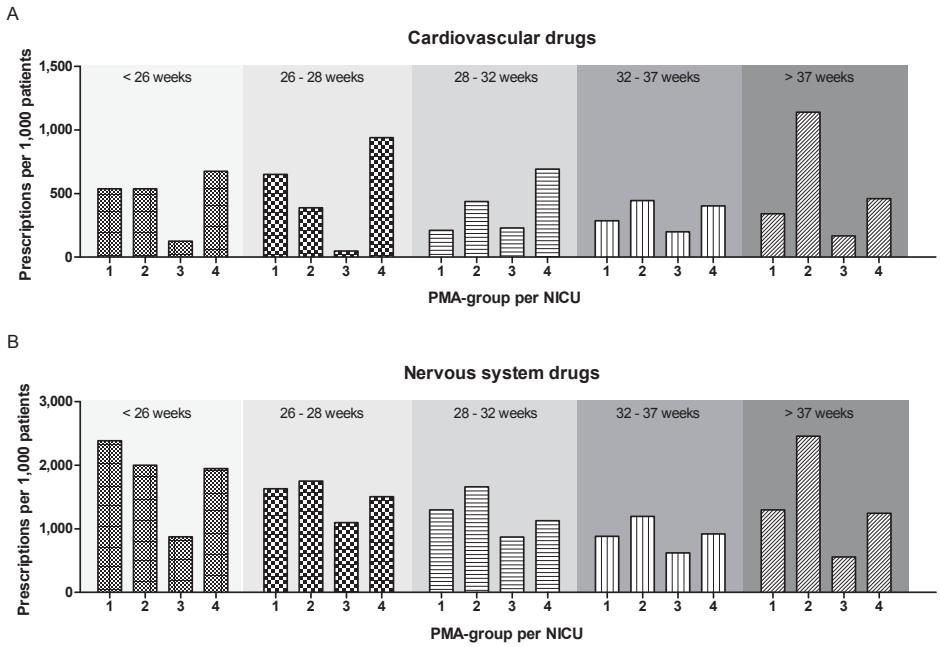


**Figure 3. Exposure of preterm neonates in four NICUs to cardiovascular and nervous system drugs at different PMA's.**

The number of cardiovascular drug prescriptions is expressed per 1,000 patients in each PMA group.
Abbreviations: PMA-postmenstrual age; NICU-neonatal intensive care unit

**Table 4. Nervous system drug prescriptions according to PMA (per 1,000 neonates per PMA-group).**

| | NICU1 | | NICU2 | | NICU3 | | NICU4 | |
|---|---|---|---|---|---|---|---|---|
| **PMA** | **Drug** | **No** | **Drug** | **No** | **Drug** | **No** | **Drug** | **No** |
| **<26** | Caffeine | 647 | Caffeine | 667 | Caffeine | 750 | Caffeine | 923 |
| | Fentanyl | 529 | Morphine | 400 | Paracetamol | 125 | Propofol | 462 |
| | Morphine | 176 | Fentanyl | 333 | | | Morphine | 231 |
| | Paracetamol | 114 | Midazolam | 133 | | | Fentanyl | 103 |
| | | | Paracetamol | 25 | | | Midazolam | 77 |
| | | | | | | | Phenobarbital | 51 |
| **26-<28** | Caffeine | 970 | Caffeine | 844 | Caffeine | 800 | Caffeine | 864 |
| | Fentanyl | 394 | Fentanyl | 469 | Paracetamol | 267 | Propofol | 492 |
| | Paracetamol | 333 | Morphine | 281 | Fentanyl | 200 | Morphine | 305 |
| | Morphine | 273 | Midazolam | 156 | Morphine | 67 | Fentanyl | 186 |
| | Phenobarbital | 91 | Paracetamol | 125 | Phenobarbital | 67 | Midazolam | 119 |
| | Midazolam | 61 | Phenobarbital | 94 | Midazolam | 67 | Phenobarbital | 102 |

**Table 4. Nervous system drug prescriptions according to PMA (per 1,000 neonates per PMA-group). (continued)**

| | NICU1 | | NICU2 | | NICU3 | | NICU4 | |
|---|---|---|---|---|---|---|---|---|
| **PMA** | **Drug** | **No** | **Drug** | **No** | **Drug** | **No** | **Drug** | **No** |
| **28-<32** | Caffeine | 857 | Caffeine | 927 | Caffeine | 902 | Caffeine | 724 |
| | Fentanyl | 304 | Fentanyl | 317 | Paracetamol | 78 | Propofol | 314 |
| | Paracetamol | 259 | Morphine | 238 | Fentanyl | 59 | Morphine | 195 |
| | Morphine | 170 | Paracetamol | 222 | Methadone | 59 | Fentanyl | 108 |
| | Propofol | 27 | Midazolam | 95 | Phenobarbital | 39 | Midazolam | 81 |
| | Midazolam | 18 | Phenobarbital | 63 | Midazolam | 39 | Phenobarbital | 49 |
| **32-<37** | Paracetamol | 427 | Morphine | 364 | Caffeine | 566 | Caffeine | 403 |
| | Caffeine | 378 | Paracetamol | 352 | Paracetamol | 226 | Morphine | 266 |
| | Morphine | 134 | Caffeine | 341 | Etomidate | 57 | Propofol | 252 |
| | Fentanyl | 122 | Fentanyl | 307 | Fentanyl | 57 | Paracetamol | 209 |
| | Phenobarbital | 98 | Midazolam | 182 | Midazolam | 38 | Fentanyl | 173 |
| | Propofol | 98 | Chloralhydrate | 80 | Dexmedetomidine | 19 | Midazolam | 165 |
| **>37** | Paracetamol | 557 | Morphine | 686 | Paracetamol | 396 | Paracetamol | 309 |
| | Morphine | 257 | Paracetamol | 512 | Phenobarbital | 73 | Midazolam | 281 |
| | Phenobarbital | 157 | Midazolam | 504 | Caffeine | 63 | Morphine | 253 |
| | Midazolam | 157 | Fentanyl | 388 | Fentanyl | 31 | Phenobarbital | 185 |
| | Propofol | 114 | Phenobarbital | 231 | Midazolam | 31 | Levetiracetam | 129 |
| | Chloralhydrate | 100 | Chloralhydrate | 149 | Morphine | 21 | Propofol | 79 |

The top 5 prescribed nervous system drugs has been shown for every PMA-group.
Abbreviations: PMA-postmenstrual age; NICU – neonatal intensive care unit

### Treatment protocol comparison

Table 5 gives an overview of the drugs and the order in which they should be prescribed according to the local treatment protocols in the different NICUs for the four selected indications per gestational age-groups.

## DISCUSSION

We evaluated drug prescriptions between NICUs during one year and found that a considerable part of the drugs is still used off-label and that large differences exist in drug prescriptions between four NICUs. The largest variability was found for drug classes with the highest proportion of off-label drugs in relation to neonatal age, i.e. cardiovascular and nervous system drugs. These differences became larger with decreasing PMA, although the proportion off-label prescriptions became smaller. Despite the new

**Table 5. Order of drugs in treatment protocols concerning four major care indications in the four NICUs.**

| | No | NICU1 | No | NICU2 | No | NICU3 | No | NICU4 |
|---|---|---|---|---|---|---|---|---|
| **Pain** | | Paracetamol | | EMLA | | Paracetamol | | Fentanyl |
| | | EMLA | | Lidocaine | | Lidocaine | | Morphine |
| | | Morphine | | Morphine | | Fentanyl | | Midazolam |
| | | Lidocaine | | Fentanyl | | Methadone | | Paracetamol |
| | | Fentanyl | | Paracetamol | | Morphine | | |
| | | | | Midazolam | | Lorazepam | | |
| | | | | Levomepromazine | | | | |
| | | | | Esketamine | | | | |
| **Hypotension** | 1 | Dopamine | 1 | Dopamine | 1 | Dopamine | | |
| | 2 | Dobutamine / Norepinephrine | 2 | Dobutamine / Norepinephrine | 1 | Dobutamine | | |
| | 3 | Hydrocortisone | 3 | Dexamethasone | 1 | Norepinephrine | | |
| | | | | Epinephrine | 1 | Epinephrine | | |
| | | | | Milrinone | 2 | Hydrocortisone | | |
| | | | | Hydrocortisone | | Methylene blue § | | |
| | | | | | | Naloxone § | | |
| **Intubation** | 1 | Atropine (<32 wk) | 1 | Atropine | 1 | Atropine | 1 | Propofol |
| | 1 | Fentanyl (<32 wk) | 1 | Fentanyl / morphine | 1 | Fentanyl/ morphine | | |
| | 1 | Rocuronium (<32 wk) | 1 | Rocuronium / vecuronium | 1 | Rocuronium / etomidate | | |
| | 2 | Propofol (>32 wk) | | | | | | |
| **Convulsions** | 1 | Phenobarbital | 1 | Phenobarbital | 1 | Phenobarbital | 1 | Phenobarbital |
| | 2 | Levetiracetam | 2 | Midazolam (+ pyridoxine) | 2 | Midazolam | 2 | Midazolam (+ pyridoxine) |
| | 3 | Lidocaine | 3 | Lidocaine | 3 | Lidocaine | 3 | Lidocaine |
| | 4 | Midazolam * (+ pyridoxine) | 4 | Levetiracetam | 4 | Pyridoxine | 4 | Levetiracetam |
| | 5 | Clonazepam | 5 | Pyridoxine | 5 | Thiopental | 5 | Pyridoxine |
| | 6 | Thiopental | | | | | 5 | Clonazepam |

The number (No) indicates the order in which drugs should be prescribed for treatment of each indication. If the same number has been used multiple times for one indication in one NICU, this means that their preference is equal, meaning that the attending physician is free to select one of the suggestions. One drug may be prescribed or a combination of drugs simultaneously. The absence of a number concerning pain treatment, indicates none of the NICUs suggest a certain order in the drugs to be prescribed for pain treatment.

Abbreviations: NICU – neonatal intensive care unit

* Preferably avoid midazolam use for premature born infants

§ Experimental drugs

FDA and EMA drug legislations many drugs are still used off-label and the variability in drugs prescriptions reflects the lack of evidence on drug use especially in the smallest newborns.



## Prescribed drugs

Of almost 11 thousand drug prescriptions for neonates, 23% was off-label in relation to neonatal age. Comparable proportions of prescriptions off-label in relation to neonatal age were found in the last decade by Neubert et al. with 38% in Germany[16], Hsieh et al. with 35% in the USA[1], and Cuzzolin et al. with 34% in Italy[17]. A comforting finding was that the proportion of off-label prescriptions in relation to neonatal age, increased with PMA at start of drug therapy. Therefore, the most vulnerable infants with the lowest PMA, were exposed to fewer off-label drugs than infants at higher PMA. This might reflect the cautiousness of clinicians in the most vulnerable patients. Dell'Aera et al. and Avenel et al. also found a higher prevalence of off-label prescriptions within the fullterm neonates compared to the preterms[5, 18].

Also comforting was the small proportion of drug prescriptions off-label in relation to neonatal age (4%) in the largest drug class of anti-infective drugs. On the other hand, the second largest class concerned the nervous system drugs, of which 31% was off-label in relation to neonatal age. These findings are in agreement with those of Cuzzolin et al. and Neubert et al. who also found that anti-infective drugs were the largest ATC-class prescribed with a proportion off-label in relation to neonatal age of 24% and 11%, respectively[16, 17]. For nervous system drugs, these studies found a proportion of 67% and 56% prescriptions off-label in relation to neonatal age, which is comparable with our results.

Nevertheless, off-label drug use does not necessarily implicate inadequate drug use, although this is generally suggested[19]. Instead of referring to the label, adequate drug use should be based on the level of evidence, with an expert interpretation. Consequently, several sources have been developed which are periodically updated and released, such as *British National Formulary*, *Pediatric Dosages* by Lexicomp, *Pediatric Injectable Drugs,* and *Micromedex*. Ceelie et al. reported on large differences between four commonly used pediatric drug formularies, which points at challenges in the availability and reliability of pediatric drug dosing guidelines in present drug formularies[20]. Recently, in the Netherlands a continuously updated online pediatric formulary has been released- the Dutch Pediatric Formulary[21]. Despite the valuable interpretation regarding dosages and safe drug use, the sources mentioned above do not suggest which drug to choose for certain indications and therefore do not help to reduce the differences in prescriptions between physicians and hospitals.

***Comparing NICUs***

Large differences between NICUs were found in neonatal drug use. Drug classes with a high proportion of off-label drugs in relation to neonatal age showed the largest differences between NICUs, i.e. cardiovascular and nervous system drugs. Also, these ATC-classes, together with ATC-class blood and blood forming organs, showed the largest range of total prescriptions per ATC-class per 1,000 patients between NICUs. As the high rank of blood and blood forming organs was driven by heparine and phytomenadion alone, this class was of limited interest for further comparison.

The large differences among cardiovascular drugs strengthen the alarming message of a severe lack in pediatric documentation, which has been reported by Pasquali et al. and Bajetic et al.[22, 23]. Nervous system drugs also showed large variability, which may be a reflection of the variation in drugs mentioned in pain treatment protocols of these four NICUs. This may be explained by the worldwide discussion on the neurodevelopmental safety of nervous system drugs such as opioids, paracetamol, benzodiazepines in the preterm brain[24]. A comparable discussion accounts for the choice of premedication for intubation[25-27]. This can be recognized in treatment protocols in our study, chosing either an opioid with a muscle relaxant, or propofol. Mehler et al. studied analgesic and sedative drug use in very low birth weight infants in German NICUs and reported large differences, as well as many changes over time in analgesic and sedative treatment[28]. On the other hand, the treatment protocol of neonatal convulsions showed less differences between NICUs, which seems the result of an existing national guideline[29]. Even though all mentioned drugs in the guideline were off-label for treatment of convulsions in neonates, this publicly accessible expert opinion appears to reduce different interpretations of sparse evidence.

Liem et al. reported a comparable approach for antibiotic drugs alone and found a considerable variation between Dutch NICUs in the number of different antibiotics used and in the total dosage of antibiotics[30]. This heterogeneity indicates that empiric antibiotic treatment varies among neonatal intensive care units and there are currently no consensus guidelines regarding the choice of empiric antibiotics.

Although all four participating NICUs were level 3, considerable differences were found in the general descriptives between the NICUs; i.e. duration of admission, gestational ages, and specific treatments (surgery, extracorporal membrane oxygenation). These may partly explain the large variability in prescribed drugs between NICUs. Another cause for differences in drug use concerns the steps by which new evidence is adapted to clinical care, which may depend on local expert opinions.

Our multicentre comparison of drug use in NICUs provides a unique view of neonatal pharmacology in practice, but is limited by some assumptions. First, our data did not allow to compare NICUs with respect to drug dosages, routes of administration, specific products, or preparation of drugs for administration. Apart from judging if a drug is

registered for use in neonatal age, each of these items could also have been related to the label if the data were available. Second, since practically all drugs were first labelled for an adult indication, their ATC code was often incorrect with respect to their use in current neonatal practice. Even for drugs where the neonatal indication has been added to the label, their ATC code remains as primarily marketed. This concerns for example sildenafil, ibuprofen, caffeine, phytomenadione. Third, differences in local practice of decision making determines treatments and drug use. In a smaller NICU it may be easier to reach consensus than in a larger NICU. Fourth, data was retrospectively collected from different prospective electronic health record systems, which may have caused some differences in definitions used for data output. Fifth, our findings from a single country cohort, cannot easily be compared to other countries or reports, as the content of the SmPCs may be different between countries, and various definitions for off-label status have been used, which has also been shown by Aronson et al.[31]. Sixth, if the SmPC mentioned an indication for infants in general without mentioning an age range, this was considered to include neonates as well and therefore on-label in relation to neonatal age (see Supplementary File 1). Nevertheless, physicians would not feel safe to prescribe these drugs in clinical practise based on this information, knowing that a general dosage for infants in not optimal and safe for (preterm) neonates. Though, if these drugs, with an on-label status for infants without mentioning an age range, would be considered off-label in relation to neonatal age instead, the overall proportion off-label prescriptions in our cohort increases from 23% to 41%. This is mainly due to changes in the ATC groups; cardiovascular drugs (from 30% to 94% off-label), anti-infectives (from 4% to 24% off-label), and nervous system drugs (from 31% to 46% off-label). In addition, an indication and dosage for neonates in the SmPC, rarely differentiates for gestational age. As the definition of a 'neonate' is limited to a newborn infant during its first 30 days of life, without referring to a certain gestational age, we considered neonates to be term as well preterm newborn infants. Nevertheless, on-label in relation to neonatal age should not necessarily mean on label for all gestational ages.

## FUTURE SUGGESTIONS

Our study shows drugs and indications on which little consensus has been reached, and should therefore be prioritized for expert-interpretation of current evidence and for future research. New investigator initiated research is urgently required as pharmaceutical companies have little benefit of incorporating new findings in pediatrics, which has led to few drug labeling changes made under pediatric legislation, including neonates[12]. Nevertheless, pharmacological trials involving neonates deal with multiple challenges. Appropriate dosing is hampered by the rapid physiological changes occurring at this

stage of development. The selection of proper end-points and biomarkers is complicated by the limited knowledge of the pathophysiology of the specific diseases of infancy. Copinni et al. have addressed possible perspectives to stimulate research in neonates and infants[32]. Furthermore, as evidence on pharmacological treatment of neonates remains thin more (inter) national guidelines on treatment of common indications should be written, following the succesfull example of the guideline for neonatal convulsions.

## CONCLUSION

We showed that drugs used for neonatal care differed importantly between four Dutch level III NICUs. Our findings form a valuable contribution to the several pooled prescription data analyses of multiple NICUs that have been reported. The drug classes with the highest proportion of off-label drugs in relation to neonatal age showed the largest differences between NICUs, i.e. cardiovascular and nervous system drugs. We believe that drug research in neonates should have high priority to ensure the use of safe and appropriate drug therapy in newborns.

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## SUPPLEMENTARY FILE

**Supplementary File 1. Age range in SmPC per active substance and off-label interpretation in relation to neonatal age.**

| Active substance | Off-label interpretation | SmPC includes the following age range with an on-label indication |
|---|---|---|
| Acetazolamide | on-label | Infants in general, not differentiated for neonates |
| Acetylcysteine | off-label | Above 2 years |
| Acetylsalicylic acid | off-label | Above 12 years |
| Aciclovir | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Adenosine | on-label | Infants in general, not differentiated for neonates |
| Albumin | on-label | Neonates, including preterm infants |
| Alimemazine | off-label | Above 2 years |
| Alprostadil | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Alteplase | off-label | No indication for infants |
| Amiodarone | off-label | No indication for infants |
| Amlodipine | off-label | Above 6 years |
| Amoxicillin | on-label | Neonates, including preterm infants |
| Amoxicillin + Clavulanic acid | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Amphotericin B | on-label | Infants in general, not differentiated for neonates |
| Antitrombin | off-label | No indication for infants |
| Argipressin | off-label | Unlicensed |
| Atropine | on-label | Infants in general, not differentiated for neonates |
| Azitromycin | off-label | Above 1 year |
| Beclomethasone | off-label | Above 5 years |
| Benzylpenicillin | on-label | Infants in general, not differentiated for neonates |
| Biotin | off-label | Unlicensed |
| Budesonide | off-label | Above 8 years |
| Bumetanide | off-label | No indication for infants |
| Bupivacaine + Epinephrine | off-label | Above 1 year |
| Caffeine | on-label | Preterm born infants below gestational age of 37 weeks |
| Captopril | off-label | Above 1 month |
| Carbamazepine | on-label | Infants in general, not differentiated for neonates |
| Cefazolin | off-label | Above 1 month |
| Cefotaxim | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Ceftazidime | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Ceftriaxone | on-label | Neonates, not differentiated for gestational age / preterm infants |

**Supplementary File 1. Age range in SmPC per active substance and off-label interpretation in relation to neonatal age. (***continued***)**

| Active substance | Off-label interpretation | SmPC includes the following age range with an on-label indication |
|---|---|---|
| Cefuroxime | on-label | Neonates, including preterm infants |
| Chloralhydrate | off-label | Unlicensed |
| Chloramphenicol | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Cholecalciferol | on-label | Neonates, including preterm infants |
| Ciprofloxacin | on-label | Infants in general, not differentiated for neonates |
| Cisatracurium | off-label | Above 1 month |
| Claritromycin | off-label | Above 6 months |
| Clemastine | off-label | Above 1 year |
| Clindamycin | off-label | Above 1 month |
| Clonidine | off-label | No indication for infants |
| Dexamethasone | off-label | Above 1 month |
| Dexamethasone + Tobramycin | off-label | Above 2 years |
| Dexamethasone + Xylometazoline | off-label | Above 2 years |
| Dextran70 + hypromellose | off-label | No indication for infants |
| Diazepam | off-label | Above 1 month |
| Diazoxide | on-label | Infants in general, not differentiated for neonates |
| Digoxin | on-label | Neonates, including preterm infants |
| Dimetindenum | off-label | Above 1 year |
| Dobutamine | on-label | Infants in general, not differentiated for neonates |
| Dopamine | off-label | No indication for infants |
| Dornase | off-label | Above 5 years |
| Doxapram | off-label | Above 12 years |
| Enoxaparin | off-label | No indication for infants |
| Epinephrine | on-label | Infants in general, not differentiated for neonates |
| Epoprostenol | off-label | No indication for infants |
| Erytromycin | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Esketamine | off-label | No indication for infants |
| Esmolol | off-label | No indication for infants |
| Esomeprazole | off-label | Above 1 year |
| Etomidate | off-label | Above 6 weeks |
| Factor VIIa | off-label | No indication for infants |
| Fentanyl | off-label | Above 2 years |
| Ferrous fumarate | off-label | Above 3 months |
| Fibrinogen | off-label | No indication for infants |
| Flecainide | off-label | Above 12 years |

**Supplementary File 1. Age range in SmPC per active substance and off-label interpretation in relation to neonatal age. (*continued*)**

| Active substance | Off-label interpretation | SmPC includes the following age range with an on-label indication |
|---|---|---|
| Flucloxacillin | on-label | Infants in general, not differentiated for neonates |
| Fluconazole | on-label | Neonates, term |
| Flumazenil | off-label | Above 1 year |
| Fluticasone | off-label | Above 1 year |
| Folic acid | off-label | No indication for infants |
| Folinic acid | off-label | Above 1 month |
| Furosemide | on-label | Infants in general, not differentiated for neonates |
| Fusidic acid | on-label | Infants in general, not differentiated for neonates |
| Ganciclovir | off-label | No indication for infants |
| Gentamicin | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Glycopyrronium bromide | off-label | No indication for infants |
| Heparin | off-label | No indication for infants |
| Hyaluronidase | off-label | No indication for infants |
| Hydrochlorothiazide | on-label | Infants in general, not differentiated for neonates |
| Hydrocortisone | off-label | No indication for infants |
| Hydrocortisone + oxytetracycline + polymyxine | off-label | No indication for infants |
| Hydroxychloroquine | on-label | Infants in general, not differentiated for neonates |
| Hypromellose | off-label | Above 1 year |
| Ibuprofen | on-label | Neonates, including preterm infants |
| Immunoglobulin | on-label | Infants in general, not differentiated for neonates |
| Indometacin | on-label | Preterm born infants |
| Insulin | off-label | Above 10 years |
| Ipratropium | off-label | Above 6 years |
| Isoprenaline | off-label | No indication for infants |
| Isosorbide | off-label | Unlicensed |
| Labetalol | off-label | No indication for infants |
| Lactitol | off-label | Above 1 year |
| Lactulose | off-label | Above 1 month |
| Lamivudine | off-label | Above 3 months |
| Levetiracetam | off-label | Above 1 month |
| Levocarnitine | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Levomepromazine | off-label | No indication for infants |
| Levothyroxine | off-label | Neonates, not differentiated for gestational age / preterm infants |
| Lidocaine | off-label | No indication for infants |

**Supplementary File 1. Age range in SmPC per active substance and off-label interpretation in relation to neonatal age. (***continued***)**

| Active substance | Off-label interpretation | SmPC includes the following age range with an on-label indication |
|---|---|---|
| Lidocaine + prilocaine | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Lorazepam | off-label | Above 12 years |
| Macrogol | off-label | Above 6 months |
| Melatonin | off-label | No indication for infants |
| Meropenem | off-label | Above 3 months |
| Methadone | off-label | No indication for infants |
| Metoprolol | off-label | Above 6 years |
| Metronidazole | on-label | Above 6 weeks |
| Miconazole | off-label | No indication for infants |
| Midazolam | on-label | Neonates, including preterm infants |
| Milrinone | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Morphine | on-label | Infants in general, not differentiated for neonates |
| Mupirocin | on-label | Infants in general, not differentiated for neonates |
| Nadroparin | off-label | No indication for infants |
| Naloxone | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Nifedipine | off-label | No indication for infants |
| Nitrofurantoin | off-label | Above 5 years |
| Norepinephrine | off-label | No indication for infants |
| Nystatin | on-label | Neonates, including preterm infants |
| Octreotide | off-label | No indication for infants |
| Omeprazole | off-label | Above 1 year |
| Oseltamivir | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Oxybutynin | off-label | Above 5 years |
| Palivuzimab | on-label | Neonates, including preterm infants |
| Pamidronic acid | off-label | no indication for infants |
| Pantoprazole | off-label | Above 12 years |
| Paracetamol | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Pentobarbital | off-label | Unlicensed |
| Pethidine | off-label | Above 1 year |
| Phenobarbital | off-label | Above 2 years |
| Phentolamine | off-label | No indication for infants |
| Phenylephrine | off-label | No indication for infants |
| Phytomenadione | on-label | Neonates, not differentiated for gestational age / preterm infants |

**Supplementary File 1. Age range in SmPC per active substance and off-label interpretation in relation to neonatal age. (*continued*)**

| Active substance | Off-label interpretation | SmPC includes the following age range with an on-label indication |
|---|---|---|
| Piperacillin | off-label | Above 2 months |
| Piritramide | off-label | Above 5 years |
| Prednisolone | off-label | No indication for infants |
| Propofol | off-label | Above 1 month |
| Propranolol | off-label | Above 1 year |
| Protamine | off-label | No indication for infants |
| Pyridoxal phosphate | off-label | Above 1 month |
| Pyridoxine | off-label | Above 1 month |
| Ranitidine | off-label | Above 3 years |
| Remifentanil | off-label | Above 1 year |
| Retinol | off-label | Unlicensed |
| Rifampicin | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Rocuronium | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Ropivacaine | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Salbutamol | off-label | Above 2 years |
| Sildenafil | off-label | Above 1 year |
| Somatropin | on-label | Above 1 month |
| Sotalol | off-label | No indication for infants |
| Spironolactone | on-label | Infants in general, not differentiated for neonates |
| Sufentanil | off-label | Above 1 month |
| Surfactant | on-label | Neonates, including preterm infants |
| Suxamethonium | off-label | No indication for infants |
| Teicoplanin | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Tetracosactide | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Thiamazole | off-label | No indication for infants |
| Thiopental | on-label | Infants in general, not differentiated for neonates |
| Tobramycin | on-label | Neonates, including preterm infants |
| Tocopherol | off-label | No indication for infants |
| Tranexamic acid | off-label | Above 1 year |
| Trimethoprim | off-label | Above 5 years |
| Trimethoprim + sulfamethoxazole | off-label | Above 6 weeks |
| Trometamol | off-label | Unlicensed |
| Tropicamide | on-label | Infants in general, not differentiated for neonates |

**Supplementary File 1. Age range in SmPC per active substance and off-label interpretation in relation to neonatal age. (***continued***)**

| Active substance | Off-label interpretation | SmPC includes the following age range with an on-label indication |
|---|---|---|
| Urokinase | off-label | No indication for infants |
| Ursodeoxycholic acid | off-label | Above 1 month |
| Valganciclovir | off-label | No indication for infants |
| Vancomycin | on-label | Neonates, not differentiated for gestational age / preterm infants |
| Xylometazoline | off-label | Above 2 years |
| Zidovudine | on-label | Neonates, term |
| Zinc oxide | on-label | Neonates, not differentiated for gestational age / preterm infants |

Abbreviations: SmPC–Summary of Product Characteristics

# QUANTIFICATION OF DRUGS AND METABOLITES

# 3

# Quantification of acetaminophen and its metabolites in plasma using UPLC-MS: doors open to therapeutic drug monitoring in special patient populations

*Therapeutic Drug Monitoring 2017 Apr;39(2):164-171*

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## ABSTRACT

### Background

Acetaminophen (APAP, paracetamol) is the most commonly used drug for pain and fever in both the United States and Europe and considered safe when used at registered dosages. Nevertheless, differences between specific populations lead to remarkable changes in exposure to potentially toxic metabolites. Furthermore, extended knowledge is required on metabolite formation following intoxication, to optimize antidote treatment. Therefore, the authors aimed to develop and validate a quick and easy analytical method for simultaneous quantification of APAP, APAP-glucuronide, APAP-sulfate, APAP-cysteine, APAP-glutathione, APAP-mercapturate, and protein-derived APAP-cysteine in human plasma by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-MS/MS).

### Methods

The internal standard was APAP-D4 for all analytes. Chromatographic separation was achieved with a reversed-phase Acquity UPLC HSS T3 column with a runtime of only 4.5 minutes per injected sample. Gradient elution was performed with a mobile phase consisting of ammonium acetate, formic acid in Milli-Q ultrapure water or in methanol at flow rate of 0.4 mL/min.

### Results

A plasma volume of only 10 µL was required to achieve both adequate accuracy and precision. Calibration curves of all six analytes were linear. All analytes were stable for at least 48 hours in the autosampler; the high quality control of APAP-glutathione was stable for 24 hours. The method was validated according to the US Food and Drug Administration guidelines.

### Conclusions

This method allows quantification of acetaminophen and six metabolites, which serves purposes for research, as well as therapeutic drug monitoring (TDM). The advantage of this method is the combination of minimal injection volume, a short runtime, an easy sample preparation method, and the ability to quantify acetaminophen and all six metabolites.

## INTRODUCTION



Acetaminophen (APAP, N-Acetyl-p-Aminophenol, or paracetamol) is the most commonly used drug for pain and fever in both the United States and Europe[1]. Acetaminophen is generally safe when used at registered dosages, thereby titrated upon effect, with a maximum of 4 g/day in four doses for adults. In children, dosage depends on age and weight as follows: with <1 month—30-60 mg/kg/day in three doses; with age >1 month—up to 90 mg/kg/day in four doses[2]. On the other hand, administration of supra-therapeutic doses of acetaminophen is the leading cause for liver failure in the United States[3], mainly influenced by its drug metabolism. This metabolism has been reported to deviate in (premature) neonates[4], obese patients[5], and following supratherapeutic doses[6]. Such variability in exposure to potentially toxic metabolites can be expected in other specific populations as well, e.g., anorexic patients, patients from different ethnical backgrounds, extreme elderly, pregnant women and their fetuses[7], and in patients with drug- or genetically driven changes in CYP1A2 activity, e.g., omeprazole induction.

Acetaminophen is largely metabolized in the liver, predominantly by sulfation and glucuronidation (Figure 1). In adults, sulfation encompasses about 30% and glucuronidation about 55%[8-10]; only 2%-5% is excreted unchanged in the urine[9, 10]. Approximately 5%-10% of acetaminophen is metabolized by cytochrome P450 (CYP), primarily by CYP2E1[11-13], to the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI)[9, 14-16]. At therapeutic doses, NAPQI is immediately inactivated by conjugation with glutathione. However, without this detoxification route, NAPQI binds covalently to cellular proteins and forms toxic protein adducts, such as protein-derived acetaminophen-cysteine (APAP-cysteine). These protein adducts may cause mitochondrial dysfunction and early oxidant stress[17-19]. Consequently, hepatotoxicity can be caused by liver cell necrosis[3]. Although it was thought that depletion of 70% of total liver glutathione would be necessary for NAPQI to begin with protein binding[20], protein-derived APAP-cysteine was detected in serum from human after therapeutic doses[21]. It is likely that either a threshold of protein-derived APAP-cysteine needs to be exceeded for the development of toxicity or that specific binding targets are spared at therapeutic doses[6].

Currently, acetaminophen concentrations have only been considered important to measure for patients who are suspected of intake of a toxic amount and for patients who show a decreased hepatic function. In that case, acetaminophen is mostly analyzed with an immunoassay, not measuring metabolites, despite their key role in acetaminophen hepatotoxicity. Considering acetaminophen-metabolic routes, further investigation for associations between exposure to acetaminophen metabolites and toxicity is warranted, as well as exposure in specific populations. In the case of acetaminophen intoxication, extended knowledge of metabolite formation will assist in optimizing (antidote) treatment. This also applies to intoxication upon chronic use of high acetaminophen dos-

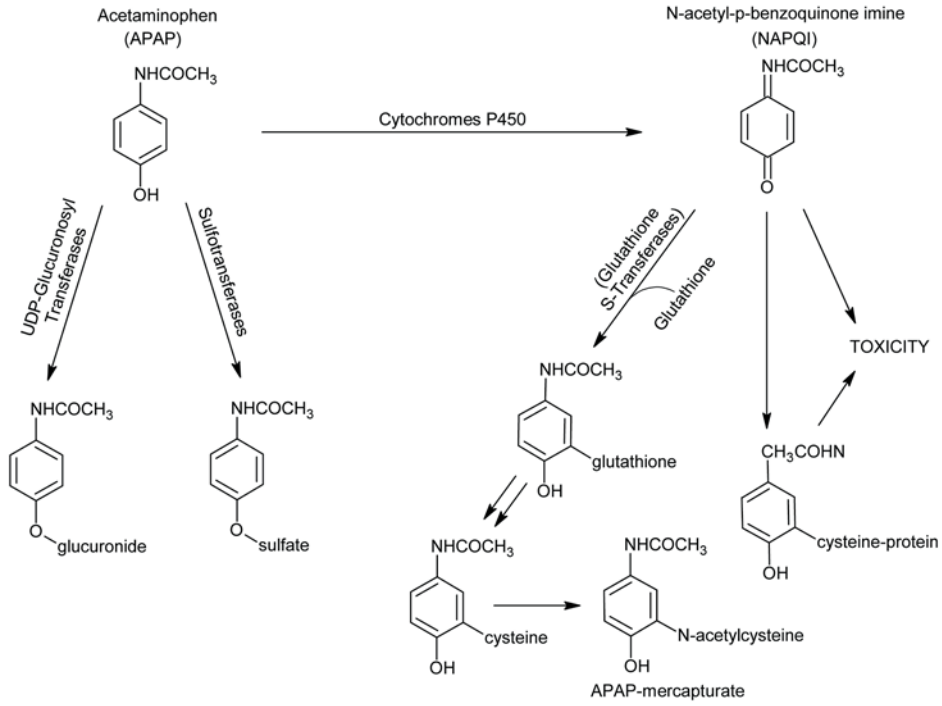


**Figure 1. Metabolic pathway of acetaminophen**
UDP: Uridine 5'-diphospho-glucuronosyltransferase

ages. Currently, there remains a knowledge gap regarding the optimal treatment with N-acetylcysteine infusion to prevent or treat hepatotoxicity. These new insights suggest Therapeutic Drug Monitoring (TDM) of metabolites in case of toxicity, or as part of standard clinical care in certain populations. Ultimately, monitoring of APAP-metabolite concentrations may prevent or reduce toxicity and optimize therapy.

We developed and validated an analytical method for simultaneous quantification of APAP, APAP-glucuronide, APAP-sulfate, APAP-cysteine, APAP-glutathione, APAP-mercapturate, and protein-derived APAP-cysteine in a minimal volume of human plasma by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-MS/MS), preceded by an easy sample preparation. We aimed to optimize the sensitivity of the assay to minimize the required sample volume, which allows measurement of the smallest volume samples from preterm infants.

## MATERIALS AND METHODS

### Chemicals and reagents

APAP, APAP-sulfate, APAP-mercapturate, APAP-glucuronide, and APAP-cysteine solution were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). APAP-glutathione was obtained from Toronto Research Chemicals (Eching, Germany) and APAP-D4 solution from Sigma Aldrich Cerilliant (Zwijndrecht, The Netherlands). Methanol absolute LC-MS grade and formic acid 99% ULC/MS grade were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was purified by using a MilliPore Advantage A10 system. External quality control samples for acetaminophen were purchased from *Stitching Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie* (KKGT, The Hague, The Netherlands) and Santa Cruz Biotechnology (Heidelberg, Germany).



### Stock solutions, calibrators, quality control samples, and internal standard

Stock solutions of APAP, APAP-sulfate, APAP-glucuronide, and APAP-cysteine were prepared at a concentration of 500 mg/L using methanol, while stock solutions of APAP-mercapturate and APAP-glutathione were prepared at a concentration of 100 mg/L using methanol. For each analyte, two separate stock solutions were prepared with the same concentration, for both calibration of standard samples and for QC samples. Stock solutions were stored at −20 °C.

The working solution, calibrator 8 (50 mg/L), was prepared by drying 500 µL of APAP, APAP-sulfate, APAP-glucuronide, and APAP-cysteine, and 2500 µL of APAP-mercapturate and APAP-glutathione in one glass tube at 40 °C under nitrogen flow until all methanol was evaporated. Subsequently, all analytes were reconstituted in 5-mL human plasma and mixed for 30 seconds. Calibrators 1 through 7 (0.05–25 mg/L) and the lower limit of quantification (LLOQ) standard (0.01 mg/L) were prepared by diluting calibrator 8 with human plasma. Quality control (QC) samples were prepared the same way, using the other stock solution. The working solution was diluted with human plasma to get three concentrations: QC Low (0.20 mg/L), QC Medium (1.5 mg/L), and QC High (15 mg/L). Then, calibrators and QC samples were transferred in 10-µL portions to 1.5-mL tubes (Eppendorf) and stored at −80 °C awaiting analysis.

The internal standard (IS) was APAP-D4. A working solution of the internal standard was prepared in methanol at a concentration of 100 µg/L APAP-D4.

### Specimens

Human blank plasma was obtained from the blood transfusion laboratory of the Erasmus Medical Center Rotterdam. Because acetaminophen is a regularly used drug, acetaminophen-free blood was collected from volunteers. Blood was centrifuged to

separate plasma from the red blood cells. Plasma was pooled and collected in smaller tubes. These tubes were stored at −20 °C awaiting analysis.

## Sample preparation

All calibrators, QC samples, blank and patient samples were thawed at least half an hour prior to preparation. Then, to 10 µL of each standard and sample, 40 µL of internal standard solution was added for protein precipitation. The samples were mixed for 15 seconds and then centrifuged for 5 minutes at 16,000 x *g*. Of about 30 µL of supernatant was taken from each sample and transferred to amber auto sampler insert vials (VWR). Next, 140 µL of 0.1% aqueous formic acid was added and the samples were mixed for 15 seconds. The ratio of the aqueous and organic solvent in the sample matched the ratio in the mobile phase at start of the gradient. The blank sample, without internal standard, was prepared by adding 40 µL of methanol instead of internal standard solution. For acetaminophen, acetaminophen-D4, and APAP-cysteine, 4 µL of sample was injected into the UPLC-MS/MS apparatus. For all the other analytes, 10 µL was injected because of the lower sensitivity for these analytes.

### *Protein-derived APAP-cysteine*

For quantification of protein-derived APAP-cysteine in patient samples, the sample preparation was preceded with one extra step after thawing the sample: The protein-bound fraction was removed by filtration of an extra 130 µL plasma through an Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane (Merck Chemicals, Amsterdam, The Netherlands) and discarded afterward, in order to collect 10 µL plasma with unbound APAP-cysteine. The concentration of protein-derived APAP-cysteine is determined by calculating the difference in APAP-cysteine concentrations before and after filtration. The sample was further prepared as described for all other analytes, continuing with the addition of 40 µL of internal standard.

## Instrumentation

The equipment used was a Dionex Ultimate UPLC system consisting of an Ultimate 3000 RS UPLC pump, an Ultimate 3000 RS autosampler and an Ultimate 3000 RS Column Compartment. The UPLC was connected to a triple quadrupole Thermo TSQ Vantage MS with HESI probe (Thermo Scientific). The software programs Chromeleon (version 6.8, Dionex, Thermo Scientific), Xcalibur (version 2.1, Thermo Scientific), and LCquan (version 2.6, Thermo Scientific) were used to control the system and analyze the data.

## UPLC conditions

Chromatographic separation, based on affinity of the analytes with the nonpolar stationary phase, was achieved with a reversed-phase Acquity UPLC HSS T3 column (1.8

μm, 2.1 × 100 mm; High Strength Silica with a bound trifunctional C18 alkyl phase). Gradient elution was performed with a mobile phase consisting of 1 mL of a 154 mg/L solution of ammonium acetate in formic acid (99%) in 1 L of Milli-Q ultrapure water (eluent A) and 1 mL of the same solution in 1 L of methanol (eluent B). Prior to the analysis, the system was equilibrated at the starting conditions of 86% eluent A and 14% eluent B until pressure was stable. The multistep gradient was as follows: from 0 to 0.8 minutes, eluent B was increased from 14% to 28%; from 0.8 to 1.0 minute, eluent B was increased to 95%; from 1.0 to 2.0 minutes, eluent B was kept stable at 95%; from 2.0 to 2.2 minutes, eluent B was decreased to 14%; from 2.2 to 5.3 minutes, eluent B was kept stable at 14%. The run ended at 5.3 minutes at starting conditions. The flow was kept at 0.400 mL/minute during the entire runtime. The temperature for the column oven was set at 40°C and for the autosampler at 15°C. In order to quantify all analytes, a volume of 4 μL as well as 10 μL is injected, which requires two runs per sample and therefore doubles the runtime to 10.6 minutes. For the quantification of protein-derived APAP-cysteine, a third run is required.



## MS/MS conditions

For the detection and quantification of acetaminophen and metabolites, settings of the MS/MS were as follows: MS runtime of 4.5 minutes, experiment type was Selected Reaction Monitoring (SRM), ionization at ESI+, spray voltage of 4000 V, vaporizer temperature at 375°C, sheath gas pressure with nitrogen at 50 psi, auxiliary gas pressure with nitrogen at 20 psi, capillary temperature at 250°C, and collision pressure at 1.5 mTorr. All other settings were specific for each analyte and were determined by infusion experiments with academic solutions of each analyte of 1 mg/L. The chosen transitions and settings are shown in Table 1.

## Assay validation

Validation of the method was performed according to the US Food and Drug Administration (2001) guidelines for bioanalytical methods[22]. The following validation parameters were investigated.

### *Linearity*

The relation between the concentration of the calibrators and response (ratio of peak areas of the analytes and the internal standard) was tested with a calibration curve. This curve should be linear across the range from 0.05 up to 50 mg/L. To make the calibration curve, eight calibrators were prepared and analyzed. Linear least square regression was used to analyze the data. It was decided to apply weighting 1/x, which means that calibrators with the lowest concentrations are more important for the calibration line than calibrators with highest concentrations[23]. The relative standard deviation (RSD)

**Table 1. Analyte-specific settings**

| Analyte | Parent ion (m/z) | Product ion (m/z) | ESI mode | Collision Energy (v) | S-Lens |
|---|---|---|---|---|---|
| APAP | 152.169 | **110.16** | + | **15** | 77 |
| | | 93.13 | | 23 | |
| | | 65.13 | | 29 | |
| APAP-D4 (IS) | 156.191 | **114.19** | + | **15** | 77 |
| | | 97.16 | | 22 | |
| | | 69.17 | | 30 | |
| APAP-D3-sulfate | 235.017 | **155.06** | + | **26** | 77 |
| | | 113.10 | | 37 | |
| | | 68.10 | | 31 | |
| APAP-sulfate | 232.046 | **152.06** | + | **13** | 77 |
| | | 110.10 | | 22 | |
| | | 65.10 | | 39 | |
| APAP-glucuronide | 328.202 | **152.14** | + | **15** | 80 |
| | | 110.07 | | 35 | |
| | | 93.03 | | 55 | |
| APAP-cysteine | 271.155 | 182.08 | + | 12 | 76 |
| | | **140.07** | | **26** | |
| | | 96.07 | | 37 | |
| APAP-mercapturate | 313.176 | 208.10 | + | 16 | 77 |
| | | 166.10 | | 27 | |
| | | **140.050** | | **31** | |
| APAP-glutathione | 457.245 | 328.18 | + | 13 | 110 |
| | | 181.89 | | 22 | |
| | | **140.01** | | **40** | |

The bold printed product ion mass-to-charge values were chosen.
ESI: Electrospray ionization

was required to be lower than 15%, and the correlation coefficient (r) together with the determination coefficient (r) were required to be at least 0.9950 and 0.9900, respectively.

### *LLOQ and ULOQ*

The LLOQ was measured by analyzing the LLOQ calibrator (0.01 or 0.05 mg/L) six times in a row. Mean and standard deviation of the response ratios of the six samples were measured. Imprecision and accuracy were calculated and should be ≤20% and between 80% and 120%, respectively. The highest calibrator of the calibration curve was used as upper limit of quantification (ULOQ).

### *Accuracy*

Accuracy was measured by analyzing three QC concentrations (n = 6 for each concentration). The percentage deviation between measured concentration and theoretical concentration was calculated, and should be lower than 15%.

***Repeatability and reproducibility***

Repeatability was tested by analyzing three QC concentrations in six-fold on the same day. For each concentration, mean and RSD were calculated. Reproducibility was tested by analyzing each QC concentration in duplicate on six different days. The mean response of the 12 concentrations for each sample with their RSD was calculated. For both tests, RSD was required to be lower than 15%.

***Stability***

In-process stability was determined by storing QC samples of three concentrations (n = 2 per concentration) at 6°C prior to preparation for 24 and 48 hours. Autosampler stability was determined by storing QC samples (n = 2 per concentration) after sample preparation in the autosampler for 24, 48, and 72 hours. Response ratios were measured and compared with response ratios of samples kept at −80°C prior to preparation. After sample preparation, samples were directly analyzed. Recovery was required to be between 90% and 110%.

***Matrix effect and recovery***

It is important to measure matrix effects and absolute recoveries in the development of an LC-MS/MS method since ion suppression and ion enhancement effects can be expected owing to interferences by matrix compounds, stable-isotope-labeled internal standards and co-eluting compounds[24]. In order to check whether the precision, the reproducibility, and the stability of the concentration-signal ratio are affected by interference of the matrix analytes, the method described by Matuszewski et al. (2003) was used[25]. Five different lots of human plasma were used. To two QC concentration levels (QC low and QC high) and a blank sample (all three in duplicate), analytes were added before extraction. The same set of QCs and blanks was prepared with the analytes added after extraction. Also, a set of six samples was evaluated with only Milli-Q ultrapure water instead of plasma. Matrix effects were calculated as follows: (peak area of analyte spiked after extraction) / (peak area of analyte prepared in Milli-Q ultrapure water) × 100%.
The recovery was calculated as the percentage ratio of the area of the analytes spiked before extraction and the ones prepared in Milli-Q ultrapure water. The mean and RSD were calculated of both matrix effects and recovery. In the ideal situation, the mean matrix effects and recovery are between 80% and 120% and the RSD of both parameters is ≤15%. Furthermore, for each analyte, the IS-normalized matrix effect should also be calculated by dividing the matrix effect of the analyte by the matrix effect of the IS. The RSD of the IS-normalized matrix effect calculated from the different lots of matrix should not be greater than 15%.

### Application to pediatric pharmacokinetic samples

For the validation of the assay for clinical practice and research purposes, the method has been applied to quantify acetaminophen and its metabolites in plasma of children participating in a pediatric clinical study. This observational prospective study was performed at the Department of Anaesthesia and Intensive Care Medicine of Our Lady's Children's Hospital, Dublin, Ireland, between January and November 2012. Children (with and without Down's Syndrome) routinely received acetaminophen post-cardiac surgery in a dose of 7.7 mg/kg for children below 10 kg bodyweight, and 15 mg/kg for children above 10 kg bodyweight. The study protocol was approved by the local ethics committee.

## RESULTS

### Linearity

Linearity was achieved for each analyte in the range between the LLOQ and the ULOQ (Table 2), with all RSDs to be lower than 15% and the determination coefficient (r) to be 0.998 at the lowest. APAP was linear from calibrator 1 up to and including 7; APAP-mercapturate and APAP-cysteine from calibrator 1 up to and including 6; APAP-sulfate from calibrator 2 up to and including 8; APAP-glucuronide, APAP-glutathione from calibrator 1 up to and including 8. Quantification performance of protein-derived APAP-cysteine is subject to those of APAP-cysteine, with the step of filtration being the single difference.

### LLOQ and ULOQ

The results of determination of LLOQ and ULOQ are shown in Table 2.

### Accuracy, repeatability, and reproducibility

The accuracy, repeatability, and reproducibility data all met the requirement of being less than 15%, except for the APAP-cysteine accuracy of 30.9% for QC high. The results are shown in Table 2.

### Stability

Except for APAP-glutathione, the recovery of all QCs was between 90% and 110%, indicating that they were stable for at least 48 hours when stored at 6°C. APAP-glutathione was only stable for 24 hours. All prepared QCs were stable for at least 48 hours when kept in the autosampler. The effect of drying showed no significant difference between the dried and non-dried standard (Mann Whitney test; $p < 0.05$).

**Table 2. Validation results**

| Analyte | QC | Accuracy[#] (%) | Repeatability RSD (%) (within-run imprecision) | Reproducibility RSD (%) (between-run imprecision) | LLOQ (mg/L) | ULOQ (mg/L) |
|---|---|---|---|---|---|---|
| APAP | QC L | −2.6 | 2.2 | 5.0 | 0.020 | 25.0 |
| | QC M | 4.7 | 2.4 | 5.4 | | |
| | QC H | 1.9 | 2.0 | 6.1 | | |
| APAP-cysteine | QC L | −4.9 | 5.9 | 8.6 | 0.020 | 10.0 |
| | QC M | −0.4 | 3.7 | 6.9 | | |
| | QC H | 30.9 | 4.1 | 11.7 | | |
| APAP-glucuronide | QC L | 1.5 | 5.1 | 5.7 | 0.047 | 47.0 |
| | QC M | −6.4 | 3.8 | 5.4 | | |
| | QC H | 4.6 | 10.4 | 12.4 | | |
| APAP-glutathione | QC L | −4.0 | 6.6 | 13.7 | 0.022 | 43.0 |
| | QC M | 7.2 | 3.4 | 6.4 | | |
| | QC H | −0.6 | 3.6 | 7.0 | | |
| APAP-mercapturate | QC L | 2.2 | 3.6 | 4.8 | 0.010 | 15.0 |
| | QC M | 1.8 | 2.5 | 4.7 | | |
| | QC H | −2.3 | 3.4 | 5.2 | | |
| APAP-sulfate | QC L | −0.6 | 3.1 | 6.4 | 0.043 | 43.0 |
| | QC M | 0.8 | 3.4 | 4.4 | | |
| | QC H | 2.2 | 3.1 | 6.2 | | |

QC: Quality control
LLOQ: Lower limit of quantification
ULOQ: Upper limit of quantification
[#] The percentage for accuracy is the error of measurement.

## Matrix effect and recovery

Matrix effects and absolute recoveries in the development of the LC-MS/MS method are shown in Table 3. The test of Matuszewski showed that APAP, APAP-cysteine, and APAP-sulfate experienced neither matrix effect nor an effect from the sample preparation. Concerning APAP-glucuronide though, 191.2% matrix effect indicates ion enhancement. On the other hand, for APAP-mercapturate, ion suppression was observed; matrix effect

**Table 3. Matrix effect, recovery, and process efficiency**

| Analyte | Matrix effect mean (%) | Recovery mean (%) | Process Efficiency mean (%) |
|---|---|---|---|
| APAP | 90.3 | 108.2 | 97.7 |
| APAP-cysteine | 104.5 | 122.2 | 127.6 |
| APAP-glucuronide | 191.2 | 105.9 | 204.0 |
| APAP-glutathione | 81.4 | 18.6 | 16.2 |
| APAP-mercapturate | 72.0 | 140.3 | 96.4 |
| APAP-sulfate | 95.8 | 104.5 | 100.2 |

was 72.0%. A good recovery was achieved for all analytes, except for APAP-glutathione with mean 18.6%.

### Application to pediatric pharmacokinetic samples

A total of 162 post-dose samples were collected from a pediatric cohort (n = 30), consisting of children with Down's Syndrome (n = 17) and without Down's Syndrome (n = 13) (data unpublished); median age at surgery was 176 days (range 92-944), median weight at cardiac surgery 6.1 kg (4-12.9).

For APAP, APAP-cysteine, APAP-glucuronide, and APAP-sulfate, only 1 of the 162 (0.6%) samples was measured below LLOQ. For APAP-mercapturate and APAP-glutathione, this was the case in 5 (3.1%) and 161 (99.4%) samples, respectively. None of the analytes was measured above the ULOQ in these samples. Due to the small sample volume, it was not possible to differentiate between protein bound and unbound APAP-cysteine.

## DISCUSSION

We have validated an UPLC-MS method for the quantification of acetaminophen and its metabolites according to US Food and Drug Administration guidelines, with an easy sample preparation, short runtime, and minimal injection volume. Therefore, the assay is very suitable for TDM. The metabolites incorporated in this method are APAP-glucuronide, APAP-sulfate, APAP-glutathione, APAP-cysteine, APAP-mercapturate, and protein-derived APAP-cysteine. Prior reported methods for the quantification of acetaminophen and metabolites in human plasma contained few metabolites, mostly acetaminophen sulfate and/or glucuronide[26-28]. Assays with more metabolites were prior validated in animal matrices[29], although Cook et al. recently published a method in human plasma and urine that comes close to the performance of our assay[30]. Our assay is distinguished by a shorter total runtime per injection of 5.3 minutes versus 20 minutes, easier sample preparation, and the ability to quantify the toxic metabolite protein-derived APAP-cysteine.

Our assay fulfilled the desired criteria for accuracy, repeatability, and reproducibility, except for the 30.9% accuracy of QC high of APAP-cysteine. This QC high concentration of APAP-cysteine was outside the linear range. The overestimation of APAP-cysteine could be caused by transformation from the instable APAP-glutathione. At therapeutic doses, the acetaminophen ULOQ is generally not exceeded, although it may be for toxicology purposes. The ranges for linearity for all other analytes were perfectly suitable for clinical pharmacology and toxicology.

Relevant matrix effects were measured for APAP-glucuronide during the experiment, resulting in an increased process efficiency. This is in line with the general problematic

behavior of glucuronide-metabolites in LC-MS/MS analyses, due to their susceptibility to interferences from the co-eluting matrix analytes[31]. On the other hand, for APAP-mercapturate, matrix effects lead to observed ion suppression. The coefficients of variation of APAP-mercapturate in the samples spiked before extraction are 5.8% and 4.7% for QC-L and QC-H, respectively. Therefore, the effect of the matrix can be considered acceptable. For all other analytes, no matrix effects were measured, which indicates the absence of interferences by matrix compounds, stable-isotope-labeled internal standards, and co-eluting compounds, that may affect ion suppression and ion enhancement. The matrix effect of plasma is relevant, although it does not influence quantification as all analytes and calibrators are prepared in the same plasma-matrix and are subject to influence to the same extent.



Except for APAP-glutathione, the stability of all analytes was good, which means they were stable for at least 48 hours when stored at 6°C. APAP-glutathione was only stable for 24 hours at 6°C, and therefore the measurement or storage of the plasma sample in a freezer should be aimed for within 24 hours. This instability has also been reported by Cook et al.[30]. Hydrolysis of APAP-glutathione quickly transforms APAP-glutathione to APAP-cysteine, presumably by gamma-glutamyl transferase and dipeptidases. This may lead to an undervaluation of the actual concentration APAP-glutathione at the time of sample collection and may lead to an increased APAP-cysteine concentration. This instability of APAP-glutathione, where APAP-cysteine is formed from APAP-glutathione, may also be responsible for the increased recovery and process efficiency of APAP-cysteine and the lower recovery and process efficiency of APAP-glutathione. For future research, the addition of peptidase inhibitors during sample collection could prevent or reduce this degradation.

The assay was successfully validated for clinical practice and research purposes, quantifying acetaminophen and its metabolites in 162 plasma samples from children. APAP-glutathione could only be quantified in one sample, as a result of rapid conversion into APAP-cysteine. This confirms the relevance of the addition of a peptidase inhibitor during sample collection. APAP-mercapturate could not yet be detected in five samples, which were all the first to be collected post dose, as APAP-mercapturate is the last metabolite to be formed. For one sample, which was drawn 4 minutes after the dose, all analytes were below LLOQ. Since acetaminophen was not yet detectable at that time, metabolites could not have been formed either. In conclusion, the assay is performing well for samples in clinical practice.

Quantification of APAP-glutathione during therapeutic as well as toxic dosages of acetaminophen may be relevant, as it plays a crucial role in the formation of toxic metabolites, although quantification of in vivo APAP-glutathione levels has only been reported in animals yet[29]. Normally, the reactive metabolite NAPQI is quickly detoxified by conjugation with glutathione and further converted to the cysteine conjugate before

it is acetylated to form APAP-mercapturate. However, when the formation of the reactive metabolite exceeds the glutathione-conjugation capacity of the liver, covalent binding of NAPQI to cellular macromolecules may result, which initiates the events ultimately leading to cytotoxicity.

Nevertheless, protein-derived APAP-cysteine can be measured with our assay if 130 µL of plasma sample is available. Generally, this allows the quantification in adults and older infants, but not in neonates. Protein-derived APAP-cysteine is mostly present in hepatocytes and is directly related to toxicity and detectable in serum at therapeutic doses[6]. The interpretation for the treatment or toxicity still remains to be investigated.

More research is needed on the toxic effects and characteristics of acetaminophen metabolites in specific populations where different metabolism may be expected. These may include patients with anorexia, patients from different ethnic backgrounds, elderly patients, pregnant women and their fetuses[7], obese adults[5], preterm infants[32-34], patients with possible pharmacokinetic interactions on CYP1A2, and patients subjected to repeated administration of acetaminophen leading to induced CYP enzymes. Repeated administration of acetaminophen at a subtoxic dose may result in an induction of hepatic CYP enzymes CYP2E1, CYP3A, and CYP1A[35].

Generally, for toxicology purposes, acetaminophen concentrations have only been considered important to measure for patients who are suspected for intake of a toxic amount, not its metabolites. Extended knowledge is required about metabolite formation following intoxication to optimize treatment by infusion of the antidote, N-acetylcysteine. TDM of metabolites may be indicated in case of toxicity, or as part of standard clinical care in certain populations where metabolites may be used as a marker for suspected liver injury.

## CONCLUSION

We have developed a method for the simultaneous quantification of APAP, APAP-glucuronide, APAP-sulfate, APAP-cysteine, APAP-glutathione, APAP-mercapturate, and protein-derived APAP-cysteine in human plasma, which greatly facilitates further research into acetaminophen and metabolites, as well as for TDM purposes, even in the smallest plasma volumes obtained from preterm infants.

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# 4

# Simultaneous quantification of fentanyl, sufentanil, cefazolin, doxapram and keto-doxapram in plasma using liquid chromatography–tandem mass spectrometry

*Biomedical Chromatography 2018 May 16:e4290*

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## ABSTRACT

### Aims

A simple and specific UPLC-MS/MS method was developed and validated for simultaneous quantification of fentanyl, sufentanil, cefazolin, doxapram and its active metabolite keto-doxapram.

### Methods

The internal standard was fentanyl-d5 for all analytes. Chromatographic separation was achieved with a reversed phase Acquity UPLC HSS T3 column with a run-time of only 5.0 minutes per injected sample. Gradient elution was performed with a mobile phase consisting of ammonium acetate, formic acid in Milli-Q ultrapure water or in methanol with a total flow rate of 0.4 mL/minute.

### Results

A plasma volume of only 50 µL was required to achieve both adequate accuracy and precision. Calibration curves of all 5 analytes were linear. All analytes were stable for at least 48 hours in the autosampler. The method was validated according to US Food and Drug Administration guidelines.

### Conclusions

This method allows quantification of fentanyl, sufentanil, cefazolin, doxapram and keto-doxapram, which serves purposes for research, as well as therapeutic drug monitoring, if applicable. The strength of this method is the combination of a small sample volume, a short run-time, a deuterated internal standard, an easy sample preparation method and the ability to simultaneously quantify all analytes in one run.

## INTRODUCTION

One of the most important issues in peri-operative and intensive care medicine is the establishment of an individual antibiotic and analgosedation drug profile for each patient with respect to the clinical situation, together with support of vital functions. In general, the analgesic and sedation dose regime will be adjusted to the clinical situation of each individual patient to shorten duration of therapy and to reduce morbidity[4]. Large knowledge gaps still exist with respect to the optimal drug therapy and covariates that determine the effect and safety, especially in neonates and infants, which requires well designed trials[6].



For certain drugs therapeutic drug monitoring (TDM) has been proven valuable to ensure drug effects, dosage regimes, monitor physiological changes, and when appropriate, adapt the medical health care of each patient in the intensive care unit. Despite many drugs are still being dosed on clinical response, the continuously expanding assortment of analytical methods improves drug safety and individual patient treatment[31]. Both for research as well as for TDM, more assays are necessary with a minimal sample volume, a short runtime, quick and easy sample preparation, and simultaneously measuring multiple analytes. Simultaneous quantification in one assay either allows to quantify multiple analytes in one sample without requiring extra sample volume, and allows to run samples containing different drugs efficiently in one assay-run.

Evidence is sparse on the use of fentanyl, sufentanil, cefazolin and doxapram for certain pediatric age-ranges and indications. Sufentanil, fentanyl and cefazolin are part of peri-operative treatments for children. Furthermore, sufentanil and fentanyl are synthetic opioid analgesics widely used in clinical anesthesia and analgesia[18, 23]. Cefazolin is a first-generation cephalosporin Beta-lactam antibiotic used for treatment of sepsis or life-threatening infections[20], where adequate individual dosing may be lifesaving. Neither has doxapram been investigated sufficiently in children, despite its frequent and promising use in neonatal intensive care for treatment of apnea of prematurity[9, 10, 23, 26].

This assay will serve future research to close the knowledge gaps on these four drugs, but may also be used for therapeutic drug monitoring (TDM) if a target concentration range may be defined. As these four drugs are commonly prescribed and combined, we aimed to develop and validate a quick and easy analytical method for simultaneous quantification of fentanyl, sufentanil, cefazolin, doxapram and its active metabolite keto-doxapram in human plasma by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-MS/MS). We optimized sensitivity of the assay so to minimize the required sample volume, which allows measurement of small volume samples, even from premature born infants.

## MATERIALS AND METHODS

### Chemicals and reagents

Fentanyl, fentanyl-d5 and sufentanil were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Cefazolin was obtained from Santa Cruz Biotechnology (Heidelberg, Germany), doxapram from Selleckchem (Munich, Germany) and keto-doxapram from Tractus (London, England). Ammonium acetate was obtained from Sigma Aldrich (Zwijndrecht, the Netherlands). Methanol, acetonitrile and formic acid were purchased from Biosolve BV (Valkenswaard, the Netherlands). All reagents were LC-MS grade, which means at least 99% purity. Water was purified by using a MilliPore Advantage A10 system. Human drug-free plasma was obtained from the blood donation center (Sanquin, Rotterdam, the Netherlands).

### Stock solutions, calibration standards, quality control samples and internal standard

Stock solutions of doxapram and keto-doxapram were prepared at a concentration of 500 mg/L using methanol. The following substance stock concentrations in methanol were prepared, sufentanil at a concentration of 20 mg/L, fentanyl at a concentration of 2 mg/L, and cefazolin at a concentration of 5000 mg/L using Milli-Q water. For each analyte two separate stock solutions were made with the same concentration, for both calibration of standard samples and for quality control samples. Stock solutions were stored at -20°C, except the stock solution of cefazolin which was stored at 2-8°C. The calibration standard 8 and Quality Control (QC) High were made from the stock solutions with drug-free human plasma. Calibration standard 1 through 7 and the lower limit of quantification (LLOQ) standard were prepared by serial diluting of calibration standard 8 with human plasma. QC Medium and QC High samples were prepared the same way, using the other stock solution (QC High) which was diluted with human plasma. The concentrations of all calibration standards are given in Table 1 and the concentrations of the quality controls are given in Table 2. Calibration standards and quality control samples were stored as 50 µL portions in 1.5 mL Eppendorf tubes at -80°C prior to analysis. The internal standard was fentanyl-d5, which was dissolved in a mixture of acetonitrile and methanol 1:1 at a concentration of 10 µg/L. The internal standard working solution was stored at -20°C.

### Sample preparation

A mixture of acetonitrile and methanol, containing 10 µg/L fentanyl-d5 (the internal standard solution) was used for protein precipitation. 50 µL of the calibration standards, quality control samples, blanks and patient samples were thawed at least half an hour prior to preparation. Then plasma proteins were precipitated by adding 200 µL of the

**Table 1: Concentrations of all calibration standards and LLOQ standard**

| Analyte | LLOQ (µg/L) | S1 (µg/L) | S2 (µg/L) | S3 (µg/L) | S4 (µg/L) | S5 (µg/L) | S6 (µg/L) | S7 (µg/L) | S8 (µg/L) |
|---|---|---|---|---|---|---|---|---|---|
| Fentanyl | 0.10 | 0.10 | 0.50 | 1.0 | 2.5 | 4.0 | 5.0 | 8.0 | 10.0 |
| Sufentanil | 0.25 | 1.0 | 5.0 | 10.0 | 25.0 | 40.0 | 50.0 | 80.0 | 100 |
| Cefazolin | 1,000 | 1,000 | 5,000 | 10,000 | 25,000 | 75,000 | 80,000 | 90,000 | 100,000 |
| Doxapram | 50 | 100 | 500 | 1,000 | 2,500 | 4,000 | 5,000 | 8,000 | 10,000 |
| Keto-doxapram | 50 | 50 | 100 | 250 | 500 | 1,000 | 2,000 | 4,000 | 5,000 |

S: Calibration standard-LLOQ: Lower limit of quantification

**Table 2: Concentrations of all quality controls**

| Analyte | QC Low (µg/L) | QC Medium (µg/L) | QC High (µg/L) |
|---|---|---|---|
| Fentanyl | 0.5 | 2.5 | 7.5 |
| Sufentanil | 2.0 | 10.0 | 30.0 |
| Cefazolin | 4,000 | 25,000 | 70,000 |
| Doxapram | 400 | 2,500 | 7,000 |
| Keto-doxapram | 150 | 850 | 3,000 |

QC: Quality control

internal standard solution. Subsequently, the samples were vortexed for about 10 seconds. After vortexing, the precipitant was separated by centrifugation for 5 minutes at 16,000 x *g*. 100 µL of each supernatant was transferred into an auto sampler insert vial (VWR, Amsterdam, the Netherlands) and diluted by adding 400 µL of mobile phase A. The autosampler vials were mixed by using the vortex for 10 seconds. For cefazolin, doxapram and keto-doxapram 1 µL was injected into the UPLC. For fentanyl and sufentanil, 10 µl was injected into the system because of the lower therapeutic range of these compounds (see Table 2).

## Instrumentation

A Dionex Ultimate UPLC system consisting of an Ultimate 3000 RS UPLC pump, an Ultimate 3000 RS autosampler and an Ultimate 3000 RS Column Compartment were used as the equipment. The UPLC was connected to a Thermo TSQ Vantage triple quadrupole MS with HESI probe (Thermo Scientific, Waltman, MA). The software programs Chromeleon (version 6.8, Dionex, Thermo Scientific), Xcalibur (version 2.1, Thermo Scientific), and LCquan (version 2.6, Thermo Scientific) were used to control the system and analyze the data.

## UPLC conditions

Chromatographic separation, based on affinity of the analytes with the nonpolar stationary phase, was achieved with a reversed phase UPLC Acquity BEH C18 column,1.7 µm, 2.1x100 mm (Waters, Milford, USA). Gradient elution was performed with a mobile

phase consisting of 1 mL of a 154 mg/L solution of ammonium acetate in formic acid (99%) in 1 L of Milli-Q ultrapure water (eluent A) and 1 mL of the same solution in 1 L of methanol (eluent B). Before the analysis, the system was equilibrated at the starting conditions of 75% eluent A and 25% eluent B until pressure was stable. The multistep gradient was as follows: from 0 to 0.6 minutes, eluent B was increased from 25% to 48%; from 0.6 to 1.5 minutes, eluent A decreased to 35% and B was increased to 65%; from 1.6 to 2.8 minutes, eluent B was kept stable at 100% and 0% eluent A; from 3.0 to 5.0 minutes, eluent A was inceased to 75% and B was decreased to 25%. The run ended at 5.0 minutes at starting conditions. Temperature for the column oven was set at 50 °C and for the autosampler at 15 °C.

The separation was performed by gradient elution using mobile phase A (1 mL of 2M ammonium acetate in formic acid 99%), in 1 L Milli-Q water and mobile phase B (1 mL of 2M ammonium acetate in formic acid 99%), in 1 L methanol with a total flow rate of 0.4 mL/min. Mobile phase B was kept at 25% from 0.0 min to 0.6 min, then at 48%, from 0.6 to 1.5 min mobile phase B at 65%, then from 1.6 to 2.8 min at 100%, from 3.0 to 5.0 min mobile phase B was kept at 25%. The run ended at 5.0 minutes at starting conditions. Temperature for the column oven was set at 50 °C and for the autosampler at 15 °C.

## MS/MS conditions

MS/MS detection was performed in positive mode by using Selected Reaction Monitoring (SRM) with electrospray ionization. To optimize the MS/MS parameters to detect the most intense signal of each analyte, solutions of 1 mg/L were directly infused in methanol by addition of the mobile phase (75% mobile phase A and 25% mobile phase B) from the LC at a flow rate of 0.4 mL/min. The MS/MS instrument was operated with a capillary spray voltage of 3 kV, vaporizer temperature at 375 °C, capillary temperature at 250 °C, sheath gas pressure at 50 (arbitrary units), auxiliary nitrogen gas pressure at 20 (arbitrary units), collision gas pressure at 1.5 mTorr. Specific parameters for each compound are given in table 3.

## Assay Validation

Validation of the method was performed according to the US Food and Drug Administration (2001) guidelines for bio analytical methods[7]. The following validation parameters were investigated; linearity, LLOQ and ULOQ, accuracy, repeatability, reproducibility, stability and matrix effect.

### *Linearity*

To investigate the linearity of the method, a blank sample (without internal standard), a zero sample (blank with internal standard) and eight calibration standards in duplicate were prepared and analyzed. Calibration curves were generated by plotting the

**Table 3: MS/MS settings**

| **Analyte** | **Parent ion** (m/z) | **Product ion** (m/z) | **Collision Energy** (V) | **S-Lens** (V) |
|---|---|---|---|---|
| Fentanyl | 337.400 | 188.200 | 22 | 124 |
| Fentanyl-d5 | 342.400 | 188.200 | 22 | 124 |
| Sufentanil | 387.300 | 238.200 | 18 | 124 |
| Cefazolin | 455.050 | 323.00 | 10 | 80 |
| Doxapram | 379.250 | 128.100 | 55 | 135 |
| Keto-doxapram | 393.241 | 214.100 | 26 | 150 |



theoretical standard concentration versus the ratio of the standard peak area to the internal standard area. The determination coefficient (R) should be at least 0.9950. The relative standard deviation (RSD) of the calculated concentrations of the standard concentrations was required to be lower than 15%, except at the LLOQ, where it should not deviate by more than 20%. It was decided to apply weighting 1/x, which means that standards with the lowest concentrations are more important for the calibration line than standards with highest concentrations (Saar, Gerostamoulos, Drummer & Beyer, 2010). The calibration curves were formed using the peak area ratios for the analytes and their corresponding internal standard (response) versus the concentrations applying linear least square regression with a weighing factor of 1/x and excluding of the origin.

***LLOQ and ULOQ***

The LLOQ was measured by analyzing the LLOQ standard six times in a row. Mean and standard deviation of the response ratios of the six samples were measured. The response of the analyte should be at least 5 times the response compared with the response of the blank. Precision and accuracy were calculated and should be ≤20% and the accuracy should be between 80-120%, respectively. The highest standard of the calibration curve was used as upper limit of quantification (ULOQ).

***Accuracy***

Accuracy was measured by measuring three concentrations (QC-H, QC-M and QC-L) in six-fold on the same day. The difference in percentage, between the measured concentration and the theoretical concentration, known as the relative standard deviation (RSD) was required to be lower than 15%.

***Repeatability and reproducibility***

The repeatability was tested by analyzing three QC levels in six-fold on the same day. The reproducibility was tested by analyzing three concentrations in duplicate on six different days. The requirement for both parameters was an RSD lower than 15%.

***Stability***

Autosampler stability was determined by storing QC samples (n=2 per concentration) after sample preparation in the autosampler for 24, 48, 72 and 120 hours. Response ratios were measured and compared with response ratios of samples kept at -80°C prior to preparation. After sample preparation, samples were directly analyzed. Recovery was required to be between 90 and 110%.

***Matrix effect***

It is important to measure matrix effects and absolute recoveries in the development of an LC-MS/MS method since ion suppression and ion enhancement effects can be expected owing to interferences by matrix compounds, stable-isotope-labeled internal standards and co-eluting compounds[32]. In order to check whether the precision, the reproducibility and the stability of the concentration-signal ratio are affected by interference of the matrix analytes, the method described by Matuszewski et al. was used[19]. Five different lots of human plasma were used. To two concentration levels (QC Low and QC High) (both in duplicate), the analytes were added before and after extraction, which served to calculated the recovery. Also, a set of six academic samples was evaluated with only Milli-Q ultrapure water instead of plasma. Matrix effects were calculated as follows: peak area of analyte spiked after extraction / peak area of analyte prepared in Milli-Q ultrapure water × 100%.

The process efficiency was calculated as the percentage ratio of the area of the analytes spiked before extraction and the ones prepared in Milli-Q ultrapure water. The mean and RSD were calculated of both matrix effects, process efficiency and recovery. In the ideal situation, the mean matrix effects, process efficiency and recovery are between 80% and 120%, and the RSD of both parameters is ≤15%. Furthermore, for each analyte, the internal standard normalized matrix effect should also be calculated by dividing the matrix effect of the analyte by the matrix effect of the IS. The RSD of the internal standard-normalized matrix effect calculated from the different lots of matrix should not be greater than 15%.

## Clinical application

The method was developed for the analysis of plasma samples from a pharmacokinetic study and may also be used for TDM, if this can be proven clinically valuable. For the validation of the assay for clinical practice, clinical application, and research purposes the method has been applied to quantify doxapram, keto-doxapram and fentanyl in plasma of preterm born infants participating in a clinical study. The Erasmus Medical Center ethics review board approved the protocol and written informed consent from parents/legal guardians was obtained prior to study initiation (MEC-2014-067, ClinicalTrials.gov by NCT02421068). This observational prospective multicenter study was performed

between September 2014 and June 2017 at the Departments of Neonatology of the Radboud University Medical Centre in Nijmegen, Maastricht University Medical Centre in Maastricht, Maxima Medical Centre in Veldhoven and Sophia Children's Hospital in Rotterdam. Neonates routinely received doxapram (Dopram®, Manage, Belgium) for treatment of apnea of prematurity starting with a loading dose of 2.5 mg/kg body-weight in 15 minutes, followed by a maintenance starting dose of 2.0 mg/kg/h, either by continuous intravenous infusion or continuous gastro-enteral administration. Fentanyl (Bipharma, Almere, the Netherlands) was indicated for comfort as an intravenous continuous infusion of 0.5–2.0 µg/kg/h or as a bolus injection of 0.5–3.0 µg/kg.



## RESULTS

### Linearity

Linearity was achieved for each analyte in the range between the LLOQ and the ULOQ (Table 2), with all RSD's to be lower than 15% and the determination coefficient (r) to be at least 0.995. The calibration curves showed that a regression with a weighting factor of 1/x best described the data set over the range for all analytes. Figure 1 shows the ion chromatograms obtained after the analysis of the lowest plasma calibrator standard for all the analytes, and the corresponding retention times of each analyte (see Table 1).

### LLOQ and ULOQ

The results for the LLOQ for cefazolin, keto-doxapram and fentanyl did not meet the initial requirements. Therefore, the LLOQ for these analytes was set to calibration standard 1, which was acceptable. The results of determination of LLOQ and ULOQ are shown in Table 4.

### Accuracy, repeatability and reproducibility

The RSD of accuracy, repeatability and reproducibility data were within the requirement of an RSD lower than 15% (Table 4).

### Stability

Except for cefazolin and keto-doxapram, the recovery of all QC's was between 90% and 110%, indicating that they were stable for at least 120 hours when stored in the autosampler at 15°C. Cefazolin was only stable for 72 hours and keto-doxapram only for 48 hours.

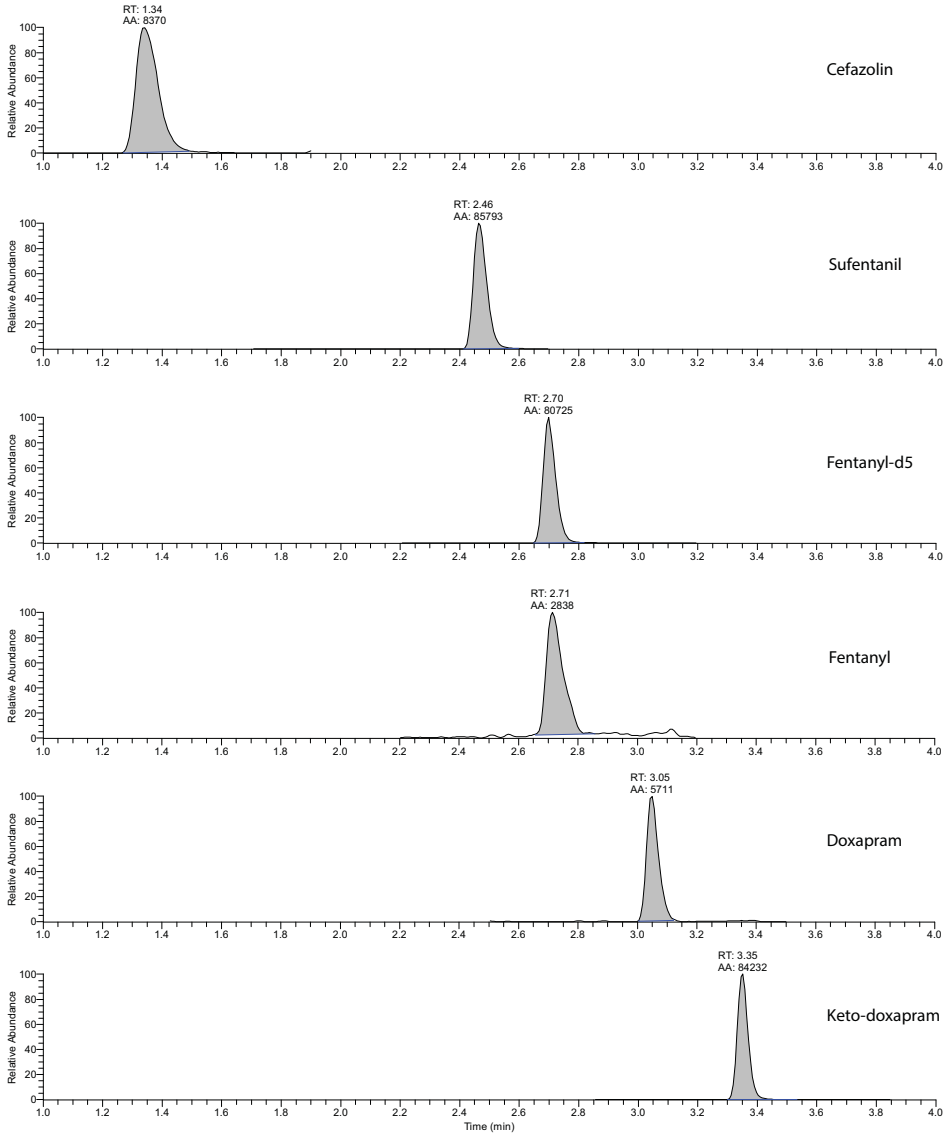


**Figure 1. Ion chromatograms of all analytes and internal standard in lowest concentration calibration standard 1 (see Table 1)**

For the ion chromatograms of sufentanil and fentanyl 10 uL was injected, and for cefazolin, doxapram and keto-doxapram the injection volume was 1 uL.

Abbreviations: RT: Retention time-AA: Automatic integrated area

**Table 4. Validation results (n=6)**

| Analyte | QC | Accuracy RSD (%) | Repeatability *(within-run precision)* | | | Reproducibility *(between-run precision)* | | | LLOQ | ULOQ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean (µg/L) | SD (µg/L) | RSD (%) | Mean (µg/L) | SD (µg/L) | RSD (%) | (µg/L) | (µg/L) |
| Fentanyl | L | -2.7 | 0.46 | 0.01 | 1.5 | 0.51 | 0.02 | 3.9 | 0.10 | 10.0 |
| | M | -2.7 | 2.1 | 0.05 | 2.4 | 2.00 | 0.03 | 1.5 | | |
| | H | -3.4 | 6.8 | 0.09 | 1.3 | 6.82 | 0.15 | 2.2 | | |
| Sufentanil | L | 3.0 | 5.7 | 0.06 | 1.0 | 5.60 | 0.14 | 2.5 | 0.25 | 50.0 |
| | M | -0.2 | 26.7 | 0.44 | 1.7 | 25.5 | 0.56 | 2.2 | | |
| | H | -1.8 | 53.1 | 1.03 | 1.9 | 52.5 | 1.47 | 2.8 | | |
| Cefazolin | L | 8.0 | 6.6 | 0.12 | 1.7 | 6.57 | 0.23 | 3.5 | 1,000 | 100,000 |
| | M | 0.4 | 32.1 | 0.39 | 1.2 | 30.9 | 0.34 | 1.1 | | |
| | H | 0.9 | 101.6 | 1.48 | 1.5 | 104.2 | 1.25 | 1.2 | | |
| Doxapram | L | 3.2 | 0.42 | 0.01 | 1.4 | 0.43 | 0.02 | 4.7 | 50 | 4,500 |
| | M | 3.0 | 2.15 | 0.04 | 1.9 | 2.22 | 0.02 | 0.9 | | |
| | H | -1.2 | 3.46 | 0.06 | 1.8 | 3.57 | 0.05 | 1.4 | | |
| Keto-doxapram | L | -4.8 | 0.16 | 0.00 | 1.5 | 0.18 | 0.01 | 5.7 | 50 | 5,000 |
| | M | 3.2 | 0.88 | 0.02 | 2.1 | 0.77 | 0.02 | 2.6 | | |
| | H | 1.7 | 3.43 | 0.08 | 2.4 | 3.43 | 0.12 | 3.5 | | |

Abbreviations: QC: Quality control-L: Low-M: Medium-H: High – SD: Standard deviation – RSD: Relative standard deviation-LLOQ: Lower limit of quantification-ULOQ: Upper limit of quantification



## Matrix effect

Matrix effects and absolute recoveries in the development of the LC-MS/MS method are shown in Table 5. The method described by Matuszewski et al. showed that fentanyl, sufentanil, cefazolin, doxapram and keto-doxapram experienced neither matrix effect, nor an effect from the sample preparation. A good recovery was achieved for all analytes.

## Clinical application

A total of 618 samples were collected from a pediatric cohort of preterm born infants (n = 157), consisting of 92 infants who received fentanyl and 65 infants with doxapram. Eleven samples were collected from six patients from the cohort of 157 infants who received fentanyl and doxapram simultaneously. The median gestational age of the fentanyl cohort was 27.1 weeks (range 24.3-31.2 weeks), median postnatal age at start of drug therapy 4.5 days (range 0-68 days), median body weight at start of drug therapy 968 g (range 465-3,000 g). The median gestational age of the doxapram cohort was 26.1 weeks (range 24.0-29.4 weeks), median postnatal age at start of drug therapy 17 days (range 1-52 days), median body weight at start of drug therapy 960 g (range 650-1,520 g).

Fentanyl was quantified in 370 samples from 92 patients, and doxapram and keto-doxapram in 248 samples from 65 patients. For fentanyl 78 (21%) of the 370 samples was measured below LLOQ, and 19 (5%) below LOD. For doxapram 29 (12%) and for keto-doxapram 33 (13%) of the 248 samples were below LLOQ, and 8 (3%) doxapram and 6 (2%) keto-doxapram measurements were below LOD. For doxapram, three (1.2%) samples were measured above the ULOQ.

**Table 5. Matrix effect, recovery and process efficiency**

| Analyte | Matrix effect mean (%) | Recovery mean (%) | Process Efficiency mean (%) |
|---|---|---|---|
| Fentanyl | 113.3 | 102.2 | 115.9 |
| Sufentanil | 108.8 | 93.5 | 101.7 |
| Cefazolin | 108.0 | 90.1 | 97.4 |
| Doxapram | 111.3 | 92.5 | 102.9 |
| Keto-doxapram | 99.9 | 99.2 | 99.1 |

## DISCUSSION

We developed a robust UPLC-MS method for simultaneous quantification of fentanyl, sufentanil, cefazolin, doxapram and its active metabolite keto-doxapram according to US Food and Drug Administration guidelines. The easy sample preparation, small required sample volume of 50 μL human plasma and short runtime of 5.0 minutes perfectly met the objectives. Herewith, we are able to analyze one plasma sample to simultaneously quantify multiple drugs that are part of one treatment, and combine samples with different drugs to be measured in one assay run.

Previously reported methods for quantification of these analytes concerned either one of these analytes (with or without their metabolites) per assay, or in a combination with other drugs. These combinations mostly concerned multiple drugs from the same drug class; i.e. sufentanil or fentanyl with other analgosedatives by Nosseir et al.[12] and by Fernandez et al.[8], cefazolin with beta-lactams by Carlier et al. (Carlier et al., 2012) and Kirziazopoulos et al.[14]. Our assay concerns four drugs from three different Anatomical Therapeutic Chemical (ATC) classes; fentanyl and sufentanil as nervous system drugs, cefazolin as an anti-infective drug, and doxapram as a respiratory drug. Herewith, our assay enables to quantify four drugs in one sample simultaneously following one sample injection. This may be valuable for TDM as well as for research, concerning patients using a combination of these drugs as part of a certain treatment protocol. The burden to the patient may be reduced compared to a separate assay per drug, which is especially important concerning vulnerable (preterm) infants. Furthermore, samples with different

drugs may be combined in one single run, which may improve efficiency of the laboratory process.

In general, for all analytes, our assay performed better than or comparable to prior reported assays, even in comparison with assays measuring only a single analyte, which makes it easier to achieve good performance on runtime, required sample volume, matrix effects[1, 3, 5, 8, 13, 14, 15, 16, 17, 22, 24 25, 29, 30]. Furthermore, most assays use a different drug as an internal standard, where we used a deuterated form of fentanyl, which shows better comparable behavior to the analytes that are measured than using a different drug. Next, our sample preparation consisted of a simple one-step protein precipitation method, where in most studies a solid phase extraction is prescribed, or a liquid-liquid extraction with an evaporation and/or ultrafiltration step, or other additional steps.

For sufentanil, other reported assays required more plasma volume and a more complex sample preparation compared to our assay[22, 24, 29]. Concerning doxapram, four of the five reported assays date from the 90's [1, 15, 21, 27] and are inferior to our assay with respect to the use a different drug for internal standard, sample preparation which requires an evaporation step, higher LLOQ, larger sample volume required, a longer runtime, and two assays could not measure keto-doxapram. The recently published assay by Suzuki et al.[30] required only 25 µL plasma volume compared to our 50 µL, and an LLOQ for doxapram of 20 µg/L compared to our 50 µg/L On the other hand, Suzuki et al. needed a 12 minutes runtime and used propranolol an internal standard, where we needed 5 minutes runtime and used deuterated fentanyl, and our sample preparation required less operational steps. Regarding cefazolin, multiple assays have been reported with comparable performance[3, 14, 16, 25]. The reported fentanyl assays require larger sample volumes except for Hisada et al., who need only 20 µL and reached a lower LLOQ of 0.05 µg/L compared to our 0.1 µg/L[13].

Except for cefazolin and keto-doxapram, the stability of all analytes was good, which means they were stable for at least 120 hours when stored in the autosampler at 15°C. Cefazolin was only stable for 72 hours and keto-doxapram only for 48 hours at 15°C. Suzuki et al. tested the stability of keto-doxapram for 48 hours at 10°C for autosampler conditions and also found them to be stable for that time[30]. Stability during 3 cycles of freezing and thawing, together with freezer stability studies of the analytes in the matrix are not presented because they were already carried out in previous published papers and do not depend on the analytical method. No relevant effects of freezing and thawing were found for all analytes, together with good stability at -20°C for 4 weeks[5, 14, 17, 22, 24, 25, 29, 30].

Our assay fulfilled the desired criteria for accuracy, repeatability, and reproducibility. Furthermore, for all analytes a good recovery was achieved and matrix effects were measured. These indicated the absence of interferences by matrix compounds, stable

isotope-labeled internal standard, and co-eluting compounds, that may cause ion suppression and ion enhancement.

The ranges for linearity for all analytes were perfectly suitable for clinical pharmacology research, as well as for possible TDM purposes. The assay was successfully validated for clinical practice and research purposes of fentanyl and doxapram. Fentanyl was quantified in 370 plasma samples from 92 preterm infants, and doxapram and keto-doxapram in 248 plasma samples from 65 preterms. The considerably high proportion of samples below LLOQ (21% for fentanyl, 12% for doxapram, 13% for keto-doxapram) was due to the objective of the study on investigating drug pharmacokinetics. Namely, to estimate clearance of the investigated drugs opportunistic sample collection was allowed up to and beyond the time at which the plasma concentrations decreased below the LLOQ. All samples collected during continuous administration of both drugs were all above the LLOQ for all three analytes, and only above ULOQ for three doxapram samples shortly after a bolus administration. In conclusion, the assay performed well for samples in clinical practice. Furthermore, investigation is currently in progress in which this method has been applied to several pharmacokinetic studies in preterm born infants up to elderly patients.

Despite the good performance, our assay has certain limitations. First, the stability of cefazolin and keto-doxapram did not reach the desired 120 hours at 15°C in the autosampler. Although as performance of the assay is finished within 48 hours, this did not create a problem in practice. Second, although the small plasma volume of 50 µL for performing the assay, this may be too much for some preterm infants, and for quantification of multiple drugs requiring the use of different assays. Third, the LLOQ of certain analytes in our assay was higher than some reported assays quantifying a single analyte. This is due to our goal to quantify multiple analytes in one run simultaneously, which makes it more difficult to achieve maximal performance for all analytes. Nevertheless, the LLOQs of our assay all meet the clinically required limits of quantification. Fourth, the assay did not include inactive metabolites as these are not relevant for clinical practice. Therefore, only keto-doxapram was included being the active metabolite of doxapram.

It has been suggested to implement therapeutic drug monitoring as a supportive tool for analgosedation for fentanyl and sufentanil which may help physicians increase patient comfort regarding intra- and inter-operative interventions[22]. Also for Beta-lactam antibiotics the contributive value of TDM has been suggested[11]. Quantification of doxapram and keto-doxapram during therapeutic dosages of doxapram may be relevant to improve successful therapy even further in treatment of apnea of prematurity[12], and for evaluation of safety[2].

## CONCLUSIONS

We have developed a method for the simultaneous quantification of fentanyl, sufentanil, cefazolin, doxapram and keto-doxapram in 50 µL human plasma within a runtime of only 5.0 minutes. This greatly facilitates further research into these drugs as well as possible TDM purposes, even in the smallest plasma volumes obtained from preterm infants.

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# PHARMACOKINETICS IN PRETERM INFANTS

# 5

# Model-based clinical dose optimization for phenobarbital in neonates: an illustration of the importance of data sharing and external validation

*European Journal of Pharmaceutical Sciences. 2017 Nov 15;109S:S90-S97*

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## ABSTRACT

### Background

Particularly in the pediatric clinical pharmacology field, data-sharing offers the possibility of making the most of all available data. In this study, we utilize previously collected therapeutic drug monitoring (TDM) data of term and preterm newborns to develop a population pharmacokinetic model for phenobarbital. We externally validate the model using prospective phenobarbital data from an ongoing pharmacokinetic study in preterm neonates.

### Methods

TDM data from 53 neonates (gestational age (GA): 37 (range: 24-42) weeks, bodyweight: 2.7 (range: 0.45-4.5) kg; postnatal age (PNA): 4.5 (range: 0-22) days) contained information on dosage histories, concentration and covariate data (including birth weight, actual weight, post-natal age (PNA), postmenstrual age, GA, sex, liver and kidney function, APGAR-score). Model development was carried out using NONMEM® 7.3. After assessment of model fit, the model was validated using data of 17 neonates included in the DINO (Drug dosage Improvement in NeOnates)-study.

### Results

Modelling of 229 plasma concentrations, ranging from 3.2 to 75.2 mg/L, resulted in a one compartment model for phenobarbital. Clearance (CL) and volume ($V_d$) for a child with a birthweight of 2.6 kg at PNA day 4.5 was 0.0091 L/h (9%) and 2.38 L (5%), respectively. Birthweight and PNA were the best predictors for CL maturation, increasing CL by 36.7% per kg birthweight and 5.3% per postnatal day of living, respectively. The best predictor for the increase in $V_d$ was actual bodyweight (0.31 L/kg). External validation showed that the model can adequately predict the pharmacokinetics in a prospective study.

### Conclusion

Data-sharing can help to successfully develop and validate population pharmacokinetic models in neonates. From the results it seems that both PNA and bodyweight are required to guide dosing of phenobarbital in term and preterm neonates.

## INTRODUCTION

Rational dosing guidelines for drugs in neonates are urgently needed. However, datasets from prospective clinical trials in children are scarce and both the number of included children and the number of samples per child are usually very small[1]. To overcome this problem data-sharing is of utmost importance and can help to make the most out of all available data[1-3]. Existing data can be utilized to determine the optimal design of prospective trials but it may also aid dose finding in ongoing trials in case the collected data is not (yet) sufficient to draw valid conclusions[2, 4].



Besides that, the application of advanced data analysis techniques, namely the population pharmacokinetic modelling approach, allows handling sparse and infrequently collected samples[3]. Moreover, it offers the possibility to quantify inter-individual variability and to identify covariates that determine the pharmacokinetics of drugs along the whole paediatric life-span and can thereby be used to optimize drug dosing[5, 6].

Phenobarbital remains the traditional first-line treatment for seizures in neonates although evidence to favour one antiepileptic agent over the other is lacking[7]. Using phenobarbital alone, only around 50 % of seizures can be effectively controlled[8, 9]. As persistence of seizures might cause permanent functional and structural damage to the brain and existing brain damage might be worsened[10, 11], safe and efficacious treatment is of primary importance. The optimal dosage of phenobarbital in term and preterm neonates remains a topic of discussion. The therapeutic effect is dose dependent with a suggested therapeutic range between 15-40 mg/L[12]. At higher concentrations sedation and feeding difficulties might occur[13]. The Dutch National Children's Formulary recommends a loading dose of 20 mg/kg and a maintenance dose of 2.5-5 mg/kg/day[14]. However, pharmacokinetic data is sparse in term[13, 15-18] and preterm[17-19] newborns.

In this analysis, we utilize therapeutic drug monitoring (TDM) data collected between 1997-2003 in the neonatal intensive care unit of the Maastricht University Medical Centre (study 1) to build a population pharmacokinetic model for phenobarbital in term and preterm newborns. We validate the model with data originating from an ongoing PK study (DINO-study: Drug dosage Improvement in preterm NeOnates, NL47409.078.14) (study 2). This study collects pharmacokinetic data of phenobarbital and eight other frequently used off-label drugs in preterm neonates to increase the knowledge on the pharmacokinetics and pharmacodynamics using sparse sampling and limited sample volumes to minimize the burden on the individual child. Using this approach, we illustrate that data sharing and external validation can lead to model-based clinical dose optimization, particularly in neonates where ethical and practical constraints limit the possibilities to perform studies.

## METHODS

### Model development dataset (TDM data, study 1)

TDM data were obtained from the database of the Maastricht University Medical Centre between 1997 and 2003 with approval from the medical ethical committee (MEC 02-204.3). Neonates younger than 35 days at the start of phenobarbital treatment were included. A total of 229 samples from 53 neonates (28 male, 25 female) were available for analysis. First dose and consecutive doses (intravenous as well as oral) varied between 4-40.7 mg/kg and 1.3-20mg/kg, respectively, and the study period ranged from 4 to 85 days (Table 1). The median first dose was 20 mg/kg, 17 children received a first dose that was higher than 25 mg/kg. The median maintenance dose was 3.9 mg/kg. Covariates were retrieved from the patient's records.

**Table 1: Patient characteristics (median (range)) of the TDM dataset (study 1) and the prospective validation dataset from the DINO study (study 2)**

| | TDM data (study 1) | Prospective data (study 2) |
|---|---|---|
| Gestational age [weeks] | 37 (24–42) | 25 (24-31) |
| Birthweight [kg] | 2.6 (0.45–4.4) | 0.94 (0.58–2.2) |
| Postnatal age (at the day of inclusion) [days] | 4.5 (0-22) | 15 (1–76) |
| Duration on study* [days] | 12 (4-85) | 5 (1–20) |
| Actual bodyweight [kg] | 2.7 (0.45-4.5) | 1.07 (0.63–4.7) |
| APGAR-Score at 5 min | 3 (0-9) | 8 (1-9) |
| First Dose [mg/kg] | 20 (4–40.7) | 10.8 (2–22) |
| Maintenance Dose [mg/kg] | 3.9 (1.3–20) | 10.8 (1.2-20) |
| Samples after intravenous dosing [n] | 226 | 56 |
| Samples after oral dosing [n] | 16 | 6 |

defined as the time between the first and the last blood sample contributing to the analysis

### External Model Validation Dataset (prospective data, study 2)

The DINO-study (NL47409.078.14, MEC-2014-067) prospectively studies a total of nine drugs including phenobarbital used as standard of care in preterm infants born before 32 weeks of gestation aiming at evidence-based individualized dosing regimen and is still ongoing. From September 2014 to September 2016, 61 blood samples from 17 children (7 female, 10 male) containing phenobarbital were evaluable for the analysis (Table 1).

### Bioanalytical Analysis

Phenobarbital concentrations of the model development dataset (study 1) were determined using a fluorescence polarization assay[20] on the COBAS INTEGRA 700 (Roche

Diagnostics; Basel, Switzerland) using COBAS INTEGRA reagent system cassettes at the pharmacy of Maastricht University Medical Centre. Fluorescein-labelled phenobarbital binds an antibody and the emitted light is polarized due to the reduction in freedom of rotation. In case phenobarbital is present in the patients serum it reduces the extent of fluorescence polarization due to antibody binding[20]. The test range of the assay was 0.6-60 mg/L.

Phenobarbital concentrations of the validation dataset (study 2) were determined using a particle-enhanced turbidimetric inhibition immunoassay (Petinia) on the Architect C4000 (Abbott Diagnostics; Hoofddorp, The Netherlands) using Architect C4000 reagent system cassettes at the pharmacy of the ErasmusMC, Rotterdam. The assay is based on competition between drug in the sample and drug coated onto a microparticle for antibody binding sites of the phenobarbital antibody reagent. The test range is 2.0-80 mg/L.



## Population Pharmacokinetic Analysis

The analysis was performed using NONMEM version 7.3 (ICON Development Solutions, Ellicott City, MD, USA), supported by Perl-speaks-NONMEM (PsN) version 3.4.2 and Xpose version 4.3.5[21]. Model development started using the intravenous data. After covariate inclusion and assessment of the model fit, oral data was added to the model. The absorption rate constant ($k_a$) was fixed to a value of 50 h obtained from literature[22]. The first-order conditional estimation with interaction method was used throughout model development. In case of missing covariate information, the last value observed in the subject was carried forward. For actual bodyweight, linear interpolation between available measurements was performed. The objective function value was used to discriminate between nested models. Standard errors obtained from NONMEM and the confidence intervals of the bootstrap analysis in PsN (n=1000) were used to evaluate the precision of the parameter estimates.

For the covariate analysis η-values (ETA) and conditional weighted residuals (CWRES) were used. ETA values are defined as the random effect describing the deviation of the individual empirical Bayes estimate of the parameter from the typical population parameter estimate of the corresponding parameter, e.g. CL or central volume of distribution (V), for a given subject. Using the base model, plots of eta values on CL and V versus covariates were used to investigate potential covariates. In a next step potential covariates were evaluated using a stepwise covariate modelling (SCM) procedure[23]. A significance level of $p \leq 0.05$ was used for the forward inclusion and a significance level of $\leq 0.01$ for the backward elimination.

### Model Evaluation

Key models as well as the final model were evaluated using goodness-of-fit plots and normalized prediction distribution errors (npde)[24] based on 1000 simulations of the model and a bootstrap analysis based on 1000 samples of the data.

### External Model Validation

Phenobarbital plasma concentrations from study 2 were predicted by fixing the parameters to the parameter estimates in the final model using post hoc Bayesian forecasting. To compare predicted and observed values, goodness-of fit was assessed and bias (mean prediction error [MPE]) was calculated using equation 1:

$$MPE = \frac{\sum^{\frac{predicted - observed}{observed}}}{n} * 100 \quad \textbf{(1)}$$

where n denotes number of observations. Predicted parameter values were population predicted values for each individual.

### Evaluation and optimization of Dosing Regimen

Based on the validated model[25], the current dosing regimen was critically reviewed for its ability to reach a target concentration between 15-40 mg/L in all children. To this end, 1000 simulations of a child with a birthweight of 0.6, 1, 2 and 3 kg were performed upon an intravenous loading dose of 20 mg/kg and an intravenous maintenance dose of 5 mg/kg daily over 60 days. The weight of the children born weighing 0.6, 1 and 2 kg was simulated to develop according to the preterm growth curves published by Anchieta et al.[26] and the weight of child born weighing 3 kg was assumed to develop as described by Thulier et al.[27].

In a next step the dosing regimen was modified in order to improve target attainment in the simulated individuals.

## RESULTS

### Population pharmacokinetic model

Modeling of plasma concentrations resulted in a one compartment model with intra-individual variability (IIV) on CL and V. A proportional error yielded the best description of the residual variability. Using this model without covariates, the plasma concentrations of the smallest children were underpredicted and concentrations of the heaviest children were overpredicted.

In the covariate analysis, birthweight, actual bodyweight, GA and height showed high correlation coefficients with CL, while only weak or no correlations could be seen for Apgar scores, serum creatinine, umbilical artery pH and PNA. V was highly correlated

with birthweight, actual bodyweight, GA and height. Other covariates showed weak correlations. The stepwise covariate modelling proposed birthweight or GA and PNA as predictors for CL and actual bodyweight as predictor of V. No other covariates were found. Model fit was comparable for the combination of PNA and GA or PNA and birthweight on CL. In line with the literature[28] the model containing birthweight and PNA on CL (Figure 1) was carried forward as dosing based on birthweight might lead to a more comprehensible dosing guideline. The ETAs of the final model showed no correlation to parameters relevant for maturation and development indicating that covariates have been correctly implemented (Supplement 1). Covariate inclusion reduced IIV on CL and V from 56 % to 30 % and from 59 % to 24%, respectively.



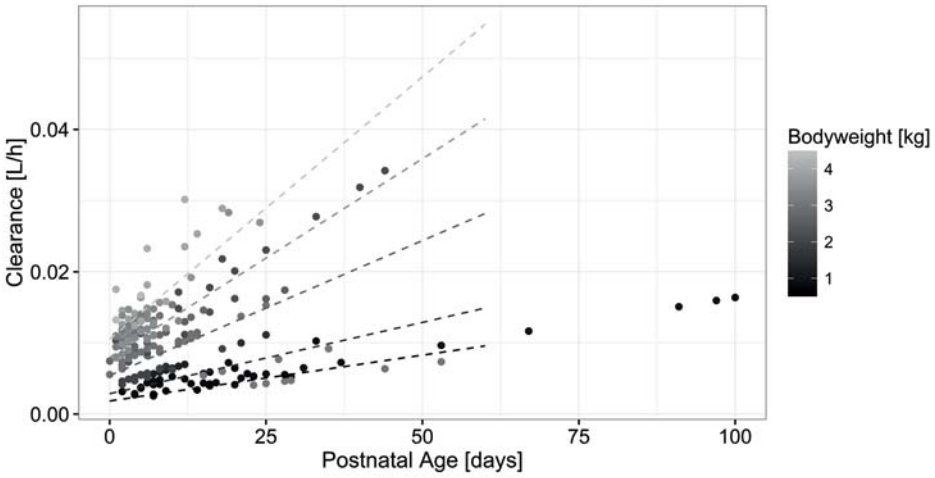



**Figure 1: Clearance [L/h] versus postnatal age [days], depicted as population values (dashed lines) for children with a birthweight of 0.6 kg, 1 kg, 2 kg, 3 kg and 4 kg and individual posthoc CL values (dots)**

The final parameter estimates as well as their respective bootstrap estimate and confidence interval (bootstrap convergence rate = 97.3 %) are displayed in table 2. Bootstrap estimates are in good agreement with the final NONMEM parameter estimates and show narrow confidence intervals for all structural parameters.

## Model evaluation (study 1)

The model was able to sufficiently describe the data of study 1 that were used to build the model (Figure 2, left panels). The npde-analysis showed no trends towards a model misspecification (Figure 3).

**Table 2: Parameter estimates of the final model and their corresponding bootstrap estimates**

| Parameter | Final Model: Estimate (RSE %) | Bootstrap-Estimate (95% CI) |
|---|---|---|
| **Fixed effects** | | |
| CL [L/h]=$CL_p$ | 0.0091 (9 %) | 0.0092 (0.0072–0.011) |
| $CL_i=CL_p*(1+\Theta_{PNA}*(PNA_i\text{-Median}))*(1+\Theta_{bBW}*(bBW_i\text{-Median}))$ | | |
| $\Theta_{PNA}$ [increase in CL per postnatal day (PNA) (fraction)] | 0.0533 (27 %) | 0.0523 (0.0201–0.0909) |
| $\Theta_{bBW}$ [increase in CL per kg birthweight (bBW) (fraction)] | 0.369 (8 %) | 0.367 (0.319–0.414) |
| V [L]=$V_p$ | 2.38 (5 %) | 2.37 (2.16–2.63) |
| $V_i=V_p*(1+\Theta_{aBW}*(aBW_i\text{-Median}))$ | | |
| $\Theta_{aBW}$ [increase in V per kg actual bodyweight (aBW) (fraction)] | 0.309 (8 %) | 0.309 (0.259–0.351) |
| $k_a$ [h] | 50 FIX | - |
| F [%] | 59.4 (10 %) | 58.4 (30.5–80.0) |
| **Inter-individual variability** (eta) | | |
| On CL [%] | 30.0 (29 %) | 28.7 (18.9–38.2) |
| On V [%] | 22.4 (40 %) | 22.0 (12.7–30.1) |
| **Residual variability** | | |
| Proportional [%] | 2.58 (22 %) | 2.55 (1.49–3.76) |

CL=clearance, V=central volume of distribution, aBW=actual bodyweight, bBW= birthweight, $k_a$= absorption rate constant, F= bioavailability, median PNA= 4.5 days; median bBW= 2.6 kg, median aBW= 2.7 kg, p=population mean value of a parameter for an individual with PNA of 4.5 days and bBW of 2.6 kg

## External Model Validation (study 2)

Model validation with the external prospective dataset (study 2) shows that the model can adequately predict the data of the included infants without bias (Figure 2, right panels). Only the peak concentrations of one individual were underpredicted by the model (Figure 2e, diamonds). The mean prediction error was -8.4 % corresponding to an adequate description of the data.

## Evaluation and optimization of Dosing Regimen

According to the final model, the current dosing regimen consisting of an intravenous loading dose of 20 mg/kg and an intravenous maintenance dose of 5 mg/kg/day resulted in distinct results in children with different birthweights (Figure 4a). The figure shows that the mean maximal plasma concentration ($C_{max}$) after loading dose is lower in smaller children. This is caused by a higher V [L/kg] in lighter and thereby less mature children, leading to a lower $C_{max}$ following an equal dose per kg bodyweight. After the loading dose the mean plasma concentration of phenobarbital keeps increasing during the first 10 days of life. Thereafter a quasi-steady state concentration is reached for about 5 days after which concentrations decrease over time due to the model predicted maturation of CL with PNA (Figure 1). At the same time the percentage of simulations below the

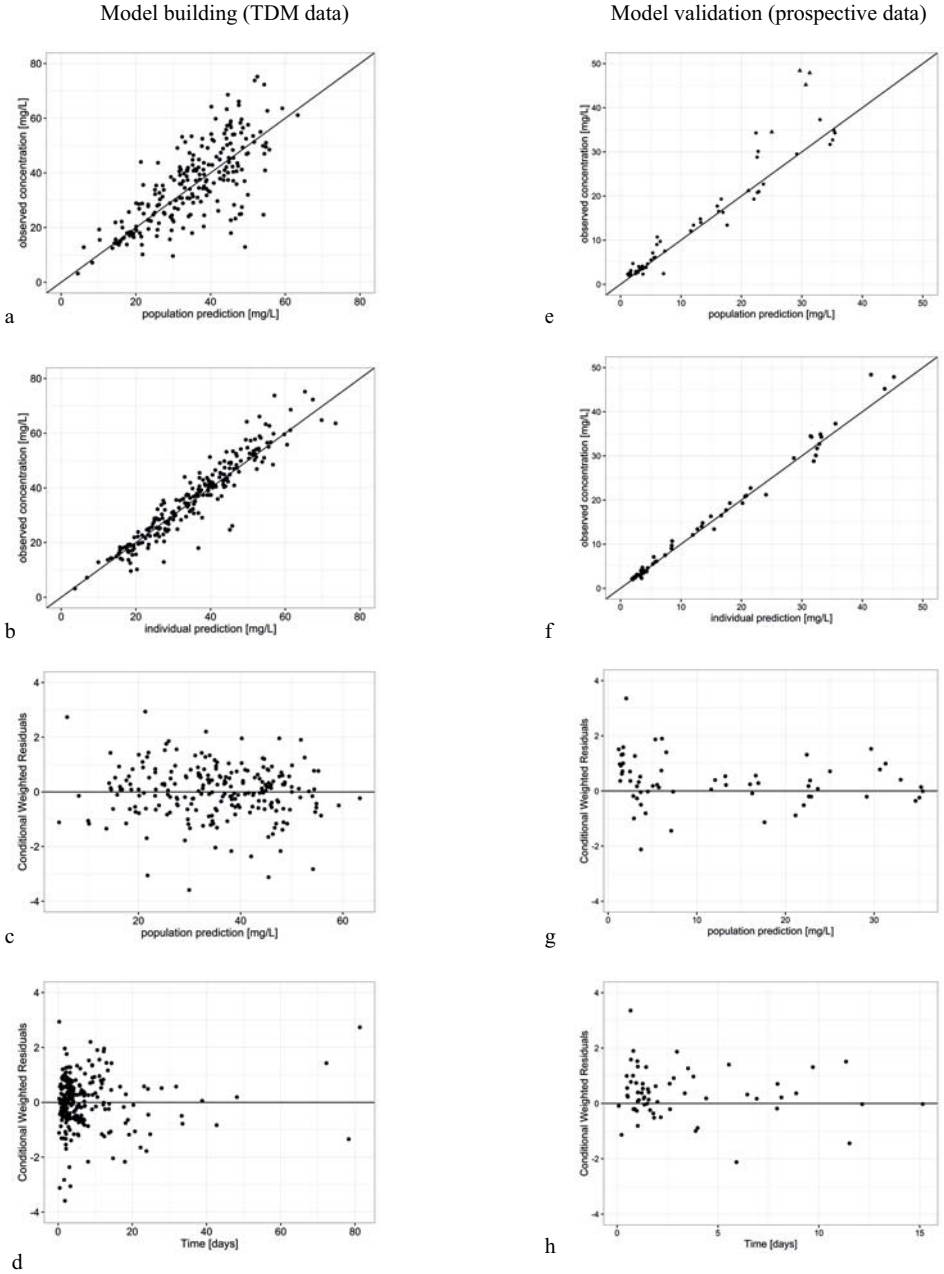



**Figure 2: Goodness-of-fit plots of the model building (TDM data, study 1) (left panels) and the model validation (prospective data, study 2) (right panels, diamonds in e= outlying subject); a/e: population predictions versus observed concentrations; b/f: individual predictions versus observed concentrations, c/g: conditional weighted residuals versus population prediction, d/h: conditional weighted residuals versus time after first dose**

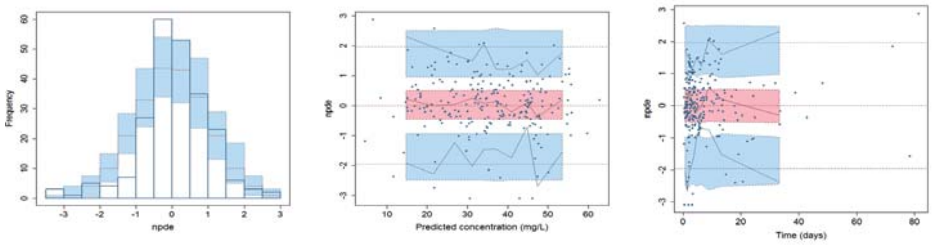



**Figure 3: Numerical prediction distribution errors (npde) of the final model: mean= 0.06501 (standard error= 0.063) and variance= 0.9018 (standard error= 0.084))**

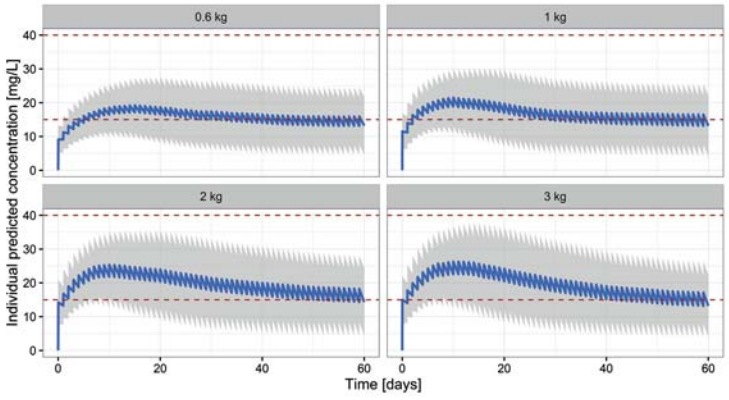
threshold of 15 mg/L increases. Lower initial $C_{max}$ values in the smallest newborn result in the highest proportion of subtherapeutic levels at the end of the observed period. The current dosing regimen did not result in any concentrations above the 40 mg/L within the 95% confidence interval of 1000 simulations. The highest concentrations were observed at a PNA of 10-15 days in the simulations of a biggest child (birthweight=3kg). The lowest concentrations were observed in the smallest child (birthweight=0.6 kg) after 6-8 weeks.

When increasing the loading dose from 20 mg/kg to 30 mg/kg (Figure 4b), the therapeutic window is immediately reached for all birthweights. If the maintenance dose is increased from 5 mg/kg/day to 6 mg/kg/d at a PNA of 15 days, the simulated median plasma concentration stays well in the desired target range (Figure 4c).

## DISCUSSION

In this study, we successfully developed and validated a population PK model for phenobarbital in neonates on the basis of existing TDM data with data from a prospective study. This is an important result as typically studies in (preterm) neonates are complicated due to ethical and practical constraints and therefore dosing regimens often off-label. The results of this study show that CL increases with both birthweight and PNA (Figure 1) while V is determined by actual bodyweight (Table 2).

Besides providing new insights into the PK of phenobarbital in term and preterm newborns this analysis highlights the advantages of data-sharing. Only due to the utilization of TDM data from a previous study (study 1) it was possible to develop a population pharmacokinetic model for term and preterm newborns. The data from the DINO-study (study 2) would have been insufficient to build such a model as it only contained data in infants born before 32 weeks of gestation. Covering the whole age range helps at identifying maturational processes and eventually aids dose finding. Furthermore, data sharing provided the possibility of external validation with an independent dataset,

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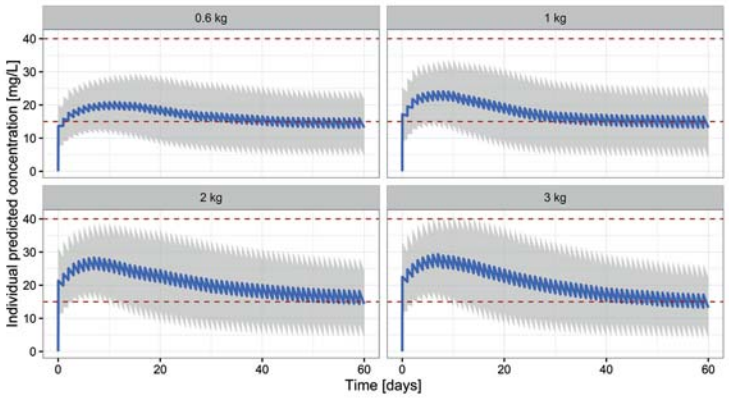


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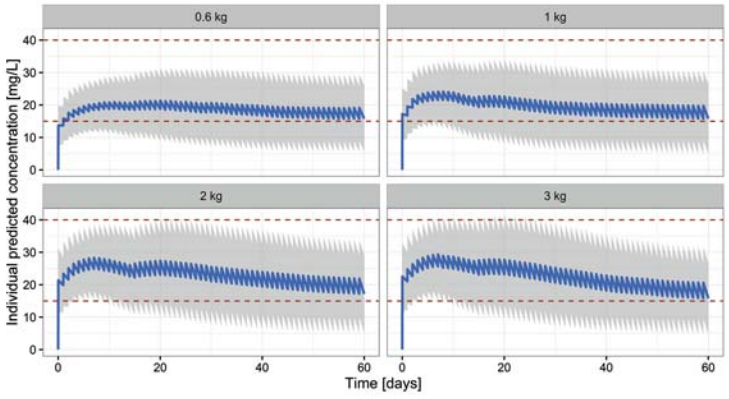


c

**Figure 4: Phenobarbital concentration time profiles (n=1000) with blue line representing median and grey shaded areas 95% confidence intervals for children with a birthweight of 0.6 kg, 1 kg, 2 kg and 3 kg (dashed red lines= therapeutic window) according to different dosing regimens. a: current dosing regimen (i.v. loading dose: 20 mg/kg; i.v. maintenance dose: 5 mg/kg/day); b: increased i.v. loading dose (30mg/mg), maintenance dose: 5mg/kg/day; c: increased i.v. loading dose (30 mg/kg) and increased i.v. maintenance dose (6 mg/kg/day vs. 5 mg/kg/day) starting at a PNA of 15 days**

however, only in the very preterm age range. Due to the excellent fit of the validations data (Figure 2) we do believe that our model is applicable for the whole age range.

So far, only few population PK models of phenobarbital in neonates have been published. Yukawa et al.[18] studied 70 neonates and infants (GA: 24.1-43 weeks, PNA:1-73 days) and found that PNA and total bodyweight were the dominant predictors of phenobarbital PK. Furthermore, the authors observed and accounted for non-linearity in the PK of phenobarbital at concentrations above 50 mg/L[18]. Data in dogs also indicate that auto-induction might be present at very high doses resulting in concentrations outside the therapeutic window[29]. As a possible mechanism leading to non-linearity, auto-induction of phenobarbital has been discussed in literature, even though it is anticipated to play a minor role in adults[30]. Our analysis does not indicate non-linearity, maybe due to the low number of concentrations above 50 mg/L and as such not be relevant when non-toxic doses are given.

Marsot et al.[17] modelled phenobarbital data of 48 newborns born at 27-42 weeks of gestation. Their covariate model is based on allometric scaling with an exponent of three quarters on CL and a linear exponent on V. Additional maturation of CL with PNA was not observed. Based on simulations of the first 12 days of life the authors recommend an intravenous loading dose of 20 mg/kg in all neonates and a weight-based maintenance dose. According to their advice, the recommended maintenance dose decreases with increasing bodyweight[17]. Our model as well as the model of Yukawa et al.[18] indicate that CL increases with PNA which would necessitate an increased maintenance dose over the treatment course (Figure 4). Based on our simulation period of 60 days we propose that the maintenance dose could be increased by 1 mg/kg at a PNA of 15 days to account for this change.

Although information on the maturation of phenobarbital metabolism in neonates is sparse, Boreus et al.[31] showed that the excretion of conjugated phenobarbital metabolite is significantly reduced in newborns when compared to adults. In addition, phenobarbital is metabolized by Cytochrome P450, mainly through CYP2C9, with minor metabolism by CYP2C19 and CYP2E1[32]. Peak concentrations of one individual were slightly under-predicted by the model. Literature suggests that inflammation as represented by high CRP values might lead to a reduced function of Cytochrome P450 (CYP) enzymes[33]. The C-reactive protein (CRP) in this child was elevated up to 100 mg/L. Reduced CYP activity might therefore have resulted in elevated (peak) phenobarbital levels.

The CYP enzymes responsible for phenobarbital metabolism may be subject to maturation processes[34] and thereby explain our findings. Ward et al. showed that the ontogeny of CYP2C19 has a profound effect on the weight-normalized CL of orally administered pantoprazole. In their analysis, the CL of pantoprazole, another CYP2C19 substrate, was positively correlated with PNA in 33 neonates aged 1 to 19 days[35].

In line with these findings, Heimann et al. found using a classical two stage approach a phenobarbital half-life of 118.6 ± 16.1 h in term newborns (0-28 days) and a half-life of 62.9 h ± 5.2 h in infants (1-12 months)[16]. Oztekin et al. observe rapidly increasing phenobarbital trough levels during the first four days of life in very low birth weight infants (<1500 g)[19], which is in agreement with our results (Figure 4). We can explain these findings because of their very low CL, upon which steady-state is reached after a long treatment period. Thus, instead of CL, V mainly determines the PK profile of phenobarbital during the first week of life.



In view of these observations, we found high volumes of distribution per kg bodyweight in newborns and thereby a high percentage of initial plasma concentrations below the therapeutic range (Figure 4). Our model thus implies that neonates require higher loading doses than the currently applied 20 mg/kg. *Donn et al.* investigated a higher loading dose of 30 mg/kg in asphyxiated term newborns[15]. The mean observed plasma concentration two hours after loading dose was 30.0 ± 3.2 mg/L and was not associated with an increased number of side effects. Safety data on higher loading doses in preterm newborns is however lacking. Therefore, safety aspects should be evaluated when administering higher loading doses in this vulnerable population.

Phenobarbital is the most frequently used treatment modality in term newborns following asphyxia and whole body hypothermia[36]. In a recent evaluation *Pokorna et al.* showed that the combination of asphyxia and hypothermia might have an effect on clearance of phenobarbital (personal communication). Therefore, it might be desirable to develop models for phenobarbital that can lead to optimized dosing in these conditions.

The results of the presented analysis indicate that the use of the currently applied bodyweight based loading and maintenance dose of phenobarbital in preterm newborns may have its limitations. Recent observations from the population pharmacokinetic field show that bodyweight is often not the only determinant of the PK[3, 37]. Our model as well as the model of Yukawa et al.[18] predicts that maturation of CL is not related to bodyweight alone but also to PNA. Therefore, preterm infants might require adjusted dosing regimen correcting for this maturation.

In conclusion, we strongly encourage data-sharing in the paediatric and neonatal PK setting in order to use the data in an optimal fashion and subsequently to minimize the burden on newborns. We show here that data-sharing can help to successfully develop and validate population pharmacokinetic models in neonates. From the results it seems that for phenobarbital both PNA and bodyweight are required to guide dosing of phenobarbital in term and preterm neonates. Based on the presented analysis the loading dose should be adjusted to 30 mg/kg in order to immediately reach the therapeutic window and maintenance dose should be increased by 1 mg/kg/day due at a PNA of 15 days due to a maturation of CL with PNA.

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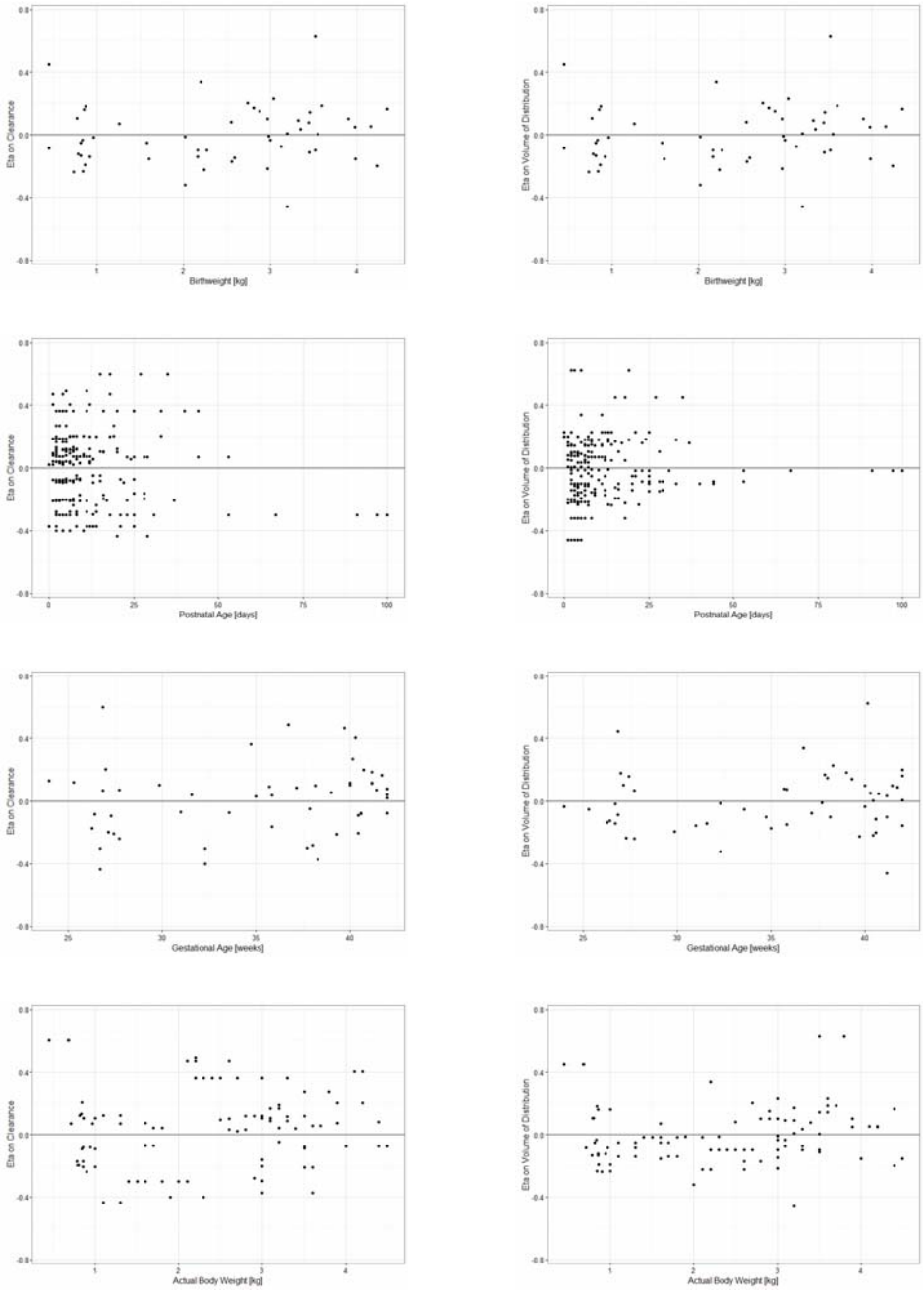


**Supplement 1: Interindividual variability (eta) on clearance (left panels) and on volume of distribution (right panels) versus relevant covariates (birthweight, postnatal age, gestational age and actuel bodyweight) for the final pharmacokinetic model**