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Determination of chlorpyrifos metabolites in human urine by reversed-phase/weak anion exchange liquid chromatography–electrospray ionisation–tandem mass spectrometry

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Abstract

A liquid chromatography–electrospray ionisation–tandem mass spectrometry (LC–ESI–MS/MS) method for the quantification of major chlorpyrifos (CP) metabolites, i.e. diethyl thiophosphate (DETP), diethyl phosphate (DEP), and 3,5,6-trichloro-2-pyridinol (TCP), in human urine was developed. Simultaneous separation of the parent compound and its primary biotransformation products was achieved within 20 min in gradient elution mode employing a mixed-mode reversed-phase/weak anion exchange (RP/WAX) separation principle. The analytical method was developed for a toxicokinetic study of an acute poisoning incidence with a CP containing pesticide formulation. An initial mass spectrometric screening performed with unprocessed urine samples revealed that CP is not excreted unchanged by the kidney. Hence, the quantitative assay was validated for DETP (quantifier transition: *m/z* 169 → 95, qualifier transition: *m/z* 169 → 141), DEP (*m/z* 153 → 79, 153 → 125), and TCP (*m/z* 196 → 35, 198 → 35) taking dibutyl phosphate (DBP) (*m/z* 209 → 79, 209 → 153) as internal standard. Clean-up of urine samples prior to LC–ESI–MS/MS analysis was carried out by a liquid–liquid extraction step with a mixture of ethylacetate and acetonitrile (70:30; v/v). Linearity was observed between 0.25 and 75 mg L^{-1}, and the signal-to-noise ratio at 0.25 mg L^{-1} was better than six for the individual analytes. Recoveries, precision, and accuracies were all adequate across the validated range of 1–75 mg L^{-1} for the present toxicological case study.

Keywords: Chlorpyrifos; Metabolites; Reversed-phase/weak anion exchange stationary phase; Liquid chromatography–tandem mass spectrometry; Human poisoning; Urine

1. Introduction

Chlorpyrifos, *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate (CP, Fig. 1), belongs to the group of organophosphorothioate insecticides. Its moderate acute mammalian toxicity together with a low persistence in environment makes it relatively safe for agricultural and domestic pest control.

Acute exposure towards CP may lead to the development of signs of neurotoxicity. Bioactivating metabolism of CP by cytochrome P450 depending monooxygenases generates a highly electrophilic intermediate, viz. CP-oxon (Fig. 1), which can easily phosphorylate the serine residue at the active site of acetylcholinesterase. In similar manner as other organophosphate type pesticides this causes a biologically virtually irreversible enzyme inhibition. Consequently, an accumulation of the neurotransmitter acetylcholine in central and peripheral nerve tissues occurs, which, in turn, can provoke a cholinergic syndrome [1,2].

Depression of plasma cholinesterase (butyrylcholinesterase) or red blood cell acetylcholinesterase, both reflections

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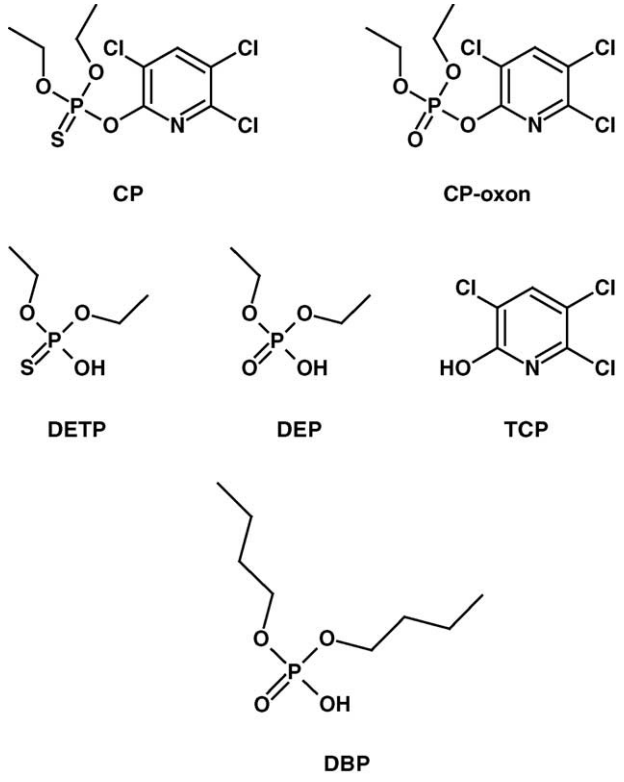

Fig. 1. Chemical structures of chlorpyrifos (CP), chlorpyrifos-oxon (CP-oxon), diethyl thiophosphate (DETP), diethyl phosphate (DEP), 3,5,6-trichloro-2-pyridinol (TCP), and the internal standard dibutyl phosphate (DBP).

of the primary toxic mechanism, can be assayed to estimate organophosphate burden. However, these methods are insensitive and in general not compound specific. They are viable means for the clinical monitoring of acute organophosphate intoxications [3–5] but may be insufficient even for assessing occupational exposure [6,7].

On the other hand, monitoring CP levels in blood and degradation products in urine, allows a more selective and sensitive determination of the actual burden with this xenobiotic. Both CP and CP-oxon are enzymatically converted primarily to phase I metabolites, i.e. diethyl thiophosphate (DETP), diethyl phosphate (DEP), and 3,5,6-trichloro-2-pyridinol (TCP) (Fig. 1). DEP may also originate from endogenous oxidation of DETP. These metabolites are eliminated chiefly by the kidney [8,9], TCP to a large extent after phase II metabolism (conjugation) [10]. Hence, urinary levels of alkyl (thio)phosphates as well as of alcoholic fragments, which are generally more compound-specific (TCP as marker for CP and CP-methyl), are suitable biomarkers of organophosphate burden [11].

Gas chromatography (GC) has previously been the method of choice for measuring DETP and DEP in urine employing mostly either mass sensitive [12–16] or flame photometric [17–22] detection. However, prior to GC analysis DETP as well as DEP need to be derivatised to volatile esters. This derivatisation step turned out to be a critical point of method reliability. Chemical instability of alkyl thiophosphates, unpredictable yields of derivatisation in the presence of water, and interferences caused by inorganic phosphate were frequently reported as potential problems. Sophisticated sample preparation protocols have therefore been suggested to avoid erroneous results. On contrary, to our knowledge only one paper reported the use of high-performance liquid chromatography (HPLC) for the direct determination of urinary DETP and DEP concentrations [23]. Ion-pairing with tetrabutylammonium acetate was employed by the authors to achieve sufficient retention and acceptable peak shape of these highly polar acidic phosphorous compounds on a reversed-phase (RP) type stationary phase. Detection was achieved by electrospray ionisation–tandem mass spectrometry (ESI–MS/MS).

TCP in urine has been quantified either by GC–MS [24–29], GC coupled with electron capture detection [25], LC–ESI–MS/MS [30,31], LC–UV [32], or immunoanalytical assays [33,34].

In general, none of the reported analytical methods proposed the simultaneous determination of the alkyl (thio)phosphate type metabolites (group specific metabolites) and TCP (compound specific metabolite), which was the primary aim of the present study. LC with a stationary phase possessing bi-modal retention characteristics hyphenated with ESI–MS/MS was employed for this purpose. The validated assay was applied in the field of analytical toxicology to measure time-dependent concentration profiles of DETP, DEP, and TCP in urine of an individual acutely poisoned with CP [10].

2. Materials and methods

2.1. Chemicals

Acetonitrile (ACN), methanol (MeOH) (both Fisher Scientific, Loughborough, UK), and ethyl acetate (EtOAc), purchased from Roth (Karlsruhe, Germany), were of HPLC grade. Bi-distilled water was used throughout. Acetic acid (HAc) and aqueous ammonium hydroxide solution (both Fluka, Buchs, Switzerland) were of analytical grade. CP (Pestanal® quality) was obtained by Riedel de Haën (Seelze, Germany) and dibutyl phosphate (DBP, purum grade) was supplied by Fluka. The metabolite standards used within this study, i.e. DETP, DEP, and TCP were prepared according to previous protocols: TCP was obtained by basic hydrolysis of CP [35] and DETP as well as DEP were synthesised from their respective acid chlorides (both purchased from Aldrich, Vienna, Austria) [23].

2.2. Preparation of standard stock solutions

From the investigated metabolites (DETP, DEP, TCP), individual stock solutions (5000 mg L^{-1}) were prepared in ACN/water (3:1; v/v). LC–UV^{DAD}–MS/MS analysis was

used to verify the purity of the individual compounds especially in terms of cross-contaminations with the other analytes. The internal standard DBP was dissolved in a mixture of ACN/water (1:1; v/v) to obtain a final concentration of 200 mg L^{-1}. Stock solutions were stored at −20 °C.

2.3. Preparation of calibration and routine-analysis standards

Blank urine collected from one individual (creatinine content 8.0 mM, determined by Birkmayer Laboratories, Vienna, Austria) was fortified with 200 μL of each CP metabolite stock solution, i.e. DETP, DEP, and TCP, in a 10 mL graduated flask. From this multi-component standard nine working calibration standards (concentration range 0.25–75 mg L^{-1} of each analyte) were prepared by adequate dilution with blank urine. For validation of the sample clean-up procedure for DETP, DEP, and TCP the absolute recovery (extraction efficiency) was assessed at three concentration levels (1, 10, 75 mg L^{-1}) in relation to spiked aqueous solutions. Also accuracy and precision were assessed at the same concentration levels which covers the practically relevant range of the intended toxicological application. A quality control sample containing DETP, DEP, TCP, and DBP each at a concentration of 10 mg L^{-1} was prepared in mobile phase A and was run embedded in the analysis sequences of the real samples. All standards were stored at −20 °C until analysis.

For a preliminary evaluation of the LC–ESI–MS/MS separation characteristics of the mixed-mode reversed-phase/weak anion exchanger (RP/WAX) stationary phase a solution containing the parent compound (CP) as well as DETP, DEP, TCP, and DBP each in a concentration of 20 mg L^{-1} was prepared.

2.4. Sample pre-treatment procedures

In order to derive information whether CP is eliminated as phase I metabolites or as products of phase II metabolism (conjugates, in particular of TCP) in urine of CP exposed individuals, two different sample clean-up strategies were utilised. One of them aimed at the determination of the amount of “free” phase I metabolites (non-hydrolysed samples) and the other of both the sum of phase I and II products (hydrolysed samples).

2.4.1. Approach A—non-hydrolysed samples

The frozen urine samples were allowed to reach room temperature and a 200 μL aliquot was pipetted in a 1.5 mL Eppendorf tube (Eppendorf, Hamburg, Germany). The aliquots were fortified with 10 μL of the internal standard stock solution, 200 μL saturated sodium chloride solution (Fluka), 200 μL concentrated hydrochloric acid (Merck, Darmstadt, Germany), and 800 μL of the extraction solvent composed of EtOAc/ACN (70:30; v/v). The tubes were vortexed (Minishaker MS 1, IKA Werke, Staufen, Germany) at 1000 revolutions per min (rpm) for 10 min at room temperature and subsequently centrifuged at 8000 rpm for 5 min (Eppendorf MiniSpin centrifuge). Four hundred microliter of the upper organic phase were carefully transferred into a glass autosampler vial. The solvent was evaporated at room temperature in a gentle stream of nitrogen and the residue was reconstituted in 500 μL of eluent A (vide infra).

2.4.2. Approach B—acidic hydrolysis of samples

Urine samples were processed according to protocol A. However, before the extraction step with EtOAc/ACN (70:30; v/v) the Eppendorf tubes were transferred into a water bath (Lauda M12, Lauda-Königshofen, Germany) maintained at 80 °C and were incubated for 120 min.

2.5. LC–ESI–MS/MS method

2.5.1. Instrumentation

For LC–ESI–MS/MS runs an Agilent HP1100 liquid chromatograph connected to a PE Sciex API 365 triple quadrupole mass spectrometer (PE Sciex Instruments, Thornhill, Canada) equipped with a pneumatically assisted electrospray ion source was employed. Eluent flow was split in a ratio of approximately 1:50 before mass spectrometric detection. Data were processed using the Analyst 1.4 software from MDS Sciex (San Francisco, CA).

2.5.2. Liquid chromatography

The stationary phase employed in this study was a recently developed mixed-mode reversed-phase/weak anion exchanger based on *N*-(10-undecenoyl)-3-aminoquinuclidine [36]. The selector was covalently attached onto 3-thiopropyl modified 5 μm 100 Å silica particles (Kromasil 100-5, EKA Chemicals, Bohus, Sweden). The chemical structure is depicted in Fig. 2.

A 100 mm × 4 mm I.D. stainless steel column was packed in-house with this material. The analytical column was protected by a Diol guard column (LiChrospher 100 DIOL, 5 μm) from Merck (4 mm × 4 mm I.D.). The flow rate was kept at 1.00 mL min^{-1} and the column oven was maintained at 25 °C. Injection volume was 15 μL throughout. Separation was achieved in gradient elution mode with a mixed organic modifier and pH gradient. The optimised mobile phases consisted of 20 mM HAc in ACN/water (30:70; v/v) adjusted to an apparent pH (pH_a) of 6.45 with ammonium hydroxide (eluent A) and 20 mM HAc in ACN/water (80:20; v/v), pH_a 7.45 (eluent B). The time program was as follows: 0 min: 0% B, 10 min: 100% B, 22.5 min: 100% B, 23 min: 0% B, 30 min: 0% B.

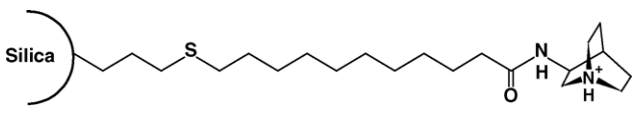

Fig. 2. Chemical structure of the utilised stationary phase.

2.5.3. Mass spectrometry

MS parameters were optimised by analyte infusion employing a syringe pump operated at a flow rate of 320 μL h^{-1} connected to the ion source of the MS. The individual compounds were each dissolved at a concentration of 10–50 mg L^{-1} in the eluent composition approximately corresponding to their retention time. All analytes used for the quantitative assay (DETP, DEP, TCP, DBP) generated pseudomolecular ions in the mass filter (Q1) by applying negative ionisation voltage and experienced specific MS dissociation pathways in Q2 (collision cell) by applying an appropriate collision energy (vide infra) and nitrogen as collision gas (CAD = 2). This enabled a selective detection via Q3 (multiple reaction monitoring, MRM) having the dwell time for each transition set to 250 ms. The total elution time window was divided into distinct detection segments, applying the respective optimised MRM conditions within the elution time frame of each analyte. The first time frame (0–6.5 min) was used for a qualitative monitoring of MS/MS traces specific for CP (positive ion mode).

For DETP, DEP, and DBP one quantification and one confirmation MRM pair each was selected. Putative dissociation pathways of DETP and DEP leading to the specified fragments can be found elsewhere [23]. TCP showed a unique fragmentation pathway with the chlorine radical anion being the sole ion that could be monitored with reasonable abundance. Hence, confirmation of the identity of this analyte was performed utilising its characteristic isotope distribution. The selected quantifier and qualifier transitions along with the applied MS/MS parameters and the respective LC retention times of the compounds are summarised in Table 1.

2.6. Toxicokinetic study

The developed LC–ESI–MS/MS method was applied to monitor the excretion profiles of CP metabolites in urine of an individual acutely poisoned with a CP-containing pesticide formulation (Dursban 2E®, 20–25% CP in a hydrocarbon mixture). After informed consent was obtained from the patient, spot urine samples were collected in 10 mL polypropylene containers at least once a day from the time of the admission to the hospital until the 14th day of hospitalisation ($n = 21$). The samples were stored at +4 °C until delivering to our laboratory (less than 5 days) and were subsequently frozen at −20 °C.

3. Results and discussion

3.1. Separation characteristics of the mixed-modal RP/WAX stationary phase

Both DETP (pK = 2.96, data from the SciFinder Scholar database) and DEP (pK = 1.42) are strongly acidic and hydrophilic in their dissociated form. Since they lack strongly hydrophobic interaction sites their LC analysis on conventional RP type stationary phases with hydro-organic eluents turned out to be problematic in terms of sufficient retention and peak performance in earlier studies, even with strongly acidic eluents.

To overcome these chromatographic limitations and avoid ion-pair agents that are prone to leading to ion-suppression [23,37] as well as to allow a simultaneous separation and analysis of acidic alkyl (thio)phosphate metabolites and the phenolic metabolite component of CP, we herein propose the use of a bi-modal retention concept employing a mixed-mode RP/WAX stationary phase (see Fig. 2). The highly polar anionic compounds (DETP, DEP, DBP) are interacting with the stationary phase primarily by anion exchange through ionic interaction with the cationic quinuclidinium moiety, while essentially non-ionised compounds like CP will be retained by hydrophobic interaction on lipophilic domains (C_{11} alkyl chain). TCP (pK = 7.5) will be retained by a combination of both anion exchange and hydrophobic interaction forces.

To illustrate the RP/WAX separation mechanism and to optimise the separation, the effect of the major influential parameters of the mobile phase (nature and amount of organic modifier, counter-ion type and concentration, pH_a) have been investigated. Mixtures of ACN/water were found superior over corresponding MeOH/water mixtures chiefly in terms of elution times, which were by a factor of more than two higher taking MeOH as modifier. Further, acetate was a counter-ion that allowed satisfying peak shapes and fulfilled the prerequisite of volatility for subsequent ESI–MS/MS detection.

The pH_a-dependency of retention of CP, DETP, and TCP with different mobile phases in the isocratic mode is discussed in the following. Two hydroorganic eluents, containing 20 mM HAc in either ACN/water (50:50; v/v) or ACN/water (80:20; v/v) were tested. The pH_a-values were adjusted in the hydroorganic mixture with ammonium hydroxide between 6.0 and 7.5. Fig. 3 depicts graphically the

Table 1
Retention times and MS/MS detection parameters of analytes and internal standard

Analyte	Retention time (min)	ESI mode	Electrospray voltage (V)	Precursor ion (*m/z*)	Collision energy (eV)	Product ion(s) (*m/z*)
CP	6.1	Positive	4250	352	31	200; 115
DETP	10.6	Negative	−4250	169	−22	*95*; 141
DEP	7.2	Negative	−4250	153	−20	*79*; 125
TCP	19.2	Negative	−3750	196	−32	*35*
				198		35
DBP	12.8	Negative	−4250	209	−26	*79*; 153

Italics *m/z* traces of product ions were used for quantification.

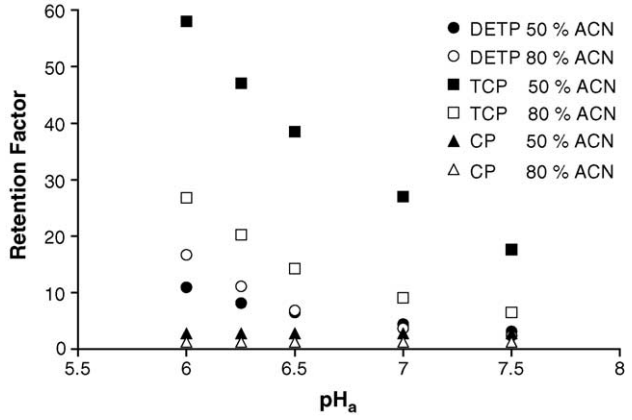

Fig. 3. pH$_a$-curve of retention factors of DETP, DEP, and TCP on the evaluated RP/WAX stationary phase employing two different hydroorganic eluents: 20 mM HAc in ACN/water (50:50; v/v) and ACN/water (80:20; v/v), respectively. Adjustment of pH$_a$ with ammonium hydroxide.

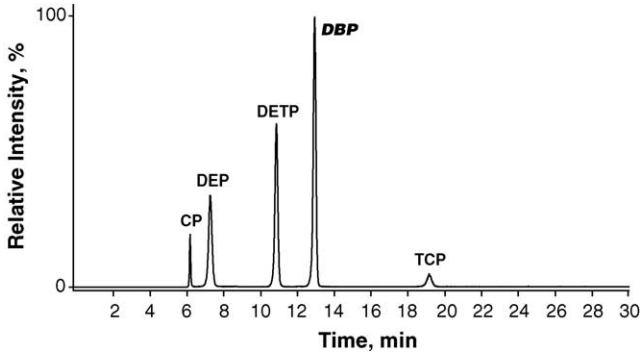

Fig. 4. LC–ESI–MS/MS sum MRM traces of quantification and confirmation ion pairs of CP (*m/z* 352 → 200, 352 → 115), DETP (*m/z* 169 → 95, 169 → 141), DEP (*m/z* 153 → 79, 153 → 125), TCP (*m/z* 196 → 35, 198 → 35), and the internal standard DBP (*m/z* 209 → 79, 209 → 153) (each 20 mg L^{-1}) simultaneously separated on the RP/WAX stationary phase; Mobile phase: (A) ACN/water (30:70; v/v), 20 mM HAc, pH$_a$ 6.45 (adjustment with NH_3); (B) ACN/water (80:20; v/v), 20 mM HAc, pH$_a$ 7.45 (NH_3); gradient elution: 0 min: 0% B, 10 min: 100% B, 22.5 min: 100% B, 23 min: 0% B, 30 min: 0% B; injection volume, 15 μL; *T*, 25 °C; flow rate, 1.00 mL min^{-1}.

dependence of the retention factors of CP, DETP, and TCP on pH$_a$.

CP follows a typical RP-type retention mechanism. Characteristically, over the investigated pH$_a$-range at constant organic modifier percentage elution times remained constant within ±3%. Increase of ACN-concentration from 50 to 80%, on the other hand, halved retention factors as expected. Such a decrease of retention upon increase of ACN percentage was similarly observed for TCP. However, also the pH$_a$ had a remarkable influence on elution time of this compound and retention was approximately three fold higher with eluents adjusted to pH$_a$ 6.0 compared to pH$_a$ 7.5. Electrostatic forces stemming from the acidic phenolic group superimposed upon hydrophobic interactions both may account for the chromatographic characteristics of TCP. Only with high amounts of ACN in the eluent and relatively high pH$_a$-values analysis times were reasonably short.

In the case of DETP, higher retention factors were typically observed at increased concentrations of ACN, which stands in sharp contrast to the elution pattern of TCP and CP. As seen from Fig. 3, at pH$_a$ 6.0 retention is approximately 1.5 fold higher for the mobile phase containing 80% ACN compared to the eluent with 50% ACN. On the other hand, at a pH$_a$ higher than 7.0 the trends return to the behaviour expected for RP-LC with higher elution strength for the ACN-rich eluents. The elution pattern of DETP may be readily explained by the primary ion exchange process, which is dominant for this solute. The shape of the pH$_a$-curve of DETP in the investigated narrow range results from weakened ionic interaction due to a decreasing ionisation of the selector entity when the pH$_a$ is increased. Moreover, the actual ion exchange capacity is also weaker in the ACN-poor eluent due to the higher total counter-ion concentration (ionic strength was not compensated for) and/or a higher dielectric constant.

From these data it became obvious that an isocratic elution of these compounds will be inappropriate if reasonably short run times for the LC–ESI–MS/MS assay are aspired. Hence, a binary gradient was developed, which not only increased linearly the content of organic modifier (from 20 to 80% ACN in 10 min) but simultaneously also the pH$_a$ (from 6.45 to 7.45), followed by isocratic elution between 10 and 22.5 min and a reconditioning interval (7.5 min). Employing these conditions a simultaneous separation of CP, DETP, DEP, TCP, and the internal standard DBP was achieved (Fig. 4).

Retention times and peak performance parameters were stable in an analysis sequence of 50 processed urine samples. Thereafter, the ion-exchange sites were regenerated by flushing the column with 2% (v/v) triethylamine in MeOH for 15 min followed by mobile phase to avoid minor shifts in retention times of the acidic solutes due to trapped ionic species. On the other hand, direct injection of urine without previous desalting has to be avoided without more frequent column regeneration.

Fig. 5 illustrates the complementary retention behaviour of CP, DETP, DEP, TCP, and TCP-*O*-glucuronide (phase II metabolite of TCP which was identified in real samples), on conventional RP (C_{12}) versus the RP/WAX system discussed herein. On the RP/WAX system retentivity, peak shapes, and MS sensitivity (due to higher ACN content at elution time) were considerably improved for DETP and DEP compared to RP stationary phases.

3.2. Mass spectrometric detection

Detection of CP metabolites by ESI–MS/MS proved to be suitable in previous studies determining urinary levels of either TCP [30,31] or DETP and DEP [23].

In the present investigation, MRM was chosen as acquisition mode due its specificity and mostly also higher sensitivity compared to selected ion monitoring. While for DETP, DEP, and DBP two specific MS/MS transitions for quantification

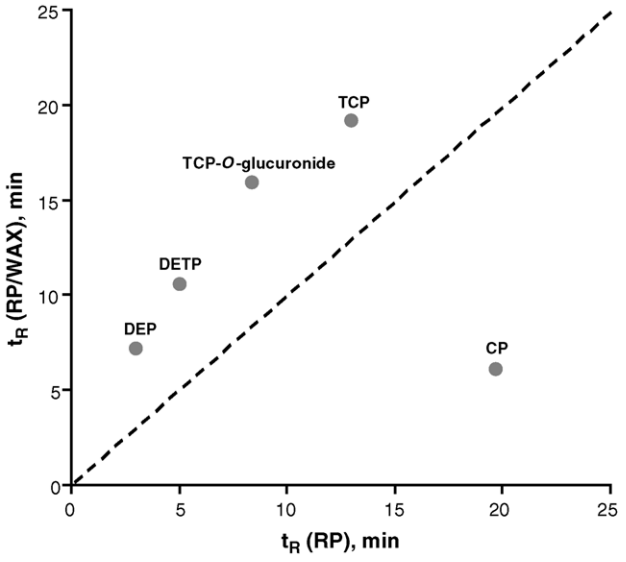

Fig. 5. Retention times (t_R, min) of CP and major urinary human metabolites, i.e. DETP, DEP, TCP, and TCP-*O*-glucuronide, on RP and RP/WAX. *Conditions RP-WAX* as specified in caption of Fig. 4. *Conditions RP*: column: Phenomenex Synergi MAX RP (C_{12}), 150 mm × 4.6 mm I.D.; mobile phase: (A) water, 0.1% formic acid, (B) ACN, 0.1% formic acid; gradient elution: 0 min: 10% B, 20 min: 100% B, 27 min: 100% B, 28 min: 0% B, 35 min: 0% B; injection volume, 15 μL; *T*, 25 °C; flow rate, 1.00 mL min^{-1}.

and confirmation were monitored, in case of TCP the chlorine radical anion was identified as the only suitable fragment (Table 1). In previous studies, pseudo-MRM conditions were applied for the quantification of TCP, where both the parent and the product ion were identical [30,31]. However, employing real dissociation pathways for TCP secures a more unequivocal analyte determination in the urinary matrix, although the trade-off is an overall slightly reduced sensitivity. The characteristic chlorine isotopomer pattern of TCP allowed quantification (*m/z* 196 → 35) and confirmation (*m/z* 198 → 35) with a single fragment.

3.3. Sample clean-up and extraction efficiency

Shortly after delivery and before developing an adequate sample pre-treatment protocol, the urine samples collected from the acutely poisoned patient were screened for the presence of the parent compound. For this purpose centrifuged and diluted urine was directly injected onto an RP column coupled to ESI–MS/MS to rule out occurrence of ex vivo conversion of CP during sample clean-up. As also reported previously [38,39] CP could not be detected in the real samples.

Hence, a sample clean-up protocol was developed specifically for the simultaneous extraction of DETP, DEP, and TCP. For that purpose previously reported protocols of liquid–liquid extraction of alkyl phosphates from urine after acidification with hydrochloric acid were adapted [16,40].

The low lipophilicity, especially of DEP, demanded polar extraction solvents. To promote phase separation the acidified urine was saturated with sodium chloride. An acceptable compromise regarding recovery of CP metabolites, presence of matrix components in the extract, and phase separation properties was found with a solvent mixture composed of 30% (v/v) ACN in EtOAc and was subsequently used for clean-up of urine samples. Absolute recovery of the optimised extraction procedure was evaluated at three concentration levels (each $n = 6$), which covered the entire range of the expected metabolite concentration, and the average recovery of the extraction procedure was calculated according to Matuszewski et al. [41]. Good extraction efficiencies were found for TCP and DETP (Table 2, procedure A). Solely for DEP extraction efficiencies were low (ca. 40%), but repeatable.

To gain information whether metabolites, in particular TCP, are also excreted as conjugates (phase II metabolites), a second sample pre-treatment protocol was developed. An aliquot of urine sample was subjected to acid-induced hydrolysis at 80 °C for 120 min before the extraction step (procedure B).

The absolute recovery of TCP was quantitative also for the second procedure (protocol B) which indicates the chemical stability of TCP under the harsh acidic hydrolysis conditions that are required to chemically cleave conjugates, e.g. glucuronides. Under these stress conditions recovery values for DETP, on contrary, decreased. This might be explained by the chemical instability of alkyl (thio)phosphates in strongly acidic media at elevated temperatures as addressed previously [16,42]. The increase of the recovery of DEP let assume that DETP is partly converted to DEP. While it was, however, possible to largely compensate the chemical instability of DETP with the calibration graph obtained from spiked urine samples processed equally, poor accuracies (76–145%, vide infra) prompted to reject this sample preparation protocol B for DETP. For DEP the figures were even worse and therefore only free DETP and DEP can be accurately measured using sample preparation protocol A.

Table 2
Recovery values (%) and their 95% confidence intervals determined for DETP, DEP, and TCP at three concentration levels (each $n = 6$) and employing both sample clean-up procedures (corrected for DBP)

Analyte	Sample treatment procedure A			Sample treatment procedure B		
	1 mg L^{-1}	10 mg L^{-1}	75 mg L^{-1}	1 mg L^{-1}	10 mg L^{-1}	75 mg L^{-1}
DETP	102.2 ± 5.1	99.6 ± 2.1	97.6 ± 2.3	47.4 ± 1.8	48.9 ± 2.4	73.6 ± 3.0
DEP	39.4 ± 1.5	41.6 ± 2.4	41.9 ± 2.5	52.0 ± 2.1	51.9 ± 1.6	47.0 ± 2.3
TCP	104.2 ± 4.9	101.4 ± 5.4	97.8 ± 2.5	107.3 ± 6.0	100.6 ± 5.7	100.7 ± 3.3

Instead of the harsh acidic hydrolysis conditions the more gentle enzymatic cleavage of conjugates (β-glucuronidase) is a frequently employed alternative. However, it has to be kept in mind that results obtained from acidic hydrolysis may not necessarily reflect solely the sum of the amount of free and conjugated TCP, but may include other metabolites such as mono-*O*-deethylated CP too (vide infra and in particular [10]). Hence, the amount of urinary TCP obtained from chemical hydrolysis may reflect better the total burden with CP than measurement of free TCP only or using more specific enzymatic cleavage.

3.4. Method validation

3.4.1. Storage stability of CP metabolites and the internal standard

The potential chemical degradation of DETP, DEP, TCP, and DBP during storage was determined at several levels. Data obtained from repetitive analysis of stock solutions of the analytes in ACN/water mixtures, fortified urine samples, as well as the processed calibration samples within three months (duration of study) did not give evidence for analyte degradation if stored at −20 °C, which is in agreement with previous studies [14,16]. Moreover, the individual analyte concentrations did not change significantly (less than 5%) in processed urine samples left in the autosampler tray for 4 days (approximately 20 °C). Studies with spiked samples that were handled analogous to the freshly collected real samples (storage at +4 °C for not longer than 5 days, thereafter at −20 °C) revealed that this treatment did not affect the measured metabolite concentrations either.

3.4.2. Selectivity and matrix effect

Selectivity of a bioanalytical LC–MS assay, i.e. the reliable identification of the target analyte in presence of potentially interfering substances like matrix components, respectively, is a crucial factor for its "real-world" suitability. In this respect, the use of compound-specific MS/MS transitions for qualitative and quantitative determination may be a viable means to achieve more reliable analysis results than with other methods, if matrix effects, interference of other metabolites, and "cross-talk" are absent [41]. In runs using the MRM acquisition mode no interfering peaks that could have deteriorated the selectivity, precision, and accuracy of the method were identified in blank and spiked urine samples within the retention time windows of the respective analytes (Fig. 6).

Further, it was investigated whether the polar liquid–liquid extraction step used for sample clean-up led to extensive carry over of matrix components that could have affected the ionisation process. Assessment of matrix effects in MS detection is mandatory especially if no isotopically labelled internal standards are available that could to a great extent compensate for ionisation suppression/enhancement processes [43]. Results obtained from measuring blank urine samples spiked after extraction were compared with data from standard solutions, i.e. the analytes dissolved in pure eluent A, both at a concentration level of 10 mg L^{-1} (close to the lower concentration end of the real samples). This ratio was calculated to be 92.2–101.6% and thus, the "absolute matrix effect" [41] was considered negligible and revealed the suitability of the employed sample pre-treatment protocol for a clean-up of urine samples prior to LC–ESI–MS/MS analysis.

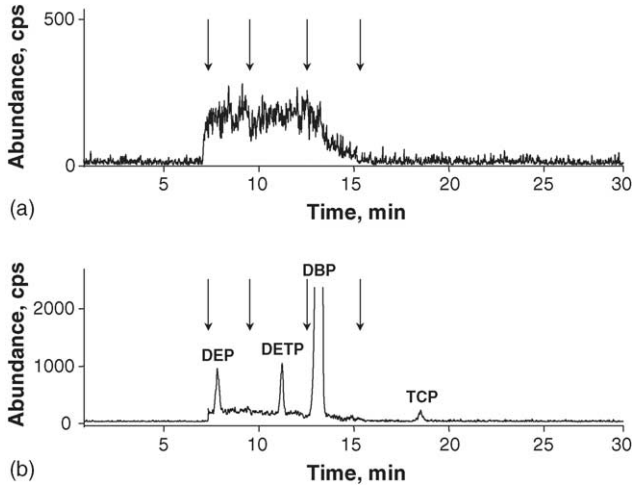

Fig. 6. LC–ESI–MS/MS MRM traces of processed urine samples: (a) blank, (b) spiked with 0.25 mg L^{-1} of DETP, DEP, and TCP. Arrows indicate changes in MS/MS conditions as specified in Table 1. In each time segment the quantification and confirmation MRM trace of the respective analyte was monitored (cf. caption of Fig. 4).

On the other hand, for the present short-term i.e. single case high-dose toxicological application the "relative matrix effect" [41] has not been evaluated, as would be desirable or even mandatory in particular for long-term studies or analysis of background levels of the metabolites due to chronic exposure at concentrations close to the quantification limit. This was supposed to be justified also to keep the workload of method validation in a reasonable extent and in balance with its application as well as the scientific impact or legal consequences of the results of the single case intoxication study.

Due to lack of knowledge of other metabolites at the time of the study and of other potentially interfering compounds that could have been spiked to the solute mixture, in order to assess the selectivity of the chromatographic method and assay, respectively, such experiments have not been performed. However, two other peaks which correspond to CP metabolites, namely TCP-*O*-glucuronide and mono-*O*-deethyl-CP, could be detected in the MRM trace of TCP in non-hydrolyse urine samples of an intoxicated person. Since these compounds were well resolved from the TCP peak, no problems with "cross-talk" evolved. This clearly emphasizes the necessity of a highly selective chromatographic separation like in the present case, in order to avoid such problems even with "intuitively specific" MS/MS. Moreover, the higher retention in the RP/WAX system was perceived to minimize the probability of co-migration of undetected matrix compo-

Table 3
Calibration graph parameters for DETP, DEP, and TCP (nine calibration samples, range: 0.25–75 mg L^{-1}, processed according to sample clean-up approach A)

	DETP	DEP	TCP	TCP[a]
Slope no. 1	0.0494	0.0135	0.0039	0.0037
Intercept no. 1	0.0274	0.0047	0.0011	0.0025
R^2 no. 1	0.9992	0.9994	0.9982	0.9982
Slope no. 2	0.0444	0.0123	0.0038	0.0037
Intercept no. 2	0.0385	0.0131	0.0014	0.0027
R^2 no. 2	0.9979	0.9934	0.9981	0.9968

[a] Processed according to sample clean up approach B.

nents and thus of matrix effects, although longer cycle times have to be accepted.

3.4.3. Calibration and linearity

"Matrix-matched" calibration and establishment of linearity of the analytical procedure for DETP, DEP, and TCP was performed by measuring nine multi-component urine calibration standards with a concentration between 0.25 and 75 mg L^{-1}. Due to the non-availability of adequate isotope-labelled compounds DBP was used as internal standard [16]. The calibration function, i.e. the mathematical relationship between the quotient (absolute analyte peak area)/(peak area of the internal standard) and the concentration of the analyte over the entire calibration range, was calculated by weighted linear regression ($1/x^2$).

The impact of sensitivity drifts of the MS detection on quantification were minimised by the measurement of spiked urine standards used for the calibration graphs embedded into each analysis sequence of real samples. For the actual analytical problem (vide infra) per sample clean-up protocol two calibration curves were obtained and showed acceptable linear correlation regarding slope and intercept. The correlation coefficients (R^2) of the obtained calibration graphs were better than 0.993 for all analytes (Table 3).

3.4.4. Precision and accuracy

Six aliquots of blank urine samples spiked with either 1, 10, or 75 mg L^{-1} of each DETP, DEP, and TCP were analysed to determine intra-assay precision (repeatability) and accuracy of the method (Table 4).

As can be seen, the results for both intra-day precision as well as accuracy are for all investigated solutes well within commonly applied acceptance criteria, even at the low concentration level [44]. The lowest accuracy was determined for DEP at the 1 mg L^{-1} level (115%), which, however, is still acceptable. At this point it is noted that in the real samples the lowest DEP concentrations were analysed to be around 2–3 mg L^{-1}, i.e. at least a factor of two higher than this lower level of accuracy validation.

Further, precision and accuracy values for TCP and use of sample preparation protocol B (acidic hydrolysis to cleave conjugates) were also conforming well regarding the acceptance criteria, which thus allows besides the determination of free TCP also the quantitation of total TCP. In contrast, the method for analysis of total alkyl (thio)phosphates (free and conjugated forms of DETP and DEP) must be rejected due to limitations regarding accuracies (e.g. 145% for DETP at 1 mg L^{-1} and even worse for DEP) originating as outlined from instabilities at acidic stress conditions and elevated temperatures of sample pretreatment protocol B.

Intra-day (run-to-run) LC–ESI–MS/MS system repeatability was determined at a concentration level of 10 mg L^{-1} (urine sample processed according to approach A) in six consecutive runs. R.S.D. values were 2.5% (DETP), 4.8% (DEP), and 1.3% (TCP).

Assessment of sequence stability in the course of ca. three days continuous operation was performed with the 10 mg L^{-1} quality control sample injected after every sixth run of calibrants/real samples ($n = 17$, totally 108 runs). Corresponding R.S.D.-values were 8.2% for DETP, 3.4% for DEP, and 1.9% for TCP. Estimates for inter-day precision (intermediate precision) were inferred from repeated measurement of the quality control sample within eight consecutive days. Absolute peak areas for analytes and internal standard had a variation of approximately 30% R.S.D. However, through calibration with the internal standard inter-day precision turned out to be better than 10% R.S.D.

3.4.5. Range

Since the response function turned out to be appropriate for DETP, DEP, TCP (sample preparation protocol A) as well as total TCP (sample preparation protocol B) in the specified calibration range as well as satisfactory precision and accuracy could be established, the assay range is validated for all these analytes to be between 1 and 75 mg L^{-1}.

Table 4
Precision and accuracy data of spiked urine samples processed according to sample clean-up approach A (each $n = 6$)

	Precision (% R.S.D.)			Accuracy (%)		
	1 mg L^{-1}	10 mg L^{-1}	75 mg L^{-1}	1 mg L^{-1}	10 mg L^{-1}	75 mg L^{-1}
DETP	10.5	2.8	2.9	97	93	91
DEP	6.9	7.5	7.4	115	97	97
TCP	12.8	1.6	2.9	100	105	97
TCP[a]	12.1	7.4	4.0	101	100	96

[a] Processed according to sample clean up approach B.

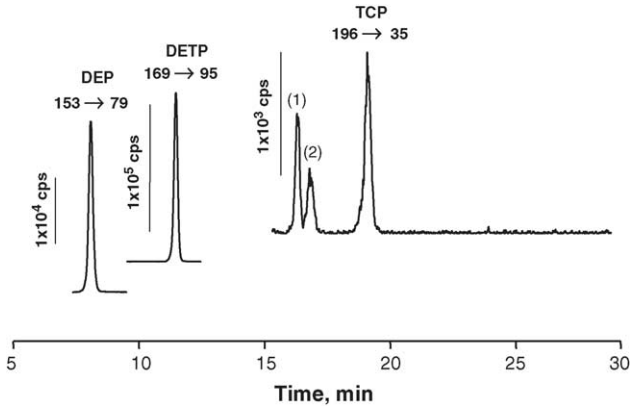

Fig. 7. LC–ESI–MS/MS MRM traces of quantification pairs of DETP (*m/z* 169 → 95), DEP (*m/z* 153 → 79), and TCP (*m/z* 196 → 35) in a processed urine sample (sample clean-up approach A) collected from a female individual approximately 27 h after an accidental ingestion of Dursban 2E®. Calculated concentrations (mean value of duplicate runs): 40.1 mg L^{-1} (DETP), 28.2 mg L^{-1} (DEP), 8.3 mg L^{-1} (TCP). (1) TCP-*O*-glucuronide and (2) mono-*O*-deethyl-CP.

3.5. Metabolite concentration in urine samples following an acute CP poisoning

The analytical assay developed was applied for a human toxicokinetic study with CP [10]. Aliquots of spot urine from a female individual who orally ingested Dursban 2E® were collected during her stay in the hospital. As discussed above preliminary analysis revealed the absence of the parent compound, which is in accordance with previous studies [38,39]. DETP, DEP, and TCP could be quantified in all samples. Concentration values of the individual metabolites reached up to 60 mg L^{-1}. Fig. 7 depicts the MRM chromatogram obtained from a processed urine sample collected 1 day post ingestion (sample treatment approach A).

From Fig. 7 it is seen that in the MRM trace of TCP two additional peaks appeared eluting before the target compound. These peaks could, however, not be detected in the corresponding hydrolysed samples nor in blank or fortified blank urine, cf. Fig. 6. It was assumed, that partial in-source fragmentation of non-identified metabolites, containing the TCP moiety, might account for this observation. This assumption was later confirmed by a precursor ion scan with the Q3 set to *m/z* 196 and 198, respectively. In these experiments the characteristic isotopomer pattern together with the observed fragmentation pathways allowed a mass spectrometric identification of the peaks as TCP-*O*-glucuronide ($[M-H]^-$: *m/z* 372; $t_R^{RP/WAX}$ = 15.9 min) and mono-*O*-deethyl CP ($[M-H]^-$: *m/z* 320; $t_R^{RP/WAX}$ = 16.8 min).

More details on the results of the urinary elimination kinetics of DETP, DEP, and TCP along with human CP biotransformation pathways can be found elsewhere [10].

4. Conclusions

An LC–ESI–MS/MS method for the determination of chlorpyrifos (CP) metabolites, viz. diethyl thiophosphate (DETP), diethyl phosphate (DEP), and 3,5,6-trichloro-2-pyridinol (TCP), in urine of acutely poisoned individuals was developed. The HPLC method made use of a mixed-mode reversed-phase/weak anion exchange stationary phase, which was run with a mixed organic modifier and pH gradient. With this method alkyl (thio)phosphate, phenolic metabolite as well as dibutyl phosphate, employed as internal standard, and the parent CP were simultaneously separated within 20 min. The direct injection of centrifuged and diluted urine collected from an acutely with CP poisoned patient revealed, however, the absence of CP, and thus the LC–ESI–MS/MS assay was adopted and validated for DETP, DEP, and TCP only. Liquid–liquid extraction was used for sample clean-up. Absence of matrix effect was shown for DETP, DEP, and TCP with a single lot of urine. Recovery was almost quantitative for all compounds except DEP (less than 50% but repeatable recovery). Linearity was determined in the range of 0.25–75 mg L^{-1} for all three metabolites and revealed correlation coefficients of $R^2 > 0.993$. Precision and accuracy were acceptable over the validated range of 1–75 mg L^{-1}. Besides this method for urinary levels of free DETP, DEP, and TCP, also a reliable method for the analysis of total TCP (i.e. free and conjugated forms) after acidic hydrolysis was presented. The analytical assay allowed to study in detail the elimination kinetics of DETP, DEP, and TCP in course of an acute CP poisoning [10].

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