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Quantitative urine spot microsamples for the chiral analysis of clenbuterol by capillary electrokinetic chromatography

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ABSTRACT

The application of quantitative volumetric dried urine spot (qDUS) sampling and analysis to the enantioselective determination of the β_2-adrenergic agonist clenbuterol is described herein for the first time. The chiral determination is obtained by capillary electrokinetic chromatography (CEKC) coupled to tandem mass spectrometry (MS/MS), using carboxymethyl-β-cyclodextrin as the chiral selector and sample stacking by electrokinetic injection. Innovative qDUS was carried out by means of Capitainer®B 50 devices, which were confirmed to be able to produce volumetrically accurate 50-μL urine spots and applying an accelerated drying protocol by dehumidified air. After solvent extraction, the samples were analysed by the CEKC-MS/MS method. The analytical workflow was validated according to current international guidelines, with good results in terms of linearity (r^2 = 0.9994 for both enantiomers), extraction yields (≥ 87 %), intra- and inter-day precision RSD (≤ 11.8 %) and matrix effect (≤ 11 %). Sample stability was high (≥ 97 %) even though qDUS were stored at room temperature for 90 days. Application to the analysis of real qDUS samples from patients undergoing therapy with racemic clenbuterol was successful, with negligible detected enantiomeric excess, as expected. The developed analytical platform based on qDUS coupled to CEKC-MS/MS is thus suitable for application to enantioselective determination of clenbuterol in different settings, including toxicological, forensic and therapeutic drug monitoring ones.

1. Introduction

Clenbuterol (1-(4-amino-3,5-dichlorophenyl)-2-[(2-methyl-2-propanyl)amino]ethanol, CBT, Fig. 1) is a widely used bronchodilator, which over the years since its introduction has proven to possess potentially useful biological activities above and beyond the approved one. In particular, it has a considerable anabolic effect on both animals and humans, although this is most often exploited for illicit uses (e.g., accelerated growth of farm animals [1], sports doping [2]). Moreover, it has shown considerable neuroprotective and neuronal growth promoting effects [3], thus being currently considered as a prospective possible therapeutic agent against neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) [4]. This latter activity seems to be mediated by β_2-adrenergic receptor agonism, while the anabolic mechanism is still debated [5].

The anabolic use of CBT is prohibited by most major food safety agencies, including the European Food Safety Agency (EFSA) [6] and the U.S. Food and Drug Administration (FDA) [7]. Of course, the World Anti-Doping Agency's (WADA) Prohibited List includes CBT as well, within group S1 (anabolic agents), sub-group S1.2 (other, not androgenic steroid agents) [8], which includes compounds forbidden in sports "at all times" (i.e., both in- and out-of-competition).

Abbreviations: AAF, adverse analytical finding; ALS, amyotrophic lateral sclerosis; ATF, atypical finding; CSP, chiral stationary phase; EFSA, European Food Safety Agency; MRL, minimum reporting level; MRPL, minimum required performance level; qDMS, quantitative dried matrix spot; qDUS, quantitative dried urine spot; TDM, therapeutic drug monitoring; FDA, U.S. Food and Drug Administration; WADA, World Anti-Doping Agency..

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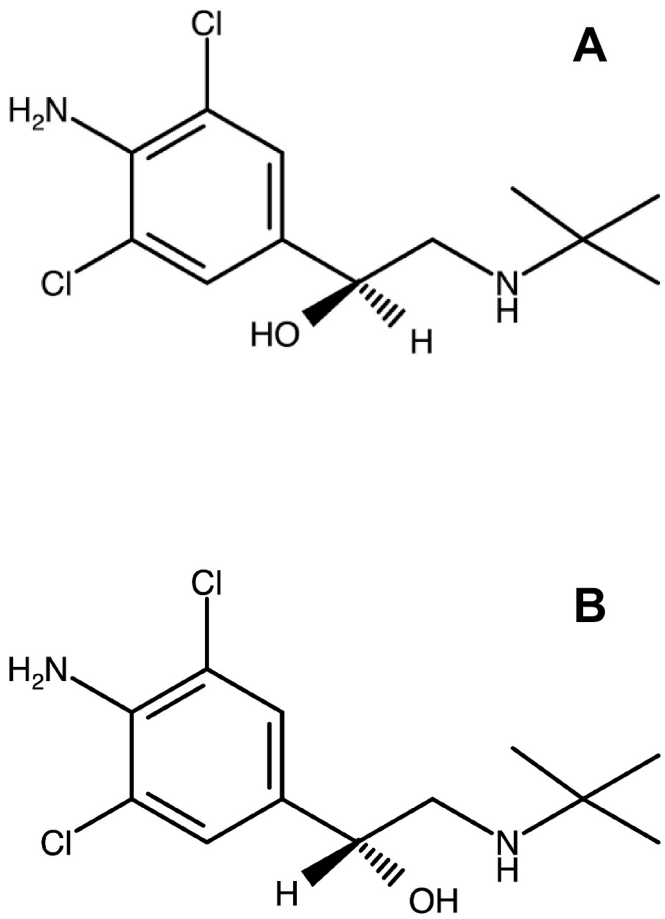

Fig. 1. Chemical structures of clenbuterol enantiomers: A. *R*-(–)-clenbuterol; B. *S*-(+)-clenbuterol.

Professional and amateur athletes alike are known to have received adverse analytical findings (AAF, i.e., positive results to an anti-doping test) from WADA laboratories for involuntarily consuming meat (or other food) contaminated with CBT [9]. This is a well-known issue, to the point that WADA has acknowledged that "disciplinary proceedings against athletes with low level urinary [CBT] concentrations, from countries known for significant risk of exposure [due to food contamination], would have little to no prospect of success and would be very unfair to the athletes concerned" in an official communication [10].

CBT is a chiral molecule, but it has been approved and is commonly sold and administered as a racemic mixture. CBT pharmacokinetics (PK) are species-specific and in particular, human PK are not enantioselective; thus, circulating and eliminated drug does not possess any significant enantiomeric excess (ee) when administered as a racemate [11]. Conversely, animal PK can be enantioselective; e.g., swine is able to interconvert CBT enantiomers; CBT administered as a racemate is found in swine meat (and other tissues) as having significant ee [12]. Given the above information, this ee is clearly not expected to change in biological fluids of humans eating contaminated food. The ee from animal food contamination is highly variable and seems not to have any evident correlation with anatomical, physiological or rearing conditions of the animals [13]. Thus, enantioselective analysis of CBT in biological samples is now considered an effective means of proving consumption of CBT-contaminated food, for either anti-doping testing, forensic, or toxicological purposes [14–16]. In perspective, it could also be a valuable tool in the investigation of modern neuroprotective and neuroregenerative applications, where the administration of pure enantiomers could be envisioned. Indeed, it appears that the *R*(−)-CBT enantiomer is responsible for most of the observed effects related to β_2 and at β_1 receptor agonism, including neuroprotection; the *S*-(+)-CBT enantiomer seems to only possess low adrenergic activity [17]. Given this activity difference, enantioselective therapeutic drug monitoring (TDM) of CBT could provide useful information, also relative to possible intake from contaminated food that could cause unwanted effects or toxicity in patients.

Obviously, many methods can be found for the enantioselective determination of CBT by either electrodriven [11,18–23] or chromatographic [9,13,16,24–34] methods. However, just some of them have been applied to biological matrices: in bovine urine [24] or tissues [13], swine urine [25] or in different kind of meats [26] by LC-MS, in human urine for anti-doping testing by GC–MS [30], LC-MS/MS [9,16,31,32] or UHPLC-MS/MS [33]. A capillary electrokinetic chromatography (CEKC) method coupled to UV detection has been developed for this purpose, which uses relatively large volumes of urine (2 mL per sample) [11]. Until now, just one paper has been published on the use of dried microsamples for the determination of CBT enantiomers in biological matrices, in this case by LC-MS/MS [34]. Microsampling is currently considered a highly viable process that can provide several advantages over traditional urine or blood/plasma sampling. For example, analyte stability is usually greatly increased by water loss, increasing drug detection times and reliability [35–38] and making storage and shipping at room temperature possible, thus significantly reducing analysis costs [39–42]. Even WADA has recently approved microsampling, in the form of dried blood spot (DBS), for anti-doping testing [43]. The previously published urine microsampling method [34] envisioned the use of volumetric absorption microsampling (VAMS) to obtain volumetrically accurate urine specimens that could then undergo miniaturised sample preparation by Stop-And-Go Extraction (StAGE) tips, followed by enantioselective LC-MS/MS analysis on a polysaccharide-based chiral stationary phase (CSP).

In the present study, alternative approaches are proposed for both microsampling and sample preparation, with application to dried urine spotting (DUS). In general, the use of dried matrix spots (DMS) is much more widespread and well-known by both medical and analytical personnel than more recent techniques such as VAMS [44]. Thus, DMS application does not require significant modifications to established, standardized, and accredited microsampling protocols, making it more readily accepted by personnel [45]. The main disadvantage of traditional DMS approaches is their lack of volumetric accuracy and thus the difficulty in obtaining reliable fully quantitative results [46]. One possible approach to solving this problem is represented by the recent development of modern, quantitative (i.e., volumetrically accurate) DMS (qDMS) platforms, mostly relying on microfluidic approaches to deliver an accurate, repeatable amount of biological fluid onto an absorbing substrate to produce DMS [47]. In this study, Capitainer®B 50 devices were used for this purpose and applied for the first time to the analysis of CBT in DUS. In addition to the innovative, alternative microsampling workflow, the present work also proposes a possible alternative approach to chiral LC, namely CEKC coupled to tandem MS detection, due to the outstanding separation efficiency and cheapness of the technique in enantioselective applications, in addition to its capacity of consuming minute (nanolitre-level) amounts of extracted sample, leaving ample possibility for further investigations and determinations [48,49].

2. Methods

2.1. Chemicals and standard solutions

Racemic CBT hydrochloride (≥ 95% purity), racemic CBT-D_9 (internal standard, IS) solution (100 μg/mL), *R*-(−)-CBT and *S*-(+)-CBT pure enantiomers (ee > 90% each), acetonitrile, methanol, formic acid (FA), sodium carbonate, carboxymethyl-β-cyclodextrin (CM-β-CD), phosphoric acid, sodium hydroxide and all the solvents used for sample preparation (all analytical grade) were purchased from Merck Italy

(Milan, Italy). Ultrapure water (18.2 MΩ cm) was obtained by means of a Milli-Q apparatus from Millipore (Milford, MA, USA). The analyte stock solutions (100 μg/mL) were prepared by dissolving suitable amounts of pure powders in methanol; the corresponding standard solutions were prepared daily by dilution with a 3 mM, pH 3.4 ammonium formate buffer. All solutions were stored protected from light in amber glass vials from Waters Corporation (Milford, MA, USA).

2.2. CEKC instrumentation and conditions

All CEKC experiments were carried out using a ^{3D}CE apparatus (Agilent Technologies, Palo Alto, CA, USA). A sheath flow CE-MS interface was used, containing an uncoated, fused silica capillary (50 μm I.D., 75.0 cm total and effective length). The separation of CBT enantiomers was performed using a background electrolyte (BGE) composed of 30 mM, pH 3.4 ammonium formate buffer containing 5.3 mM carboxymethyl-β-cyclodextrin (CM-β-CD). Injection was carried out by voltage at the anodic end at +30 kV for 10 s. The applied voltage was set at +25 kV and the capillary temperature was thermostatted at 25.0°C.

Quantitative analyte determination was carried out by coupling the ^{3D}CE apparatus to a Waters Micromass Quattro Micro triple-quadrupole mass spectrometer equipped with an electrospray ion source (ESI) through the above referenced CE-MS interface cassette. Multiple reaction monitoring (MRM) mode was applied, acquiring in positive ionisation mode (ESI+) and using two transitions for both CBT and the IS: the most abundant one for quantitative purposes, the second one for identity confirmation. The optimised parameters were as follows: ion source voltage, 3 kV; cone voltage, 13 V, ion source temperature, 120 °C; desolvation temperature, 300°C; desolvation gas flow, 600 L/h; −1.8 kV; extractor potential, 3 V; collision exit potential, 1 V; scan duration: 0.3 s. (nitrogen as the desolvation gas, argon as the collision gas). The precursor ions and the product ions, with dwell time, cone voltage and collision energy, were applied as shown in Table 1.

Data processing was carried out using Waters MassLynx 4.1 software.

Before use, the new capillary was conditioned with 1 M sodium hydroxide, water and then with the BGE for 10 min each. After each run, the capillary was rinsed with BGE for 2 min. For storage overnight, the capillary was washed with water and additionally with 1 M sodium hydroxide and again with water (rinsing time 5 min each).

2.3. Compliance with ethical standards

Urine samples, used as blank matrices, were obtained from drug-free healthy volunteers. Real urine samples were from patients receiving CBT as part of their standard treatment and had already been collected for general needs related to the therapy; all subjects provided informed consent prior to their participation.

2.4. Microsampling: Quantitative DUS (qDUS)

Urine aliquots of 100 μL were spiked with 5 μL of analyte standard and/or IS mixtures at known concentrations. Capitainer®B 50 cards (50 μL) were used: each card includes a paper support and a microfluidic polymer arrangement with a single-valve design to obtain two fixed 50-μL dried spots [50].

Using a common dropper, two or more drops of biological fluid are put into contact with the device inlet slot, until it is completely full. In a few tens of seconds, the fluid flows into the dosing channel, reaching the sample disc and changing its colour, thus confirming the correct sampling. In the case of urine qDBS, the colour change is very subtle as opposed to qDBS, so some degree of attention is required during this step. The process takes place through capillary action and using a dissolvable membrane as a valve. When a sufficient volume of fluid is put into the inlet slot, the microchannel fills automatically through capillary action so that it contains an accurately measured volume. The matrix will then be absorbed onto the paper pad and allowed to dry to generate a volumetric, quantitative dried matrix spot (qDMS); in this case, a quantitative DUS (qDUS).

Following a 15-min drying time exposed to air current at 35±5% RH (or 90 min at room temperature and humidity), qDUS samples were taken from the device with the help of tweezers and subjected to ultrasound-assisted extraction (UAE) at 50 kHz for 5 min in 200 μL of toluene, then to microwave-assisted extraction (MAE) at 1000 W for 3 min. The resulting extract was quantitatively transferred into another vial and then brought to dryness under a nitrogen stream, re-dissolved with 50 μL of diluted BGE and injected.

Table 1
MRM optimized parameters.

Compound	Parent ion $[M + H]^+$ (m/z)	Daughter ions (m/z)	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)
CBT	277.13	259.1	13	12	300
		203.1[a)]	13	18	300
IS	286.20	204.0	13	18	300
		268.1[a)]	13	13	300

[a)] confirmatory product ion

2.5. Method validation

Guidelines from the European Medicines Agency (EMA), Food and Drug Administration (FDA), World Anti-Doping Agency (WADA) and International Conference on Harmonization (ICH) [33–37] were used for method validation.

2.5.1. Linearity

Spiked samples from healthy volunteers, 7 racemic CBT concentration levels (constant IS concentration: 5 ng/mL), 3 replicates. After qDUS sampling and solvent extraction, microsamples were analysed by CEKC-MS/MS. Calibration curves were set up plotting CBT enantiomer / IS peak area ratios as a function of concentration and applying the least square method with $1/x^2$ weighing. LOQ, LOD: analyte concentrations producing peak heights 10 and 3 times the baseline noise, respectively.

2.5.2. Extraction yield

Spiked samples as above, 3 racemic CBT concentration (low, middle, high range of the calibration curves); constant IS concentration. Percentage yields calculated as CBT enantiomer / IS peak area ratios in spiked matrix divided by CBT enantiomer / IS peak area ratios in standard solutions.

2.5.3. Precision

Repeat the extraction yield assays six times in the same day (*intraday* precision) or six times over six different days (*interday* precision), express the result as percentage relative standard deviation (RSD%).

2.5.4. Matrix effect

Procedure as introduced by Matuszewski *et al.* [51] and recently discussed by Fu *et al.* [52]. Blank sample extracts from 6 different sources, fortified post-extraction with racemic CBT standard solutions; same 3 concentrations as per extraction yield assays; CEKC-MS/MS analysis. Matrix effect was calculated as the percentage difference between Analyte/IS peak area ratios of post-extraction fortified samples and Analyte/IS peak area ratios of standard CBT solutions.

2.5.5. Stability

Blank samples spiked with racemic CBT; 2 concentration levels (high and low range of the calibration curve); sampled by qDUS, then stored at RT, in the dark, in sealed polyethylene bags containing desiccant; total storage time: 3 months. qDUS extracted and analysed every month ($n = 3$). Stored sample analyte concentrations were compared to those of

samples extracted and analysed immediately after biosampling and drying. Autosampler stability of processed qDUS: extract fresh spiked samples (at 2 levels as above), then store them in the autosampler at RT for 24 h, then re-analyse them ($n = 3$).

2.5.6. Accuracy

After analysis, real patient samples were spiked with racemic CBT (3 concentration levels as per extraction yield assays) and re-analysed ($n = 3$). Accuracy is calculated as the recovery of spiked CBT enantiomers.

3. Results and discussion

3.1. Chiral separation

CEKC enantioseparation of CBT (most basic $pk_a = 9.63$ [53]) was attempted using CD-based chiral selectors at acidic pH (3.4). Unmodified β-CD, sulphobutylether-β-CD, dimethyl-β-CD, CM-β-CD and hydroxypropyl-β-CD were tested. As expected, those CD that are negatively charged at the working pH (sulphobutylether-β-CD and CM-β-CD) provided the most intense interaction with CBT (which is positively charged) and thus the largest enantioresolution. The interaction of CM-β-CD, which is partially charged at pH 3.4, was weaker than that of sulphobutylether-β-CD. This proved to be an advantage, since the strong interaction of the latter with the analytes caused long run times and efficiency loss. On the contrary, the former selector provided sufficient resolution without negatively impacting run times and efficiency. Different CM-β-CD concentrations in the 2–10 mM range were tested, then the test was refined in the 4–6 mM range. 5.3 mM produced the best compromise between resolution and retention times, while keeping good efficiency. More detailed data on enantioseparation performances of the different CDs tested can be found in Table S1 (supplementary material).

The CEK separation of CBT enantiomers has been previously obtained by Gausepohl and Blaschke [11] using hydroxyethyl-β-CD and by Zhou *et al.* [18] using CM-β-CD. With respect to the former method, the present one is faster (12.5 vs. 9.5 min), requires lower CD amounts (5.3 vs. 20 mM) and produces better efficiency; moreover, the use of a negatively charged cyclodextrin and a volatile BGE avoids the need to adopt precautions to avoid MS source contamination (which was not a problem in Gausepohl's and Baschke's work since UV detection was used). Regarding the latter method, it uses a higher concentration of selector and obtains longer run times; moreover, the buffer is nonvolatile (phosphate), making it unsuitable for MS coupling. It also includes a mechanistic study of the interactions between CBT and CD by 2D-ROESY-NMR, but without determining the enantiomer elution order.

Regarding the expected CBT enantiomer elution order, a previous paper on molecular interaction mechanisms of CDs has established that the charged group on the primary rim of CD acts mostly as a separation "amplifier", without impacting on affinity (and thus elution) order. The most important factor for affinity order of CBT enantiomers is the presence or absence of a neutral, bulky substituent (e.g., acetyl) on the secondary CD rim [39]. Since carboxymethyl-β-CD has no substituents on the secondary rim, the expected enantiomer elution order is the same as that consistently observed in native β-CD, as well as in heptakis(6-sulfo)-β-CDs substituted with non-bulky groups: i.e., the first eluting enantiomer should be *R*-(−)-CBT and the second eluting enantiomer should be *S*-(+)-CBT. This elution order was then verified by injection of the pure enantiomers, which confirmed that *R*-(−)-CBT elutes first and *S*-(+)-CBT elutes second.

Regarding the interaction mechanism between the selector and CBT, a credible model was proposed by Zhou *et al.* [18]. This model suggests that clenbuterol interacts with CM-β-CD in a tilted orientation, facilitated by the formation of intermolecular hydrogen bonds between CBT and CM-β-CD.

3.2. Detection

Due to the intrinsic limited sensitivity of CE techniques, sample stacking by electrokinetic injection (+30 kV, 10 s) was applied. In addition to sample dissolution in diluted BGE, the creation of a water plug before the sample plug accomplished the needed stacking. An approximate 50× sensitivity increase was achieved in this way, albeit at the expense of decreased repeatability (e.g., RSD for intraday precision went from 2.0 to 4.2% for a high-concentration sample).

Coupling to MS/MS was also used to obtain satisfactory method sensitivity and selectivity. Most working MS/MS conditions were kept identical or similar to those already reported in our previous paper on the chiral analysis of CBT by LC-MS/MS [34], thus granting comparable performance.

It should be noted that the use of nonvolatile CDs in the BGE could be a problem for MS detection due to ionisation suppression and source contamination. However, CD entry into the source can be effectively prevented by the combination of low BGE pH values (which reduce EOF mobility) and charged cyclodextrins (whose electrophoretic mobility is directed toward the anode), as demonstrated by G. Schulte *et al.* [54] and other authors [55] and as applied in the present study.

3.3. Urine microsampling: Preliminary assays

Due to the novelty of the qDUS approach using Capitainer®B 50 cards, two basic performance indicators were tested, namely: sampling volume accuracy and repeatability, and drying time. In particular, the cards are not currently validated for urine sampling, so this is the first experimental report of performance assessment when dealing with urine. Although of course the preparation of volumetrically accurate DUS is easily done in a laboratory by measuring the desired amount with automatic pipettes and then spotting, in this case a common dropper was used to measure two drops and to introduce them into the device's inlet slot, to make the procedure easily applicable to self- and home-sampling. It was verified by repeated sampling that two drops of urine are almost always enough to completely fill the device slot, thus ensuring the formation of the 50-μL spot. Anyway, the operator should use as many drops as needed to completely fill the card's dosing channel.

Sampling volume accuracy and repeatability assays produced very satisfactory results. After 10 independent sampling procedures, volume accuracy was 97.2%; volume repeatability RSD was 3.6%. These results are in very good agreement with those reported by other studies for qDBS samples [56,57]. So, the use of qDUS as outlined here makes the procedure possible, and highly advantageous, at home and by self-sampling, because it does not require the intervention of qualified and trained personnel.

Drying time is one of the limiting steps of dried microsample preparation; drying times of about 60–90 min are common for most forms of urine microsamples. Although the Capitainer®B 50 cards use a specific protection mechanism that makes sample smearing and contamination unlikely, shorter drying times would make the procedure both faster, safer and more reliable. For these assays, six freshly sampled cards were exposed to low-humidity air current coming from a commercial portable dehumidifier set at maximum power (i.e., set at minimum outgoing air humidity, corresponding to 35±5% RH). Under these conditions, after repeated weighing at regular intervals, constant weight was reached after 15 min. As one can see, this corresponds to 4–6 times faster than usual for urine. Although not strictly essential, this kind of time saving can be useful and can also be obtained at home by self-sampling subjects using common household equipment. However, it is acknowledged that untrained subjects could irreparably spoil microsamples when subjecting them to accelerated drying. So, for self-sampling purposes, normal drying at room humidity is generally suggested.

3.4. Microsample extraction

As reported in our previous study on CBT [34], obtaining satisfactory results when microsampling DMS on a cellulose support is difficult, since the drug is strongly retained by the cellulose matrix, making analyte extraction complicated. For this reason, further study of the extraction conditions was undertaken. Solvents with lipophilicity similar to that of CBT (LogP = 2.6), were tested, such as 1-heptanol (LogP = 2.4), toluene (LogP = 2.7), 1-octanol (LogP = 2.8) and dibutylketone (LogP = 3.0). Moreover, different extraction means were also studied, including ultrasound- (UAE), vortex- (VAE) and microwave-assisted extraction (MAE) and their combinations.

After extensively testing different associations of solvents and extraction means (see Table S2), use of toluene with a combination of UAE (50 kHz, 5 min) and MAE (1000 W, 3 min) proved successful in satisfactorily extracting the analytes from the dried matrix. Two hundred microlitres of solvent were enough to obtain quantitative extraction. After bringing to dryness the extract, it was redissolved into 50 μL of diluted BGE and injected. Using lower volumes did not provide any sensitivity increase, due to increase in both matrix effect and baseline noise.

3.5. Method validation

The validation study was performed for both enantiomers utilizing a single calibration curve. The robustness of this methodological approach is substantiated by the nearly identical kinetic characteristics of the two enantiomeric peaks, as clearly illustrated in the electropherogram presented in Fig. 2. This finding is noteworthy, as it is often observed, particularly in HPLC analyses, that the two enantiomeric peaks exhibit markedly different shapes, with the later-eluting enantiomer typically displaying broader peaks due to band broadening effects. Therefore, it is generally necessary to generate two separate calibration curves to enable accurate determination of the two different LOQs.

Linearity: 0.3–200 ng/mL ($r^2 = 0.9994$); LOQ = 0.3 ng/mL; LOD = 0.1 ng/mL. Data refers to each enantiomer.

Extraction yield & precision: Complete results in Table 2. Mean extraction yields 88–96%, precision RSD ≤ 7.1%.

Matrix effect: Complete results in Table 2. Mean matrix effect ≤ 11%.

Stability: RT storage: 1 month, recovery ≥ 98.5%; 2 months, recovery ≥ 97.8%; 3 months, recovery ≥ 97.0%. Autosampler stability, 24h, recovery ≥ 99.1%. As expected, the preparation of dried microsamples produces remarkable stability even at RT. The accelerated drying protocol could also have further enhanced this parameter, since it has led to a faster water loss and thus to a faster decrease (or outright stop) in chemical and enzymatic reactions. No significant stability differences were observed between the two enantiomers, which are confirmed to be stereochemically stable in the chosen storage and working conditions.

Table 2
Extraction yield, precision and matrix effect results.

Compound	Enantiomer concentration (ng/mL)	Extraction yield (%)[a] [b]	Intraday precision (RSD %)[a],[b]	Interday precision (RSD %)[a],[b]	Matrix effect (%)[a],[b]
CBT	0.3	96	6.5	7.1	11
	100	90	5.9	6.1	10
	200	88	3.9	4.2	6
IS	5	93	5.0	5.6	8

[a] $n = 6$.
[b] Mean of the two enantiomers.

3.6. Analysis of real samples and accuracy

The developed CEKC method was applied to the analysis of qDUS samples from patients undergoing therapy with racemic CBT; all subjects provided informed consent prior to their participation. An example of electropherogram obtained from a qDUS sample from a patient undergoing therapy with CBT fortified with the IS at 5 ng/mL and analysed by CEKC-MS/MS is reported in Fig. 2. As can be seen, chiral separation and peak shapes are satisfactory and no interference is present.

Table 3 reports the quantitative data obtained from the analysis of real samples from patients. As expected, human metabolism did not introduce any significant ee, thus the two enantiomers were present at almost identical levels and this is true for all patients. This is in excellent agreement with current knowledge in the field [18]. Good accuracy was also obtained, with recovery values always higher than 93%. As can be seen from Table 3, urine CBT levels were in the 0.9–2.7 ng/mL range. It should be noted that reference ranges (RR) for urine CBT concentrations

Table 3
CBT enantiomer concentrations found in patient samples.

Subject	Concentration found ± SD (ng/mL)[a]	
	R-(−)-CBT	*S*-(+)-CBT
1	0.9 ± 0.1	0.9 ± 0.1
2	1.5 ± 0.1	1.5 ± 0.2
3	1.5 ± 0.2	1.5 ± 0.3
4	2.0 ± 0.3	2.1 ± 0.1
5	2.1 ± 0.3	2.2 ± 0.2
6	2.7 ± 0.2	2.5 ± 0.3

[a] $n = 3$.

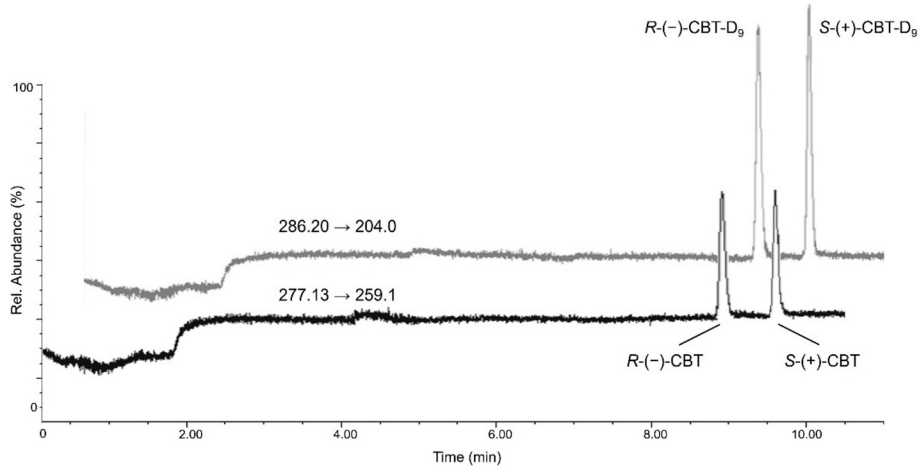

Fig. 2. Electropherogram of a quantitative dried urine spot (qDUS) sample from a patient undergoing therapy with CBT and fortified with the IS.

are currently unavailable. However, reported CBT urine concentrations in forensic samples range in the tens of ng/mL [40]. Thus, the sensitivity of this CEKC-MS/MS method is suitable for most applications, including the TDM of patients or the monitoring of volunteers undergoing systemic therapy, or general forensic applications. For prospective anti-doping applications, CBT is considered a non-threshold substance prohibited at all times (both in- and out-of-competition); as specified in Technical Letter *TL23 Growth Promoters*, just the parent drug, and none of its metabolites, markers or degradation products, should be considered for possible adverse analytical findings (AAF) [58]. The present method easily reaches the minimum reporting level (MRL, 5 ng/mL [59], or 2.5 ng/mL for each enantiomer) required by WADA. Above this urine concentration, the analysis result generates an AAF; identification of the substance below this limit produces an atypical finding (ATF) [58]. The proposed method sensitivity is still barely too low to meet the minimum required performance level (MRPL, 0.2 ng/mL or 0.1 ng/mL for each enantiomer), i.e., "the minimum concentration of a Non-Threshold Substance […] that Laboratories shall be able to detect (Initial Testing Procedure) and identify (Confirmation Procedure) in routine operations" [59].

4. Concluding remarks

An innovative, cutting-edge analytical method has been developed for the chiral analysis of CBT enantiomers in human dried urine microsamples by CEKC-MS/MS. The analytical procedure includes the generation of 50-μL qDUS microsamples from urine using Capitainer®B 50 cards. Volume accuracy and drying times were verified and an accelerated drying procedure by dehumidified air was implemented. The microsampling procedure is so reliable and feasible as to be also suitable for qDUS production in domestic or self-sampling settings.

After solvent extraction, the microsamples were electrokinetically injected into an enantioselective, high-efficiency CEKC system using carboxymethyl-β-cyclodextrin as the chiral selector.

Method validation was highly satisfactory, in terms of both precision, extraction yield and matrix effect. Thanks to the dried state of the samples, 3-month stability at RT was also outstanding.

This is the first application of qDUS for the chiral analysis of CBT by CEKC, which has been successfully used for the determination of CBT enantiomers in patients taking racemic CBT. Although application to anti-doping workflows is still not possible due to insufficient sensitivity, this goal is not unreachable: MRL compliance has been already achieved and possible, relatively small future improvements could bring the method within the MRPL applicability range. The qDUS micromatrix can be considered a good prospect for future, possible clinical trials, TDM and forensic applications, including self-sampling by patients or volunteers, due to its high performance coupled to ease of application, reliability and reduced storage, shipping and processing costs.

CRediT authorship contribution statement

Michele Protti: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Roberto Mandrioli:** Writing – review & editing, Writing – original draft, Project administration, Methodology. **Roberta Di Lecce:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Maria Tereza Cartaxo Muniz:** Writing – review & editing, Visualization, Validation, Conceptualization. **Williams Taurino De Paula Junior:** . **Roccaldo Sardella:** Writing – review & editing, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Mercolini:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2025.112940.

Data availability

Relevant data is available at the following URI: https://amsacta.unibo.it/id/eprint/7901 (AMS Acta Institutional Research Repository by almaDL University of Bologna Digital Library

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