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Single-calibrant quantification of drugs in plasma and whole blood by liquid chromatography–chemiluminescence nitrogen detection

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Abstract

Poor availability of drug reference standards may severely complicate clinical and forensic toxicology investigations. To overcome this problem, a new approach is introduced for drug analysis without primary reference standards. Liquid chromatography–chemiluminescence nitrogen detection (LC–CLND) was employed as the analytical technique, based on the detector's equimolar response to nitrogen and using caffeine as single secondary standard. Liquid–liquid extraction recoveries for 33 basic lipophilic drugs were first established by LC–CLND in blood specimens spiked with the respective reference substances. The mean recovery by butyl chloride–isopropyl alcohol extraction for plasma and whole blood was 90 ± 18 and 84 ± 20%, respectively. The validity of the generic extraction recovery-corrected single-calibrant LC–CLND was then verified with proficiency test samples, including 20 different analyses. The mean accuracy was 24 and 17% for the plasma and the whole blood samples, respectively, and the maximum error was 31% for both specimens. All 20 analyses results by LC–CLND fell within the confidence range of the reference concentrations. LC–CLND proved to be an easy-to-use and robust technique, allowing analysis of 1000 injections of biological extracts without a need for major maintenance operations.
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1. Introduction

Quantitative analysis in clinical and forensic toxicology is fundamentally dependent on the availability of reference standards for drugs and poisons. Drug action and the degree of intoxication are normally dependent on the concentration of the active substance in plasma or whole blood, and consequently, many compilations of therapeutic, toxic and fatal drug concentrations in these specimens have been published. Unfortunately, several factors limit the availability of reference substances, including administrative requirements set by authorities and pharmaceutical companies, the high expense of purchase and delayed delivery; sometimes the compounds required are not available at all. A well-equipped forensic toxicology laboratory is expected to possess approximately 1000 reference substances with continuous updating of the assortment. Management of the standards archive is one of the most important maintenance operations of this type of laboratory.

The availability problems can at least partly be resolved by using appropriate instrument technology. Liquid chromatographic (LC) detectors capable of producing a more consistent response than the UV detector include the evaporative light scattering detector (ELSD) [1] and the corona charged aerosol detector (CAD) [2]. Chemiluminescence nitrogen detection (CLND) represents a unique approach for quantification of nitrogenous substances without primary reference standards because the detector possesses an equimolar response to nitrogen. This is particularly valuable in human toxicology, since 90% of drugs contain nitrogen [3]. An advantage of LC–CLND compared with other techniques is that calibration with a single nitrogen-containing secondary standard is sufficient, and the standard need not be chemically similar to the analytes. Equimolar response follows from the principle of LC–CLND: in the first step, the LC mobile phase is evaporated and the analytes are quantitatively pyrolysed at a high temperature in the presence of oxygen to yield carbon dioxide, nitric oxide and water [4]. In the second step, after the removal of water, the resulting nitric oxide is oxidized with ozone to excited nitrogen dioxide. During transition to the ground state, a photon is emitted, which is multiplied in a photon multiplier.

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Single-calibrant quantification by LC–CLND is straightforward in cases of relatively simple materials requiring no extraction, such as combinatorial chemistry library products [5], nitrogen-containing anions in seawater [6] or seized street drugs [7]. However, there are currently very few LC–CLND applications for biological samples, and in these experiments, protein precipitation followed by direct LC injection has been the sample work-up method of choice [8,9]. A benefit from an extraction step is a cleaner chromatographic background, but quantification without primary reference standards presumes known extraction recoveries. Many factors affect solid-phase extraction (SPE) recoveries, and, in our preliminary experiments, repeatability of the tested procedures in blood was not satisfactory. Liquid–liquid extraction (LLE) involves fewer variables than SPE, and therefore it is easier to master in search for constant recoveries.

In this study, single-calibrant LC–CLND was applied for the first time to drug bioanalysis with use of LLE and correction by a mean recovery factor. The mean extraction recovery for basic lipophilic drugs in plasma and whole blood was assessed by determining LLE recoveries individually for over 30 drugs at two concentrations using butyl chloride–isopropanol. Further, plasma and whole blood drug proficiency test samples were analysed by the single-calibrant LC–CLND method, and the results based on the mean extraction recoveries were compared with the respective reference values.

2. Materials and methods

2.1. Chemicals and reagents

n-Butyl chloride and isopropyl alcohol were analytical grade from Fluka (Buchs, Switzerland) and sodium hydroxide from J.T. Baker (Deventer, The Netherlands). Methanol was HPLC grade from Rathburn (Walkerburn, UK). All other solvents and reagents were analytical grade from Merck (Darmstadt, Germany). Water was Direct-Q3-purified (Millipore, Bedford, MA, USA). Caffeine was from Sigma–Aldrich (Steinheim, Germany). The reference standards of drugs were obtained from various pharmaceutical companies.

2.2. Blood, plasma and serum samples

Pooled blank human plasma was obtained from the Finnish Red Cross Blood Service and blank whole blood was bovine blood. Certified reference serum samples for drugs were purchased from LGC Promochem (Teddington, UK), and reference blood samples for drugs were from the Nordquant proficiency test program (Oslo, Norway), involving 13 participants.

2.3. Apparatus

LC–CLND analysis was performed with an Agilent (Waldbronn, Germany) Hewlett-Packard 1090 series liquid chromatograph equipped with an autosampler, three-channel gradient pumping system, column oven and UV diode array detector (DAD). The nitrogen-specific detector was an Antek (Houston, TX, USA) 8060 CLND, coupled online after the DAD. The detector was interfaced with the computer using an HP (Agilent) analogue to a digital converter.

2.4. Sample preparation for LC–CLND

To a 5 mL plasma sample, 0.5 g of sodium chloride and 2 mL of 1 M tris(hydroxymethyl)amino methane (TRIS) buffer (pH 11) was added. pH was adjusted to 11 by 5 M sodium hydroxide. After addition of 10 mL of *n*-butyl chloride–isopropanol (98 + 2), the sample was shaken for 30 min in a rotor shaker. Following phase separation by centrifugation (10 min at 4000 rpm), 7.5 mL of the organic layer was transferred into a conical test tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in the same tube with 100 μL of methanol–0.1% formic acid (1 + 1), and after vortexing and ultrasonication (15 min), the samples were centrifuged for 5 min. The supernatant was transferred to an Eppendorf tube and, after centrifuging again for 5 min at 12,000 rpm, the clear supernatant was transferred into an autosampler vial for LC–CLND analysis.

2.5. LC–CLND analysis

LC separation was performed in gradient mode at 40 °C using a Phenomenex (Torrance, CA, USA) Gemini C-18(2) 150 mm × 2 mm (3 μm) column, equipped with a 4 mm × 2 mm pre-column. Mobile-phase components were 0.1% formic acid and methanol, and the flow rate was 0.20 mL/min. The proportion of methanol was increased from 5 to 30% in 7 min, to 60% in 12 min, to 90% in 15.5 min, and held at 90% for 5.5 min. Post-time was 7 min and injection volume was 25 μL. The diode array detector signal was recorded at 230 nm, and peak controlled spectra were recorded at 210–400 nm.

For the CLND analysis, oxygen flow was 250 mL/min, helium 50 mL/min and make-up helium 50 mL/min. Ozone flow was 25 mL/min and furnace temperature 1050 °C. The photo multiplier tube voltage was set at 750 V, and the amplification factor was 25.

LC–CLND data were processed using HP Chem Station A.06.01 software (Agilent). External calibration was performed at the beginning of the data acquisition sequence with caffeine standards. A stock solution of 1.0 mg/mL caffeine in methanol, corresponding to 7225 ng of nitrogen per injection, was diluted to obtain a solution containing 30 ng of nitrogen per injection in methanol–0.1% formic acid (1 + 1). Calibration points at 0.75, 1.0, 1.5, 3.0, 10 and 30 ng of nitrogen per injection were used to quantify the proficiency test samples (Tables 2 and 3). The curve fit was found to be linear with $R^2 > 0.997$ up to 300 ng of nitrogen per injection.

2.6. Extraction recovery measurement

Extraction recoveries for the drugs were determined in four parallel LC–CLND analyses from plasma and whole blood, spiked at concentrations of 0.2 and 1.0 mg/L. Methanolic standard solutions of these drugs were evaporated to dryness at

40 °C, the residue was reconstituted with blank plasma or whole blood and the sample preparation was carried out as described above. For a reference sample representing 100% recovery, the extraction procedure was as detailed above, but the drug standard solutions were added post-extraction to the organic phase.

3. Results and discussion

Table 1 shows the extraction recovery for 33 basic lipophilic drugs in plasma and whole blood at two concentration levels. This selection includes representatives from toxicologically relevant drug categories, such as antidepressants, antipsychotics, cardiovascular drugs, antihistamines and opioids. The blood concentrations of the selected compounds are typical for basic drugs; particularly low-dose drugs were not included. In this study, a drug was considered basic and lipophilic when the compound's calculated log *D* value (1 − octanol/water) at pH 11 was greater than 1.5 and it possessed an aliphatic amino group. Amphoteric drugs were not included in the study, except for pentazocine, which contains a phenolic hydroxyl group but is still sufficiently lipophilic to be extracted outside of the optimal pH. The recoveries were determined by LC–CLND in four parallel experiments in samples spiked with the reference substances in a conventional manner. The mean recoveries in plasma at concentrations of 0.2 and 1.0 mg/L were 88 ± 16 and 92 ± 16%, respectively, and in whole blood 80 ± 17 and 87 ± 16%, respectively. The grand mean of all extraction recoveries in plasma and whole blood samples was 90 ± 18 and 84 ± 20%, respectively. The mean repeatability of four parallel extractions was below 15%. The theoretical nitrogen amount per injection, assuming 100% recovery, ranged from 6 ng (aripiprazol 0.2 mg/L) to 161 ng (clozapine 1.0 mg/L).

Table 1
Extraction recoveries for basic lipophilic drugs in plasma and whole blood at two concentrations

Compound	log *D*[a]	Extraction recovery (%)							
		Plasma				Whole blood			
		0.2 mg/L	R.S.D.[b] (%)	1.0 mg/L	R.S.D.[b] (%)	0.2 mg/L	R.S.D.[b] (%)	1.0 mg/L	R.S.D.[b] (%)
Amitriptyline	4.91	78	3	79	8	84	5	66	9
Aripiprazol	5.59	87	4	100	3	68	6	61	14
Bisoprolol	2.13	–[c]	–[c]	–[c]	–[c]	89	11	95	2
Chlorpromazine	5.19	51	12	107	2	103	5	98	2
Citalopram	2.50	102	7	87	11	97	4	85	4
Clomipramine	5.51	96	2	95	3	62	4	94	7
Clozapine	3.48	96	4	80	5	85	4	72	10
Desipramine	4.04	98	6	95	5	75	10	81	8
Dibenzepin	1.76	57	5	99	6	70	3	82	10
Diphenhydramine	3.66	98	3	97	2	68	4	98	1
Fluoxetine	4.04	64	9	118	5	71	2	86	14
Fluvoxamine	3.10	108	3	58	10	116	11	111	6
Imipramine	4.79	90	2	116	1	82	4	99	10
Levomepromazine	4.93	89	3	96	3	42	10	38	14
Methadone	4.19	99	2	98	3	88	9	86	11
Metoprolol	1.78	86	14	69	8	88	9	98	2
Mianserine	3.67	68	1	85	9	72	11	61	10
Mirtazapine	2.75	–[c]	–[c]	–[c]	–[c]	68	8	72	9
Norcitalopram	3.13	92	6	98	2	105	5	112	3
Norclomipramine	4.77	97	5	96	5	74	10	88	9
Normethadone	2.76	88	2	101	2	97	2	95	7
Nortramadol	1.88	98	2	91	3	99	3	102	1
Nortrimipramine	4.38	99	5	102	4	72	9	99	3
Nortriptyline	5.61	109	5	82	5	89	4	92	7
Pentazocine	3.58	–[c]	–[c]	–[c]	–[c]	76	4	68	9
Promazine	4.62	66	5	72	7	47	5	78	7
Propranolol	3.09	96	4	93	6	98	9	98	8
Quetiapine	1.57	96	2	97	4	70	12	104	4
Thioridazine	6.11	57	9	47	11	63	6	84	11
Tramadol	2.49	101	5	94	8	93	9	109	2
Trimipramine	5.14	85	3	121	5	68	4	73	15
Venlafaxine	2.90	–[c]	–[c]	–[c]	–[c]	95	5	94	1
Verapamil	3.89	110	3	99	3	65	5	93	4
Mean		88	5	92	5	80	6	87	7
Median		94	4	96	5	76	5	92	7

[a] Calculated values of the partition coefficient for dissociative mixtures (log *D*) at pH 11 using Advanced Chemistry Development ACD/Labs software Version 8.15 (Toronto, Ontario, Canada).
[b] Based on four parallel extractions.
[c] Co-elutes with caffeine present in plasma.

Table 2
Comparison of single-calibrant LC–CLND analysis results with certified values for plasma drug proficiency test samples[a]

Sample	Substance	LC–CLND		Reference		Accuracy[d] (%)
		Mean (μg/L)	R.S.D.[b] (%)	Certified value (μg/L)	Confidence range[c] (μg/L)	
Medidrug TCA 1/05-D S-plus	Desipramine	181	33	205	121–288	−12
	Imipramine	158	28	202	120–284	−22
Medidrug TCA 1/06-B S-plus	Amitriptyline	244	13	354	222–487	−31
	Nortriptyline	156	18	214	127–300	−27
	Doxepine	269	16	369	232–506	−27
	Nordoxepine	358	17	307	190–424	17
Medidrug TCA 1/06-D S-plus	Desipramine	325	13	413	262–564	−21
	Imipramine	249	15	358	224–492	−31

[a] TCA External Proficiency Testing of GTFCH (Association of Toxicological and Forensic Chemistry) by Medichem Diagnostica und Verfahresentwicklung, Steinenbronn, Germany.
[b] Relative standard deviation based on four parallel extractions.
[c] Equal to two times the standard deviation of the certified reference value.
[d] Difference between the LC–CLND analysis result and the certified value.

The validity of the approach involving LC–CLND analysis without primary reference standards based on the mean extraction recovery values (90% for plasma and 84% for whole blood) was verified with proficiency test samples from two different sources (Tables 2 and 3). The mean accuracy, calculated from the absolute error values, was 24 and 17% for 8 plasma and 12 whole blood analyses, respectively, and the maximum error was 31% for both specimens. The mean repeatability of four parallel extractions of plasma and whole blood samples was 19 and 17%, respectively. All 20 analyses results by LC–CLND fell within the confidence range of the reference concentrations obtained from the plasma and blood proficiency test samples (Tables 2 and 3). Evidently, the mean accuracy of this LC–CLND approach is within the uncertainty of measurement of many customary analytical toxicology methods, for which the uncertainty may approach 30% even with calibration with primary reference standards.

LC–CLND chromatograms showed a relatively clean background following the butyl chloride–isopropyl alcohol extraction (Fig. 1). A more consistent response was obtained by CLND than by DAD for the nitrogen-containing drugs, as expected. The chromatographic separation method was developed for basic lipophilic drugs in general, without optimizing the resolution of any particular set of compounds. The organic modifier in the gradient elution was methanol instead of acetonitrile, because the LC mobile phase must be free of nitrogen to suit LC–CLND. Emulsion formation during the extraction procedure was observed with individual cases, especially with plasma samples. This phenomenon was found to significantly affect extraction recoveries, particularly for the most lipophilic, late-eluting compounds. In cases where visible emulsion was observed, sample preparation was repeated. Overall, this study was limited to compounds for which no co-eluting interferences from the matrix were detected in the standard procedure.

Table 3
Comparison of single-calibrant LC–CLND analysis results with certified values for whole blood drug proficiency test samples[a]

Sample	Substance	LC–CLND		Reference		Accuracy[d] (%)
		Mean (μg/L)	R.S.D.[b] (%)	Certified value (μg/L)	Confidence range[c] (μg/L)	
Nordquant 1/2005	Amitriptyline	113	17	160	54–266	−30
	Citalopram	474	15	420	336–504	13
	Methadone	424	18	340	238–442	25
	Dextropropoxyphene	506	2	500	250–750	1
Nordquant 2/2005	Amitriptyline	183	23	190	133–247	−4
	Citalopram	230	6	190	84–296	21
	Methadone	305	15	270	178–362	13
	Dextropropoxyphene	316	9	320	90–550	−1
Nordquant 1/2006	Amitriptyline	153	16	220	110–330	−31
	Citalopram	316	15	300	114–486	5
	Methadone	394	47	330	172–488	19
	Dextropropoxyphene	332	25	330	244–416	1

[a] Nordquant proficiency testing scheme, Norwegian Institute of Public Health, Division of Forensic Toxicology and Drug Abuse, Oslo, Norway.
[b] Relative standard deviation based on four parallel extractions.
[c] Equal to two times the standard deviation of the certified reference value.
[d] Difference between the LC–CLND analysis result and the certified value.

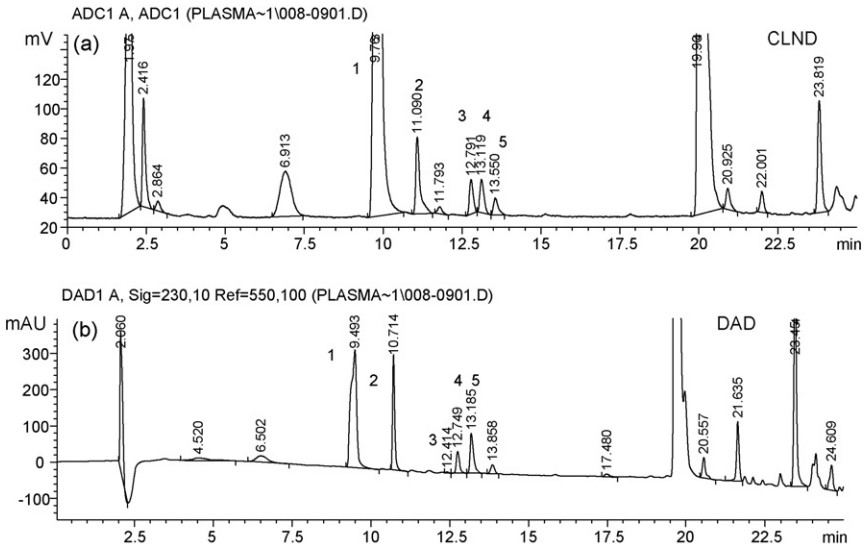

Fig. 1. LC–CLND (a) and LC–DAD (b) chromatograms of an extracted proficiency test blood sample (Nordquant 1/2006) showing caffeine (1), citalopram (2), dextropropoxyphene (3), methadone (4) and amitriptyline (5). Dextropropoxyphene gives a weak signal in DAD.

Clomipramine and norclomipramine, for example, were not quantified by the present method in the plasma proficiency test samples due to co-eluting interferences in this particular matrix.

The LC–CLND technique has rarely been used in bioanalytical studies. This is because an extraction procedure giving a high and constant extraction recovery for a wide range of compound structures is required to utilize the detector's equimolar response to nitrogen. In earlier published methods, protein precipitation has mainly been used in sample preparation [8–10]. A separate SPE method has been reported for the determination of imidacloprid in fruits and vegetables with a relatively constant extraction recovery, but the study utilized CLND solely as a nitrogen-specific detector [11]. Our study is the first applying LLE systematically to sample work-up following single-calibrant LC–CLND analysis. The choice of butyl chloride as an extraction solvent was based on its proven applicability to basic lipophilic drugs in analytical toxicology. Recognized already in the 1970s [12], butyl chloride has been shown to provide clean chromatograms and efficient extraction, [13] with a list of recoveries reported for over 200 toxicologically relevant substances from an aqueous buffer at pH 9 [14]. In our study, isopropyl alcohol was added to the organic phase to improve the extraction of the more polar drugs. To avoid emulsion formation, a rotary shaker was used instead of a vortex mixer, but even then occasional emulsion problems were not avoided. Obviously, supported liquid extraction technology is a further means of diminishing the interference from incomplete phase separation.

LC–CLND has been extensively used in the field of drug discovery, in quality assessment of combinatorial libraries [5,15,16] and in solubility determinations [17]. In forensic chemistry, our group was able to directly quantify the active components of seized street drug samples at an accuracy better than 11% without using primary reference standards [7]. In bioanalysis, sample preparation still presents a challenge because the recovery must be established. Chromatography plays a key role in obtaining reliable results and should be optimized for special applications, e.g., separating parent drugs from their normetabolites. DAD prior to CLND tended to broaden chromatographic peaks, and consequently, bypassing the UV cell resulted in more narrow peaks. We found LC–CLND to be an easy-to-use and robust technique, and after almost 1000 injections of biological extracts, the instrument continued to function without the need for major maintenance operations. LC–CLND appears to be a promising tool for major clinical and forensic laboratories aiming at widening their repertoire of quantitative analysis. However, to attain sufficient sensitivity for basic drugs, a 5 mL sample volume is generally required.

4. Conclusions

The current approach involving the establishment of the mean extraction recovery followed by single-calibrant LC–CLND analysis proved to be feasible for the analysis of basic lipophilic drugs in plasma and whole blood samples in a toxicological context. The results obtained by LC–CLND without primary reference standards deviated on average 20% from the certified reference values of proficiency test samples. While high-throughput LC–CLND analysis of biological extracts was found to be relatively trouble-free, further attention should be paid to developing generic extraction methods with predictable and steady recovery.

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