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TECHNICAL NOTE

TOXICOLOGY

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A Direct Aqueous Derivatization GSMS Method for Determining Benzoylecgonine Concentrations in Human Urine

ABSTRACT: A sensitive and reliable method for extraction and quantification of benzoylecgonine (BZE) and cocaine (COC) in urine is presented. Propyl-chloroformate was used as derivatizing agent, and it was directly added to the urine sample: the propyl derivative and COC were then recovered by liquid–liquid extraction procedure. Gas chromatography–mass spectrometry was used to detect the analytes in selected ion monitoring mode. The method proved to be precise for BZE and COC both in term of intraday and interday analysis, with a coefficient of variation (CV) <6%. Limits of detection (LOD) were 2.7 ng/mL for BZE and 1.4 ng/mL for COC. The calibration curve showed a linear relationship for BZE and COC (r^2 >0.999 and >0.997, respectively) within the range investigated. The method, applied to thirty authentic samples, showed to be very simple, fast, and reliable, so it can be easily applied in routine analysis for the quantification of BZE and COC in urine samples.

KEYWORDS: forensic science, aqueous derivatization, propyl-chloroformate, benzoylecgonine, cocaine, human urine, method validation

The use of illicit drugs still represents an important social medical problem all over the world, and among them, cocaine (COC) is one of the most used. This explains the interest in the development and optimization of analytical rapid methodology, simple, high reliability, and accuracy to quantify COC and its metabolites on many biological specimens (1). In particular, is very important the use of urine as matrix for its easy availability and for the noninvasive nature of the test to evaluate recent intake of drugs. Furthermore, drug concentrations are much higher than those detected in other matrices and nonchromatographic methods can be easily used (2). The current method of detection used by laboratories that provide drug-testing services to employers is often a two-level approach. The initial screening test for COC is usually an immunoassay on urine to identify potential positive samples that will be confirmed (on request) by gas chromatography–mass spectrometry (GC-MS) (3). Benzoylecgonine (BZE), one of the main metabolites of COC, which has a half-life six times longer than COC, is of great toxicological and analytical interest (4), and it may be detected in urine for several days following cessation of cocaine use (5). BZE is polar and hydrophilic and extraction procedures must address the problem of low recovery. One of the most common procedure for the determination of BZE in urine by GC-MS provides a cleanup steps (a solid phase extraction) to remove the interferences from the samples and concentrate the analyte before the final derivatization step (6). Usually, acylation or trimethylsilylation is performed as a derivatization procedure, while conflicting results are reported about aqueous derivatization of BZE with chloroformates (7,8). This derivatizing agent was first introduced in an earlier work by Husek and Macek (9) and then widely investigated and developed for a quantitative and reproducible derivatization of amino acids in different matrices (10–16). This procedure offers many experimental advantages, including reacting in an aqueous medium and being practically instantaneous.

Hall et al. described the aqueous alkyl-chloroformate derivatization of BZE. In their method, BZE was directly derivatized in urine, using hexyl-chloroformate and a mixture containing acetonitrile: water: hexanol: 2-dimethylaminopyridine (5:2:2:1, v/v/v/v) to yield hexyl-benzoylecgonine as the final product. Solid phase microextraction (SPME) was used as extraction procedure prior to GC-MS analysis, yielding an Limits of detection (LOD) of 30 ng/mL while Limits of Quantification (LOQ) was not reported (10).

Another group tried to adapt this procedure to detect BZE in human hair using butyl-chloroformate as the derivatization reagent; however, they reported that the attempt to derivatize BZE directly in the aqueous solution was unsuccessful, and butyl-chloroformate was added after evaporation of the extraction solvent (methanol) directly to the residue obtained, using acetonitrile as solvent (11).

The authors also attempted to apply the procedure used by Hall et al. (11) for detection of BZE in urine human samples, using liquid–liquid extraction or solid phase extraction instead of SPME but unsuccessfully.

The aim of this work was to develop and validate a simple, fast, and reliable procedure to directly derivatize BZE in aqueous phase (urine) using propyl-chloroformate (PCF) as derivatizing

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agent and a liquid–liquid extraction step with dichloromethane: n-propanol (6:4) before GC-MS analysis.

Materials and Methods

Chemicals and Reagents

Pure BZE-d3 and COC-d3 (internal standard) were purchased from S.A.L.A.R.S. Spa (Como, Italy). Triethylamine (TEA) and propyl-chloroformate (PCF) were purchased from Aldrich (Sigma-Aldrich, Milan, Italy). All solvents and other reagents were of analytical or HPLC grade and purchased from Carlo Erba Analyticals (Milan, Italy).

Calibrators and Quality Control Samples

Stock solution of BZE-d3 (0.1 mg/mL) and COC-d3 (1 mg/mL) were diluted to working concentration (10 μg/mL) with methanol and stored at 4°C. Working calibrator solutions at 25, 50, 100, 200, and 500 ng/mL were prepared by fortifying 0.5 mL of drug-free urine, with BZE and COC. In the same way, control samples were prepared adding all analytes (50, 100, and 150 ng/mL) into drug-free urine. BZE-d3 and COC-d3 (100 ng/mL) were added as internal standards. Each point was repeated three times.

Sample Derivatization and Extraction

Calibration standards, quality controls, and collected samples (0.5 mL) were transferred to glass tubes, and 1 mL of distilled water was added to each tube, followed to 1 mL of a saturated solution of Na_2CO_3. The derivatization was performed under agitation adding 30 μL of TEA and 30 μL of PCF to each tube. The extraction of BZE derivative and COC (Fig. 1) was performed by liquid–liquid extraction with 1 mL of dichloromethane/n-propanol (6:4 v/v). After centrifugation, the organic phase was evaporated under a gentle stream of nitrogen and the residue was reconstituted with 50 μL of ethyl acetate and analyzed by GC-MS.

Instruments and GC-MS Conditions

All samples were analyzed using a Shimadzu GC-MS QP2010 Ultra coupled to an AOC-20i autosampler (Shimadzu, Milan, Italy). Analyte separation was achieved using a 15 m Rtx® -5MS column (Crossbond® 5% diphenyl/95% polysiloxane, 0.25 mm internal diameter, and 0.25 μm film thickness) from Restek (Bellefonte, PA).

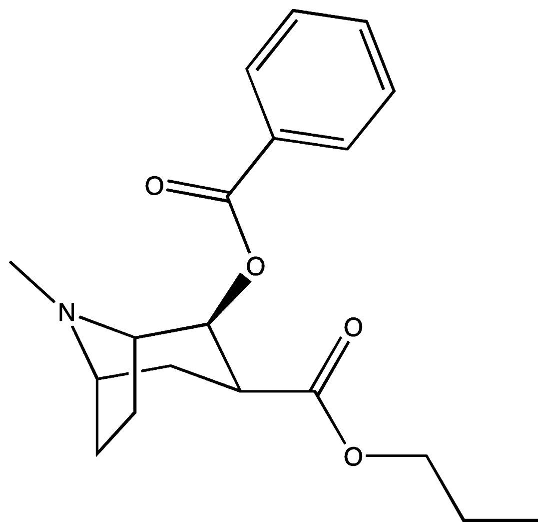

FIG 1—*Chemical structure of benzoylecgonine-propyl ester.*

The injection port was set at 270°C (injection volume 1 μL in splitless mode); the initial oven temperature was 100°C held for 1.5 min and then increased at 17°C/min to reach 320°C held for 1 min. The transfer line was set at 250°C, and helium at 1.5 mL/min was used as carrier gas.

Electron ionization (EI) was used as ionization mode with a temperature of the ion source set at 200°C (emission current 250 μA). The ions monitored in SIM mode were (m/z) as follows: 331 (27%), 272 (24%), and 210 (100%) for BZE; 82, 182, and 303 for COC; 334 (27%), 275 (24%), and 213 (100%) for BZE-d3; 85, 185, and 306 for COC-d3 with quantification ion being underlined (Fig. 2).

Validation Procedure

Method Linearity—Working calibrators at 25, 50, 100, 200, and 500 ng/mL for BZE and COC were analyzed in triplicate, injecting three different extracts and using BZE-d3 and COC-d3 at 100 ng/mL as internal standard. The linearity was determined evaluating the regression curve and expressed by the squared determination coefficient (R^2).

Limits of Detection and Quantification—The LOD, the lowest analyte concentration that can be detected, was calculated progressively diluting with blank urine at 10 ng/mL working solution containing BZE and COC, and deuterated internal standard until signal-to-noise ratio of 3 was reached. Similarly, a signal-to-noise ratio of 10 was considered acceptable to determine the lowest LOQ for all components. The values of LOD and LOQ were reported as mean of three different extracts. The acceptable criteria followed for the determination of these parameters were both the retention time within 2% and ion ratio within 20% of calibrator as reported by Ambruster et al. (17).

Precision and Accuracy—Intraday precision expressed as coefficient of variation (CV%) was determined by evaluating the repeatability of the method to three different concentration levels of 50, 100, and 150 ng/mL for a total of six repetitions at each concentration (n = 18) in the same day. The same concentrations were assessed in six replicates and conducted on three different days for interday precision evaluation. Every sample was injected once. The accuracy of the method (A%) was determined using the formula A% = (mean calculated concentration/nominal concentration) × 100. Both precision and accuracy were considered acceptable when the CVs and A% were <15%.

Selectivity—To evaluate peak purity and selectivity, ten different blank samples (no analyte or internal standard added) were analyzed to check for peaks that might interfere with detection of the analyte or internal standard (IS). Also, the same negative samples (blank samples + IS) were analyzed, to verify the absence of native analytes in the IS solution. Absence of interfering components is accepted where the response is <20% of the lower limit of quantification for the analyte and 5% for the internal standard.

Stability—Short-term and long-term stability of BZE-propyl ester was assessed. The short-term stability was studied by analysis of QC sample (cutoff value) being left at room temperature

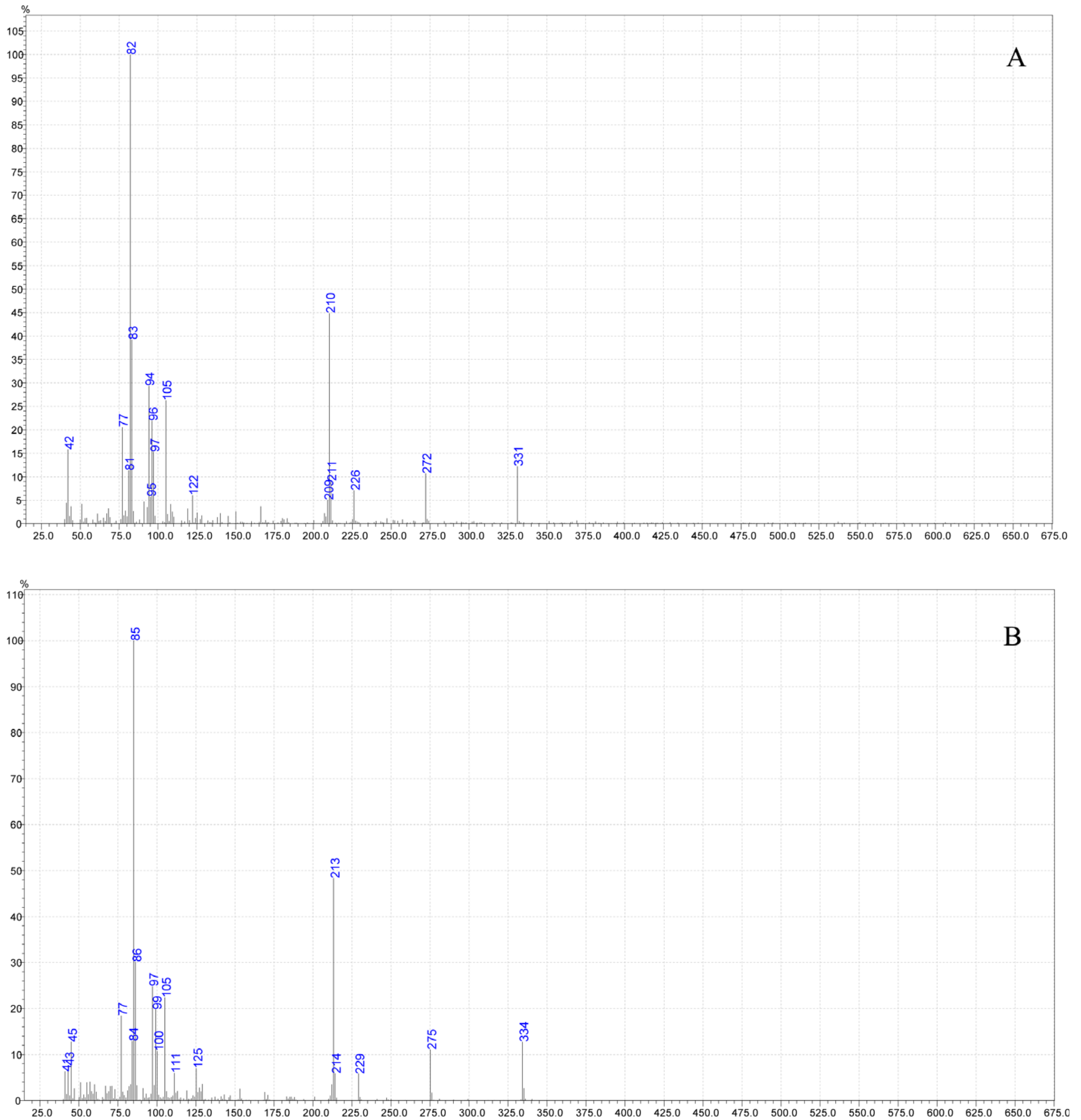

FIG 2—*Mass Spectra for benzoylecgonine-propyl ester (A) and benzoylecgonine-d3-propyl ester (B).*

for 24 h and the long-term stability by analysis of QC (cutoff value) sample 7 days after storage at −20°C.

Results

Method Validation

Linearity and Range—The linear range in urine was 25–500 ng/mL for both BZE and COC. The calibration curves and correlation coefficients were as follows: $y = 1.0130X$

TABLE 1—*Validation data for benzoylecgonine (BZE) and cocaine (COC) in urine.*

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Accuracy* (%)	CV* (%)	
				Intraday	Interday
Urine					
BZE	2.7 ± 0.4	8.9 ± 1.3	107.8 ± 4.7	2.1 ± 0.9	3.3 ± 2.2
COC	1.4 ± 0.3	4.7 ± 0.8	100.8 ± 7.2	4.1 ± 1.8	5.2 ± 2.5

*Mean of three levels of control.

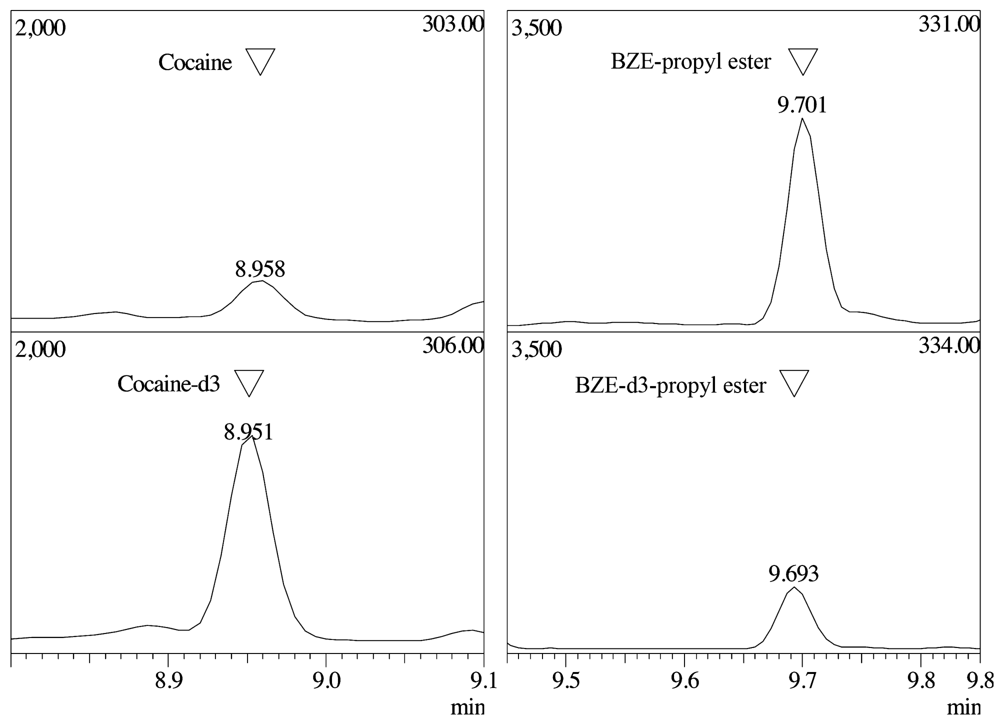

FIG 3—*A urine sample derivatized with propyl-chloroformate (PCF) for benzoylecgonine and cocaine determination.*

(SD ± 0.0073) − 0.0087 (SD ± 0.0164), R^2 = 0.999 (BZE) and $y = 0.6819X$ (SD ± 0.0105) − 0.0546 (SD ± 0.0236), R^2 = 0.997 (COC) indicating a good linear relationship from five-point calibration curves (three replicates for each level of concentration).

Limits of detection and LOQ—Limits of detections were 2.7 ± 0.4 ng/mL for BZE and 1.4 ± 0.3 ng/mL for COC, while LOQs were 8.9 ± 1.3 ng/mL for BZE and 4.7 ± 0.8 ng/mL for COC (Table 1).

Precision and Accuracy—Intraday precision (CV%) was 2.1% (SD ± 0.9) for BZE and 3.3% (SD ± 2.2) for COC, whereas the interday precision, determined after injection of six replicates at three concentrations in three different days, was 4.1% (SD ± 1.8) and 5.2% (SD ± 2.5) for BZE and COC, respectively (Table 1).

The accuracy was 107.8% (SD ± 4.7) for BZE and was 100.8% (SD ± 7.2) for COC. The acceptance limits for these parameters were within 15%, so the method showed satisfactory CV% and accuracy (Table 1).

Selectivity—Ten different blank urines were analyzed to evaluate interferences from the biological matrix; no peaks were detected at the retention time of BZE and COC.

Stability—Five samples analyzed after 24 h and 7 days showed no differences compared to the first determination (CV % < 5), and this confirmed that BZE-propyl ester was stable under the storage condition used.

Analysis of Forensic Case Samples

After validation, the procedure was applied to thirty forensic urine samples, which were found positives to the immunoassay screening (cutoff = 300 ng/mL) and already confirmed by GC-MS by derivation with pentafluoropropionic anhydride (cutoff = 100 ng/mL). Figure 3 shows a typical chromatogram of an authentic sample.

Conclusions

The use of urine in forensic toxicology laboratories is useful because of its easy availability and the noninvasive nature of the collection. Cocaine and metabolites appeared in urine rapidly following administration of single doses of cocaine. In this study, a new method for direct aqueous derivatization of BZE using propyl-chloroformate as derivatizing agent was proposed. The main benefits obtained from the use of alkyl-chloroformate, in addiction of the negligible reagent cost, are the instantaneous reaction which course does not require the exclusion of water and therefore reducing the loss of analytes due from the extraction phase before the derivatization, as is the case of SPE. The proposed method, in our opinion, is much more advantageous for simplicity, rapidity besides giving a LOD about ten times lower than the one reported in a previous work where aqueous alkyl-chloroformate derivatization of BZE and SPME extraction were used. Also, the method shows to be reliable with both precision and accuracy lower than 15% showing a very good specificity and sensitivity too. These results, combined with the simplicity of the protocol, indicate that this optimized method can be widely applied to routine analyses.

References

1. Rosario Brunetto M, Delgado Y, Clavijo S, Contreras Y, Torres D, Ayala C, et al. Analysis of cocaine and benzoylecgonine in urine by using multisyringe flow injection analysis-gas chromatography-mass spectrometry system. J Sep Sci 2010;33(12):1779–86.
2. Wu AH, Forte E, Casella G, Sun K, Hemphill G, Foery R, et al. CEDIA for screening drugs of abuse in urine and the effect of adulterants. J Forensic Sci 1995;40(4):614–8.

3. Chericoni S, Battistini I, Dugheri S, Pacenti M, Giusiani M. Novel method for simultaneous aqueous in situ derivatization of THC and THC-COOH in human urine samples: validation and application to real samples. J Anal Toxicol 2011;35(4):193–8.
4. Benowitz NL. Clinical pharmacology and toxicology of cocaine. Pharmacol Toxicol 1993;72(1):3–12.
5. Cone EJ, Sampson-Cone AH, Darwin WD, Huestis MA, Oyler JM. Urine testing for cocaine abuse: metabolic and excretion patterns following different routes of administration and methods for detection of false-negative results. J Anal Toxicol 2003;27(7):386–401.
6. Clauwaert KM, Van Bocxlaer JF, Lambert WE, De Leenheer AP. Liquid chromatographic determination of cocaine, benzoylecgonine, and cocaethylene in whole blood and serum samples with diode-array detection. J Chromatogr Sci 1997;35(7):321–8.
7. Gerlits J. GC/MS quantitation of benzoylecgonine following liquid-liquid extraction of urine. J Forensic Sci 1993;38(5):1210–4.
8. Ortuño J1, de la Torre R, Segura J, Camí J. Simultaneous detection in urine of cocaine and its main metabolites. J Pharm Biomed Anal 1990;12:911–4.
9. Husek P. Gas chromatography of amino acids. J Chromatogr 1975;113 (2):139–230.
10. Hall BJ, Parikh AR, Brodbelt JS. Aqueous phase hexylchloroformate derivatization and solid phase microextraction: determination of benzoylecgonine in urine by gas chromatography-quadrupole ion trap mass spectrometry. J Forensic Sci 1999;44(3):527–34.
11. Crossi Pereira de Toledo F, Yonamine M, De Moraes Moreau RL, Alves Silva O. Determination of cocaine, benzoylecgonine and cocaethylene in human hair by solid-phase microextraction and gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2003;798(2):361–5.
12. Wang J, Huang ZH, Gage DA, Watson JT. Analysis of amino acids by gas chromatography-flame ionization detection and gas chromatography-mass spectrometry: simultaneous derivatization of functional groups by an aqueous-phase chloroformate-mediated reaction. J Chromatogr A 1994;663(1):71–8.
13. Husek P. Chloroformates in gas chromatography as general purpose derivatizing agents. J Chromatogr B Biomed Sci Appl 1998;717 (1–2):57–91.
14. Villas-Bôas SG, Delicado DG, Akesson M, Nielsen J. Simultaneous analysis of amino and nonamino organic acids as methyl chloroformate derivatives using gas chromatography-mass spectrometry. Anal Biochem 2003;322(1):134–8.
15. Cimlová J, Kružberská P, Švagera Z, Hušek P, Šimek P. In situ derivatization-liquid liquid extraction as a sample preparation strategy for the determination of urinary biomarker prolyl-4-hydroxyproline by liquid chromatography-tandem mass spectrometry. J Mass Spectrom 2012;47 (3):294–302.
16. Jurado-Sánchez B, Ballesteros E, Gallego M. Determination of carboxylic acids in water by gas chromatography-mass spectrometry after continuous extraction and derivatization. Talanta 2012;93:224–32.
17. Arinbruster DA, Tillman MD, Hubbs LM. Limit of detection (LOD)/limit of quantitation (LOQ): comparison of the empirical and the statistical methods exemplified with GC-MS assays of abused drugs. Clin Chem 1994;40(7 Pt 1):1233–8.

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