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Concurrent estimation of metabolite concentrations along with parent drug quantification in post-mortem blood

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ABSTRACT

There is a constant demand for the quantification of drug metabolites within post-mortem toxicology. Especially electrospray ionization–mass spectrometry techniques necessitate that calibration is carried out using primary reference standards due to the non-uniform ionization efficiency between parent drugs and their metabolites. As reference standards for metabolites are not readily available and their costs are high, alternative methods for immediate quantification are required. In this study, ultra-high performance liquid chromatography coupled with photodiode array detection and corona charged aerosol detection was utilized for the concurrent quantification of 23 drug metabolites using the corresponding parent drug for calibration. Based on this secondary calibration, the quantitative results for the *N*-demethylated metabolites by each detector were similar to those obtained by the ordinary calibration using reference standards. For *O*-demethylated metabolites, the differences in detector response caused somewhat larger biases using the secondary calibration. Using the validated secondary calibration, the blood sample data gathered from 633 post-mortem cases was retrospectively re-processed to discover the combined metabolite–parent concentrations and metabolite to parent ratios for six toxicologically relevant drugs. These results, representing all causes of death, were compared to published data from therapeutic drug monitoring and post-mortem toxicology.

1. Introduction

Drug metabolite plasma concentrations are informative in therapeutic drug monitoring (TDM), as several drugs have pharmacologically active metabolites. The therapeutic ranges of some antipsychotics and antidepressants are commonly expressed as the combined concentration of the parent drug and its active metabolite [1]. In the guidelines for TDM in psychiatry, the ranges of normal metabolite to parent ratios for several drugs have been established [2]. Even the determination of less active metabolites is recommended in order to ascertain compliance or the patient's capability to metabolize drugs [1,2]. In post-mortem toxicology, however, related data on metabolite blood concentrations of statistical relevance is limited to only few publications [3], while most of the data is scattered in miscellaneous case notes and case series [4]. The lack of data is largely due to the fact that reference standards for many less active metabolites, for example hydroxyl derivatives, are not readily available and their costs are high, which in turn does not motivate the effort required to routinely analyze metabolites in casework. In the absence of reference standards, the two most important obstacles in quantification are the lack of a suitable analytical tool that possesses a uniform response to the parent drug and its metabolite, and the possible difference in the extraction recovery between the parent and metabolite [5].

Liquid chromatography-mass spectrometry (LC-MS) techniques are widely used in post-mortem toxicology due to their selectivity and sensitivity. However, the electrospray ionization (ESI) efficiency can be significantly dependent on analyte structure [6,7]. Furthermore, LC gradients affect the MS response, as compounds with different retention times are sprayed into the mass spectrometer in different solvent compositions [6]. Using nanospray ionization (NSI) [5,6,8,9] or captive spray ionization (CSI)-LC-MS [10,11] reduces differences in the ionization efficiency between drugs and their metabolites, but these techniques are currently not robust enough to be a strong option in post-mortem toxicology.

UV detection has previously been used for the quantification of drug metabolites using the parent drug for calibration [12,13]. However, even with apparently similar UV spectra the quantification can be erroneous if the chemical changes take place in the

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vicinity of a chromophore. Chemiluminescent nitrogen detector (CLND) and evaporative light scattering detector (ELSD) have traditionally been used for quantification without reference standards as their response is relatively independent of the analyte [14,15]. CLND has been proven to be suitable for single-calibrant quantification of nitrogen containing drugs using caffeine for calibration [16]. However, CLND provides an equimolar response only with compounds containing nitrogen, and the response is proportional to the number of nitrogen atoms in a molecule [15]. With ELSD, the accuracy of single-calibrant quantification is improved when calibration is performed with chemically similar compounds [14,15].

Another universal detector, the corona charged aerosol detector (CAD) is a mass-dependent detector. The LC mobile phase is converted into droplets that are dried forming particles consisting of analyte molecules, and a stream of positively charged nitrogen collides with the particles. The charge is then transferred to the particles and measured. As the amount of analyte increases, the size of the particles increases as well. Consequently, the charge of the particle is increased along with particle size, generating a signal directly proportional to the quantity of the analyte present. Unlike UV detection, which is concentration-dependent, the signal of CAD does not depend on the concentration of the analyte in the eluent [17]. In the analysis of pharmaceutical compounds CAD was more sensitive than ELSD and approximately as sensitive as the UV detector [18]. The response to different analyte structures was more uniform with CAD than with ELSD [19]. As with MS and ELSD, the CAD response depends on the composition of the mobile phase but this phenomenon can be compensated by applying an inverse post-column gradient [20].

In our previous study, we developed and validated a quantitative method for straightforward monitoring of basic drugs in blood samples, based on ultra-high performance liquid chromatography with two consecutive detectors: a photodiode array detector (DAD) and a CAD [21]. This UHPLC-DAD-CAD method utilized the response ratio of DAD to CAD, which provided the additional identification efficiency required, while the high stability of identification and quantification allowed the use of facile historic calibration. In the present study, our objective was to assess the UHPLC-DAD-CAD method for concurrent quantification of drug metabolites using the parent drug for calibration. Consequently, we applied the validated parent drug calibration to retrospectively re-process the blood sample data gathered from 633 post-mortem cases to discover the distribution of metabolite to parent ratios and combined metabolite–parent concentrations for six toxicologically relevant drugs.

2. Materials and methods

2.1. Instrumentation and chemicals

The UHPLC-DAD-CAD method, including instrumentation, sample preparation and materials used, has been described earlier in detail [21]. Briefly, blood samples were extracted at a basic pH with a mixture of ethyl acetate and butyl acetate. The analytical column was a HSS C18 of 2.1 mm × 150 mm equipped with a HSS C18 precolumn of 2.1 mm × 5.0 mm, both with a particle size of 1.8 μm (Waters, Milford, MA). UHPLC separation was performed using a mobile phase gradient consisting of 0.1% aqueous solution of trifluoroacetic acid and methanol. Analyte detection was performed using the two consecutive detectors, DAD and CAD. UV spectra were collected over the range 210–400 nm at collection speed 20 point/s and the quantification wavelength was 230 nm. CAD was operated using a nitrogen pressure of 35 psi. Calibration samples were prepared in sheep whole blood, and historic one-point calibration on both detectors was used. Reference standards for drugs and their metabolites were obtained from pharmaceutical companies and they were of pharmaceutical purity.

2.2. Validation of the method

The UHPLC-DAD-CAD method has been previously validated using the primary calibration method with use of the reference standards [21]. In this study, a secondary calibration method using the parent drug as calibration standard was tested for 23 metabolites of 21 drugs for which the reference standards were available in the authors' laboratory (Table 1). Selection of the

Table 1
Comparison of metabolite quantification by CAD and DAD between ordinary calibration and calibration using parent drug.

Parent drug	Metabolite	Linear range[a] [mg/L]	Calibration point [mg/L]	R_t (metabolite) − R_t (parent) [min]	Average quantification difference CAD [%][b]	Average quantification difference DAD [%][b]
Amitriptyline	Nortriptyline	0.1–5	1	0.150	14.4	14.3
Citalopram	Norcitalopram	0.05–5	0.5	0.076	16.2	9.5
Clobazam	Norclobazam	0.1–5	1	−0.515	14.3	21.8
Clomipramine	Norclomipramine	0.1–5	0.5	0.136	5.7	28.6
Dextromethorphan	*O*-desmethyldextromethorphan	0.05–5	0.5	−2.103	18.4	35.9
Dextropropoxyphene	Nordextropropoxyphene[c]	0.05–5	1	−0.579	7.7	
Diltiazem	Nordiltiazem	0.1–5	0.5	0.107	12.0	5.1
Doxepin	Nordoxepin	0.05–5	0.5	0.125	22.3	16.2
Fluoxetine	Norfluoxetine	0.1–5	1	0.012	13.0	20.2
Hydroxyzine	Cetirizine	0.05–5	0.1	0.487	24.9	6.1
Levomepromazine	Norlevomepromazine	0.1–3	0.5	0.143	1.1	7.7
Mianserin	Normianserin	0.1–3	0.5	0.205	7.3	25.2
Mirtazapine	Normirtazapine	0.05–5	0.5	−0.176	14.5	17.8
Olanzapine	Norolanzapine	0.05–5	0.1	0.078	14.9	12.7
Quetiapine	OH-quetiapine	0.05–5	5	−3.214	40.0	8.3
Sertraline	Norsertraline	0.05–5	0.5	−0.176	2.6	12.4
Sildenafil	Norsildenafil	0.1–5	0.5	0.056	21.5	6.8
Tramadol	Nortramadol	0.05–5	1	0.691	3.9	17.1
Tramadol	*O*-desmethyltramadol	0.1–5	1	−1.337	21.2	52.9
Trimipramine	Nortrimipramine	0.05–3	0.5	0.163	18.1	22.9
Venlafaxine	Norvenlafaxine	0.1–3	0.5	0.075	9.4	14.8
Venlafaxine	*O*-desmethylvenlafaxine	0.1–3	0.5	−1.988	30.9	12.5
Verapamil	Norverapamil	0.05–1	0.5	−0.021	19.4	12.3

[a] Based on secondary calibration with parent drug, LOQ being lowest point of linear range.
[b] Difference in metabolite quantification between ordinary one-point calibration using metabolite reference standard (primary calibration) and one-point calibration using parent drug reference standard (secondary calibration).
[c] Detectable only above 1.0 mg/L by DAD.

Table 2
Distribution of parent drug and combined parent–metabolite concentrations in post-mortem case blood samples representing all causes of death[a]

Parent drug	Metabolite	*N*	Percentile of parent drug concentration [mg/L]				Percentile of metabolite concentration [mg/L]			
			Median	90th	95th	97.5th	Median	90th	95th	97.5th
Amitriptyline	Nortriptyline	143	0.4	2.2	2.8	10.5	0.21	1.1	1.6	2.1
Citalopram	Norcitalopram	191	0.4	1	1.4	2	0.1	0.3	3.4	0.6
Mirtazapine	Normirtazapine	160	0.2	0.6	1.3	2.9	0.1	0.3	0.5	0.7
Olanzapine	Norolanzapine	119	0.3	1.1	1.5	2.9	0.1	0.4	0.7	0.8
Tramadol	*O*-desmethyltramadol	108	0.9	6.4	11.5	22	0.1	0.8	1.7	2.4
Venlafaxine	*O*-desmethylvenlafaxine	100	0.5	5.7	14.4	191.1	0.4	1.5	2.7	9.6

[a] Based on re-processed UHPLC-DAD-CAD data using parent drug calibration.

compounds was based on their toxicological relevance, prevalence in casework, and the availability of the reference standard at the authors' laboratory. Blank sheep blood was spiked with the parent drugs and the metabolites and the samples were analyzed by the UHPLC-DAD-CAD method. For the metabolites, four parallel samples at seven different concentration levels ranging from 0.05 to 5.0 mg/L were prepared. For the parent compounds, two parallel calibration samples at the calibration concentration presented in Table 1 were prepared. Primary calibration for the metabolites was performed with the metabolite itself and secondary calibration with the parent drug. One-point calibration was used with both calibration methods. The calibration samples containing the metabolites were quantified using both calibration methods and the results were compared. The linear range and limit of quantification (LOQ) for the metabolites was determined using the secondary calibration method. The precision criterion for the linear range was 15% RSD (20% at the LOQ) from four parallel samples and bias less than 15% (20% at the LOQ) from the theoretical value.

2.3. Drug and metabolite concentrations in post-mortem blood samples

Post-mortem blood samples were femoral venous blood taken at medico-legal autopsies for toxicological analysis, and the samples were stored refrigerated with a preservative (1% of NaF) before analysis. A total of 633 cases were selected that had previously been found positive by UHPLC-DAD-CAD for the 6 drugs and 8 metabolites in Tables 2 and 3. The UHPLC-DAD-CAD data was retrospectively re-processed using the calibration of the parent drug for both the parent and the metabolite. The combined parent drug–metabolite concentrations and metabolite to parent ratios were calculated from these results. If the concentration of either the parent or metabolite was below the LOQ, the case was excluded from the study. The post-mortem cases included in our study represented all causes of death and they were not sorted according to the cause or manner of death or to any other attribute.

2.4. Statistical analysis

All statistical analyses were performed using IBM SPSS version 23. The medians were compared using the non-parametric median test and Kruskal–Wallis *H* test for independent samples.

3. Results

Table 1 shows the relative difference in the quantification of 23 metabolites between the primary calibration method using the metabolite reference standards and the secondary calibration method using the corresponding parent drug reference standards. The mean difference between the two calibration methods by CAD and DAD was 15% and 17%, respectively. In some cases, the bias of the two calibrations was divergent, and consequently the difference became greater than the criterion for the linear range. For *N*-dealkylated metabolites the calibration methods gave very similar results, the mean difference by CAD and DAD being 12% and 15%, respectively. By CAD, a difference in the retention time between the parent drug and metabolite was associated with poorer accuracy. By DAD, marked differences were observed with the *O*-demethylated metabolites. This finding was consistent with our preliminary tests with standard solutions, where the response difference between *O*-demethylated metabolites and the corresponding parent drugs on DAD was on average 35% due to different UV spectra.

Table 2 shows the distribution of the parent drug and metabolite concentrations in case blood samples following re-processing of the UHPLC-DAD-CAD data from 633 post-mortem cases by the calibration method utilizing the parent drug for both the parent and the metabolite. The concentrations are expressed as

Table 3
Distribution of median metabolite to parent drug ratios for three concentration groups in post-mortem case blood samples representing all causes of death[a]

Parent	Metabolite	Median ratio of metabolite to parent drug[b]			*p*-Value	Reference metabolite to parent drug ratio in TDM [2]	Reference median metabolite to parent drug ratio in post-mortem cases [3]	
		Q_1	$Q_3 - Q_1$	$Q_4 - Q_3$			Control cases	Single drug poisoning cases
Amitriptyline	Nortriptyline	1.27	0.46	0.43	0.000	0.2–1.8	0.7	0.3
Citalopram	Norcitalopram	0.46	0.30	0.26	0.002	0.31–0.6	0.33	0.06
Mirtazapine	Normirtazapine	0.54	0.44	0.34	0.001	0.2–1.2	0.7	0.3
Olanzapine	Norolanzapine	0.49	0.41	0.32	0.009	0.1–0.3 (non-smokers), 0.2–0.4 (smokers)	na	na
Tramadol	Nortramadol	0.23	0.11	0.08	0.010	na	na	na
Tramadol	*O*-desmethyltramadol	0.27	0.15	0.10	0.001	na	na	na
Venlafaxine	Norvenlafaxine	0.12	0.12	0.14	0.751	0.46–1.48	na	na
Venlafaxine	*O*-desmethylvenlafaxine	2.00	1.14	0.17	0.001	0.3–5.2 (EM or IM CYP2D6)[c]	1.0	0.1

[a] Based on re-processed UHPLC-DAD-CAD data using parent drug calibration.
[b] Quartiles (*Q*) based on combined parent–metabolite concentration.
[c] Extensive (EM) or intermediate (IM) metabolizer related to cytochrome P450 2D6 (CYP2D6) enzyme.
na: not available.

medians and upper percentiles (90th, 95th, and 97.5th). The median combined parent drug–metabolite concentration was on average 46% (range 29–80%) higher than the median parent drug concentration, the difference being statistically significant ($p < 0.05$).

Table 3 shows the median metabolite to parent drug ratios across three concentration groups according to the combined parent–metabolite concentration. The differences of the medians between the three groups were statistically significant ($p < 0.05$) for all compounds, except for norvenlafaxine, for which the number of positive cases was small. Table 3 also shows literature data for the normal ranges in TDM [2], for fatal single drug poisoning cases, and for the post-mortem control cases in which incapacitation by drugs was excluded [3]. Several other drugs not listed in the tables were found but not included in the study.

4. Discussion

The UHPLC-DAD-CAD method using the secondary calibration proved feasible for the concurrent quantification of *N*-dealkylated and, selectively, other drug metabolites without applying the corresponding reference standards. The differences of quantification shown in Table 1 generally fell within the typical range of expanded uncertainty (30%) encountered in post-mortem toxicology. This outcome relies on the fact that between the studied parent drugs and their metabolites the extraction recoveries and detector responses on both detectors were sufficiently similar. Interestingly, the generic calibration approach using non-MS detection has not been previously highlighted in post-mortem toxicology.

As for LC-MS techniques, several attempts to manage the variable response have been presented in recent years. Correcting the LC-ESI-MS response with calibration factors obtained from LC-NSI-MS or CLND has provided promising results. When drug metabolites were quantified using the parent for calibration, the results were within 20% of those achieved by ordinary calibration [5,7]. Using correction based on UV improved the quantitative results compared to LC-ESI-MS, but e.g. for buspirone metabolites a three-fold difference was obtained compared to ordinary calibration [22]. Even though LC-CSI-MS provided equimolar responses for buspirone and its metabolites [10], for several other drugs and their various metabolites the standard curves were statistically different [11]. Furthermore, no trend was seen in the response differences with respect to different metabolic pathways.

Even with universal detectors the issue of different extraction recoveries still remains [7]. In a study using CLND, recovery correction with secondary reference standards enabled quantification of basic drugs in plasma and blood [23]. In the present study, the extraction recoveries of parent drugs and their dealkylated metabolites did not differ significantly, which allowed the use of the secondary calibration.

There are limitations concerning the present method. Identifying a metabolite cannot rely solely on the UV spectrum but should be confirmed by a MS-based method. Only a metabolite that shares a chromophore with the parent drug possesses a similar UV spectrum to the parent, provided that the transformation does not take place in the close vicinity of the chromophore [13]. As the extraction recovery of the metabolite cannot be exactly determined, merely an estimation of the metabolite concentration can be given. For example the present type of *O*-demethylated metabolites possess, according to the predicted values of pK_a and log *D* calculated with ACD/Labs software (Toronto, Canada), comparable lipophilicity at pH 9, which is the extraction pH of our method. Consequently, at the present conditions these aminophenols are likely lipophilic enough to be extracted with similar recovery to the parent drugs. All toxicology case results obtained using the secondary calibration should be reported with added information on the calibration method regardless of the uncertainty of measurement.

In TDM, such parent drug–metabolite pairs for which the combined concentration is recommended include e.g. amitriptyline–nortriptyline, clomipramine–norclomipramine, doxepin–nordoxepin, fluoxetine–norfluoxetine, imipramine–desipramine, risperidone–9-hydroxyrisperidone, and venlafaxine–*O*-desmethylvenlafaxine [1]. At the same time, use of therapeutic ranges for the combined concentration has been criticized, as the metabolites may exhibit different distribution in the body and different affinities on target proteins [24]. Still metabolite to parent ratios outside the normal ranges are considered as a signal pointing to non-adherence to treatment or altered metabolism caused by drug–drug interactions, genetic polymorphism in the cytochrome P450 (CYP) enzymes, or altered liver function [2]. Especially with amitriptyline [25], doxepin [26], risperidone [25,27], tramadol [28], and venlafaxine [25,27,29] the metabolite to parent ratios are significantly affected by the poor metaboliser genotype.

In post-mortem toxicology, attention has traditionally been paid to the parent drug concentration. The concentrations defined for TDM cannot be directly applied to post-mortem toxicology, as post-mortem redistribution (PMR) affects the concentrations of many drugs in post-mortem blood. Extensive tables of median and upper percentile concentrations of parent drugs in post-mortem blood samples have been published earlier [30,31], but related data for metabolites is scarce. The metabolite to parent ratios (Table 3) generally fell within the normal ranges defined in TDM [2], even when the concentration exceeded the therapeutic range. The metabolite to parent ratios measured in our study were rationally comparable to those reported previously for fatal poisoning cases and post-mortem controls [3]. The metabolite to parent ratios can be especially important in post-mortem toxicology with drugs that undergo significant post-mortem redistribution. For example with amitriptyline, the nortriptyline to amitriptyline ratios in our study were within the normal range even with fairly high amitriptyline concentrations, whereas the *O*-desmethylvenlafaxine to venlafaxine ratios rapidly fell below the normal range with moderate venlafaxine concentrations. One possible explanation is the difference in PMR behavior between amitriptyline and venlafaxine. Amitriptyline has been reported to exhibit considerable PMR [32], whereas venlafaxine has been found to be less prone to PMR with therapeutic concentrations in post-mortem samples close to those defined in TDM [33,34]. It has been previously shown, that in acute venlafaxine poisoning cases the *O*-desmethylvenlafaxine to venlafaxine ratio is significantly lower, approximately 0.1, than in other post-mortem cases where the ratio is between 1 and 4 [3,34]. Hence, slightly elevated venlafaxine concentrations along with significant concentrations of *O*-desmethylvenlafaxine are more likely to indicate therapeutic use of venlafaxine than overdose [34]. A low metabolite to parent ratio can, however, indicate either acute or toxic ingestion, so the results should be interpreted with caution. A limitation of our study was the lack of associated information about the cause of death, and consequently the post-mortem concentrations reported here represent all causes of death, including approximately 10–20% of fatal poisonings due to the drugs studied [31].

5. Conclusions

We have presented a practical approach for the analysis of drug metabolites without their reference standards by using a non-MS approach. Identification is based on retention time, UV spectrum and the response ratio from the two detectors, DAD and CAD. Quantification relies on one-point historic calibration for the parent drug and its metabolites concurrently, with use of the

parent drug reference standard only. For *N*-demethylated metabolites, the quantitative results are very similar to those obtained with ordinary one-point calibration using the reference standards. With *O*-demethylated metabolites, the biases in the quantitative results are larger due to differences in UV spectra and retention times. The present method shows no ionization related matrix effects and requires less workload and associated costs than the heavily reference standard dependent MS methods, while MS by nature is enhanced in terms of sensitivity and specificity. In post-mortem toxicology, utilization of combined parent drug–metabolite concentrations and metabolite to parent ratios is currently not commonplace due to lacking reference data. We anticipate that the readily accumulating metabolite data by the present method will, when connected to cause-of-death information, provide a more useful reference for interpretive post-mortem toxicology than the parent drug concentrations alone.

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