Annales de Toxicologie Analytique, vol. XVII, n° 4, 2005

Validation d'une procédure analytique pour la détermination simultanée de cocaïne et de trois de ses métabolites dans les cheveux par GC-CI/MS^2 en utilisant une détection à piège d'ions

Validation of an analytical procedure for the simultaneous determination of cocaine and three of its metabolites in hair by GC-CI/MS^2 using an ion-trap detection

Christèle WIDMER-GIROD, Emmanuelle COGNARD, Christian STAUB*

Institute of Forensic Medicine, Av. de Champel 9 - CH-1211 Geneva 4 - Switzerland

*Corresponding Author: Christian STAUB, Institute of Forensic Medicine, Av. de Champel 9
CH-1211 Geneva 4 - Switzerland - Tel : +41 22 702 56 08 - Fax : +41 22 789 24 17

(Reçu le 7 novembre 2005 ; accepté le 17 décembre 2005)

RÉSUMÉ

Une part importante de l'accréditation est de prouver la qualité et la fiabilité de la méthode analytique utilisée en routine. Le but de la validation est d'établir que ces méthodes analytiques sont appropriées pour leur utilisation. Notre laboratoire de toxicologie médico-légale est accrédité depuis 2003. La stratégie de validation de la procédure analytique a été basée sur l'approche proposée par la Société Française des Sciences et Techniques Pharmaceutiques (SFSTP).

Dans ce travail, la validation d'une méthode, pour la détermination simultanée de cocaïne (COC), d'anhydroecgoninemethylester (AEME), d'ecgoninemethylester (EME) et de cocathylène (COET) dans les cheveux par GC-CI/MS/MS en

SUMMARY

One of the important parts of the accreditation is to prove quality and fiability of the analytical method employed in routine analysis. The aim of validation is to establish that these analytical methods are suitable for their intended use. Our laboratory of forensic toxicology is accredited since 2003. The strategy of validation of the analytical procedure was based on the approach proposed by the "Société française des Sciences et Techniques Pharmaceutiques" (SFSTP).

In this work, the validation of a method for the simultaneous determination of cocaine (COC), anhydroecgoninemethylester (AEME), ecgoninemethylester (EME) and cocathylene (COET) in hair by GC-CI/MS/MS using ion-trap detection

utilisant une détection à piège d'ions, après une hydrolyse acide et une extraction en phase solide automatisée, a été présentée comme application à cette directive.
On a trouvé une régression linéaire dans une gamme de concentrations de 0,05 - 5,00 ng/mg pour la COC, l'EME et le COET, 0,10 - 5,0 ng/mg pour l'AEME. On a estimé la limite de détection (LOD) à 0,005 ng/mg pour la COC et le COET, 0,025 ng/mg pour l'EME et 0,05 ng/mg pour l'AEME. L'efficacité de la méthode, comme la justesse et la précision, ont été évaluées en utilisant des échantillons de contrôles qualités sur la gamme étudiée.

after an acid hydrolysis and an automated solid phase extraction was demonstrated as an application of this guideline. Linear regression was found in a concentration range of 0.05 - 5.00 ng /mg for COC, EME and COET, 0.10 - 5.0 ng/mg for AEME. The limit of detection (LOD) was estimated at 0.005 ng/mg for COC and COET, 0.025 ng/mg for EME and 0.05 ng/mg for AEME. Method performances, like trueness and precision, were evaluated using quality control samples over the investigated range

Introduction

Cocaine (COC) is an alkaloid found in the plant *Erythroxylum Coca* and grows in South America. This compound is commonly used and sold on the street. Its principals metabolites are benzoylecgonine (BZE) and ecgonine methylester (EME) obtained by chemical and enzymatic hydrolysis respectively (1, 2). Another metabolite, cocaethylene (COET), could be found when cocaine is used in the presence of alcohol. This compound is a pharmacologically active analogue of cocaine but more potent than COC (3). When cocaine is smoked, a pyrolysis product, anhydroecgonine methylester (AEME), is formed. This compound was reported in the urine of crack smokers (4, 5). (see figure 1).

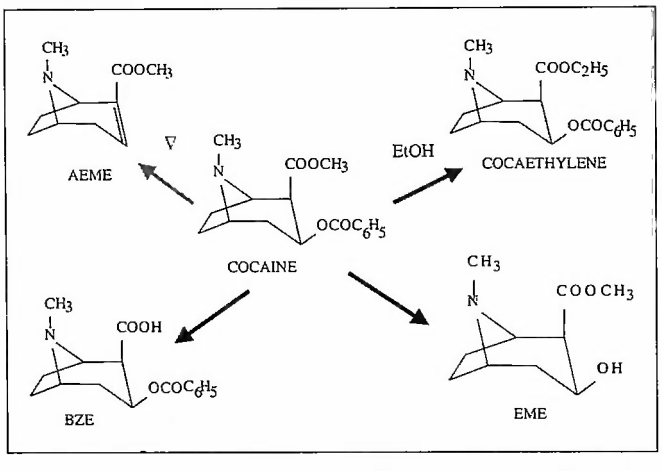

***Figure 1** : Metabolic pathway of cocaine (non exhaustive cocaine metabolism).*

One of the attractions of hair analysis is the greater positive rate of the technique over urine analysis. The presence of these metabolites in hair could offer more information potentially of benefit in distinguishing drug presence due to use versus external contamination. They could become suitable markers of cocaine use (6).

Nowadays, hair analysis is not only use in forensic toxicology but also in clinical toxicology or in traffic medicine. Several authors have considered the problem of the washing step, others have performed separation technique and used complex analytical instrumentation to increase the sensitivity and the specificity of the method. The aim of using such analytical method is to allow the detection of traces of drugs present in the hair.

The present work was conducted to quantify simultaneously COC and three of its metabolites in hair of COC abusers. As the parent drug is present predominantly in hair, when COC is detected, it's important to confirm the presence of metabolites to distinguish a consumption to a passive contamination. COC decomposes spontaneously to BZE, so the presence of this metabolite in hair is not sufficient to prove COC consumption. Then, EME, AEME and COET were chosen to confirm COC consumption (7).

After acid hydrolysis, hair samples were extracted with an automated solid phase extraction and analyzed by GC/MS/MS with an ion-trap spectrometer in positive chemical ionization. The method was validated to provide evidence that the analytical procedure is suitable for its intended use. The validation was done according to the new strategy proposed by the Commission of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) for the validation of quantitative analytical procedures (8). In a first time, the selection of the most appropriate regression model was done and in a second time, the assessment of method precision, trueness at different concentration levels over the range assay as well as the limit of quantification (LOQ) and the linearity were given (10-13).

Materials and methods

Chemical reagents

Methanol, toluene, acetic acid (100%), ammonium hydroxide solution (25%), sodium hydroxide, potassium hydroxide, sodium hydrogenophosphate, and potassium dihydrogenophosphate were supplied by Merck (Darmstadt, Germany). Methylene chloride and

isopropanol were obtained from Fluka (Buchs, Switzerland).

Acetonitrile solutions of COC, CET, AEME, EME and deuterated cocaine and ecgonine methylester (COC-d_3 and EME-d_3) were purchased from Cambridge Isotope Laboratories Inc.

Instruments and GC-MS/MS parameters

Hair samples were pulverized in a ball mill provided by Retsch (Schieritz, Hauenstein, Switzerland).

Automated solid phase extraction was performed on an ASPEC (Gilson Medical Electronics, Villiers-le-Bel, France). Isolute HCX cartridges were provided by IST (Hengoed, U.K.) and used for the extraction.

Hair sample analyses were carried out with a Varian Star 3400 CX gas chromatograph (Walnut Creek, CA, USA) equipped with a Varian Saturn 2000 ion- trap detector (Walnut Creek, CA, USA). Helium was used as the carrier gas with an inlet pressure of 0.069 Mpa (10 PSI). A J & W Scientifics (Folsom, CA, USA) capillary column of 15 m x 0.25 mm I.D. was used with a stationary phase of 5%-phenyl-methyl-polysloxane (DB-5MS, film thickness 0.25 µm) and prior connected to an inert retention gap of 1-5 m x 0.53 mm I.D. The column oven temperature was programmed as followed: 75°C maintained for 1 min to 170° C at 15°C/min, to 210°C at 5°C/min. and to 310°C at 30°C/min. The injector temperature was programmed from an initial temperature of 75°C held during 1 min, then increased to 280°C at 50°C/min and held during 1.40 min. Injections (3 µl) was made in cool on-column mode using the Varian 8200 CX autosampler (Walnut Creek, CA, USA).

The GC/MS system was operated in positive chemical ionization (CI) with isobutane as gas reagent. The trap, manifold and transfer line temperatures were 240, 120 and 290°C, respectively. Instrument control and data acquisition were carried out using the Saturn GC/MS Workstation Varian version 6.3. For the tandem mass spectrometry, the collision induced dissociation (CID) conditions used in non-resonant mode were reported in table I. In Table II, the ions used for the quantification are reported.

Table I : *CID conditions used for each compound.*

Compounds	Parent ions (*m/z*)	Excitation storage Levels (*m/z*)	Excitation amplitude (V)
COC	304.1	83.6	46
AEME	182.1	49.9	32
EME	200.1	54.9	34
CET	318.2	87.5	46
COC-d_3	307.1	84.5	46
EME-d_3	203.1	55.7	34

Table II : *Parent ions and principal product ions for each compound.*

Compounds	Parent ions -> product ions [a]
COC	304 -> 182
AEME	182 -> 105, 118, 122, 150
EME	200 -> 150, 182
CET	318 -> 196

[a] Ions used for quantification are shown underlined

Sample preparation

Hair decontamination

Before analysis, the totality of hair obtained from a person was washed successively with 10-50 ml of methylene chloride, 10-50 ml of water and finally 10-50 ml of methanol according to the size of hair tuft. This step is very important to eliminate possible external contamination. Then, the hair tufts were dried for a few minutes at 60°C in a heating block. When it is possible, tufts were cut in three segments (root-3 cm for the first segment, 3-6 cm for the second and 6 to the tip) and pulverized separately 5 minutes at 70 s^{-1} (cycles/second) in a ball mill.

Digestion and extraction

Because drugs are fixed inside the hair matrix, a digestion procedure is required before extraction. About fifty mg of powdered hair samples were placed in a glass tube of 10 ml and 1 ml of hydrochloric acid 0.1 M was added. After incubation overnight at 60°C, the solution was neutralized with 1 ml of NaOH 0.1 M, buffered with 1 ml of phosphate buffer pH 7.0 1/15 M, and 25 µl of the internal standards (COC-d_3 and EME-d_3) solution at 1 µg/ml were added. After centrifugation at 5000 r.p.m. for 10 min, the supernatant was transferred into a special glass tube for extraction.

The ASPEC system was programmed to extract the hair samples in the following steps: (1) the cartridges column were conditioned successively with 2 ml of methanol and 2 ml of water; (2) 3 ml of the supernatant solutions were dispensed on the column; (3) the latter were rinsed successively with 2 ml of water, 1 ml of acetate buffer pH 4 and 2 ml of methanol; (4) after drying the column with air, the compounds were eluted with 2 ml of (80:20:2) methylene chloride/isopropanol /ammonia hydroxide. The extracts were then evaporated to dryness under nitrogen at room temperature, reconstituted with 50 µl of toluene and finally analyzed by GC/CI/MS[2].

The complete procedure has already been described in an earlier article (9).

Calibration and quality control samples

The calibrators (CAL) and the quality control (QC) used to determine the response function and the linea-

rity respectively were prepared by adequately spiking hydrolyzed hair solution after incubation (50 mg of blank hair added with 1 ml of HCl 0.1 M and placed at 60°C overnight) with appropriate volumes of standard COC, AEME, EME and COET solutions. COC-d_3 was used as internal standard (IS) for COC and COET, whereas EME-d_3 was used as internal standard for AEME and EME. Each CAL and QC samples contained the equivalent of 0.5 ng/mg of each IS.

Validation procedure

Protocol of validation

The strategy applied for the validation of the method, based on the SFSTP guide, was adapted for a laboratory of forensic toxicology. The following validation criteria were chosen to validate our method:

- Selectivity
- Response function (calibration curve)
- Linearity and assay range
- Trueness
- Precision (repeatability and intermediate precision)
- Limit of quantification (LOQ)

Some other validation parameters could be applied in bioanalysis like absolute recovery or analyte stability in biological matrix.

Selectivity

To demonstrate the selectivity of the method, hair without any compound were incubated following the digestion procedure. The hydrolysate was then spiked with deutereted internal standard and after the extraction, injected. The aim was to show there are any interferences with endogenous substances.

The selectivity has to be studied by analyzing different sources of the matrix but in hair analysis, it is not possible to have several pool of matrix if we consider all the external component i.e. color, race etc....

Response function

The first step is to establish, within the concentration range, the relationship between the response (signal) and the concentration (quantity) of the analyte in the sample 11. The daily calibration curves for each compound of interest were established at six concentrations levels (m=6), 0, 0.05, 0.1, 0.2, 0.5, 1.0 and 5.0 ng/mg, and three replicates (n=3) for each level. These calibration curves were prepared each validation day (number of validation day k=3). The blank sample allowed to give the selectivity of the method and with the calibrators, we obtained the calibration curve.

Each day, the QC were prepared at five concentrations levels (m=5), 0.05, 0.1, 0.5, 2.5 and 4 ng/mg and four replicates (n=4) at each level.

The response function can be linear (straight line) but non-linear models, sometimes related to by detection method or the particularly wide concentration range, can also be observed. In mass spectrometry, the response function is often not strightly linear so we choose an appropriate statistical model for the evaluation of the calibration curve. When a significant difference exist between variances at lowest and highest concentration levels, the data should mathematically be transformed (8, 14, 17).

Linearity

The linearity is defined inside a range of concentration and corresponds to its ability to obtain results directly proportional to the concentration of analytes in the sample (8, 10, 13). The linearity was calculated by fitting the back-calculated concentrations of the QC versus theoretical (introduced) concentrations by applying the linear regression model based on the least squares method (14).

Trueness

Trueness is defined to the closeness of agreement between a conventionally accepted value and a mean experimental one (18). Each day, the recovery is calculated at five concentration levels between 0.05 and 4 ng/mg (n=4) that correspond to the QC concentrations. The trueness is given by the mean recovery over 3 days and could be expressed in terms of bias.

Repeatability and intermediate precision

The precision is usually measured in terms of imprecision expressed as an absolute or relative standard deviation (RSD %) and is estimated by calculating repeatability and time dependent intermediate precision at each concentration levels of the QC. The RSD of the repeatability and the intermediate precision are obtained by using variances analysis (10).

Limit of quantification (LOQ)

The LOQ is the lowest concentration of a sample that can still be quantified with acceptable precision and trueness.

In a previous study, the LOQ was estimated by the analysis of soaked hair at different concentration of the four compounds of interest. Ten replicated samples were analyzed for each concentration studied. LOQ was determined as the concentration where the relative standard deviation (RSD) is inferior to 20 % (15).

The accuracy profile is a representation of the trueness versus the concentration with an indication of the sys-

tematic and random errors comprised within the acceptance limits. Then, the LOQ is the smallest concentration level investigated inside these limits (19).

Results and discussion

For a long time, it was known that drugs incorporated body matrix, like hair, after their ingestion. Generally, the parent drug is the predominant analyte detected in hair sample, then, the possibility of external contamination is not excluded on people innocently in contact with abusers during their everyday lives. Fortunately, metabolites could be detected to provide evidence of use or no use, even if they are present in low concentration. The problem with cocaine is that it major metabolite, BZE, could be formed without ingestion of cocaine. That is why it was of interest to develop a method to detect other metabolites and give an aid to the interpretation of hair sample test cocaine positive.

A specific method was developed for the quantitative determination of COC, EME, COET and AEME and validated using the SFSTP guide.

Figure 2 show the calibration curve obtained for COC on the three days. The validation results of the response function are presented in Table III. Weighted linear regression ($1/x^2$) with six concentration levels was used. The stability of the calibration curve must be checked each time that the analyses are conducted. Good linearity is obtained with a slope close to 1 ± 0.005 and good closeness R^2 above 0.998 for all the analytes. The regression equation was reported in Table III.

The acceptance limits fixed in the laboratory of toxicology in Geneva depend on the matrix and the compound of interest. In the case of hair analysis, trueness has to be within (30%, the repeatability inferior to 20% and the intermediate precision inferior to 25%. The trueness was expressed in term of relative bias (%) and presented in Table III. The R.S.D values, presented in Table III are between 3.5 and 16.6% for the repeatability and 3.5 and 26.4% for the intermediate precision for COC and its metabolites. Two values obtained for AEME were unacceptable and outside the limits: A repeatability at 57.7% obtained for the concentration 0.05 ng/mg and an intermediate precision at 96.1% obtained at the same concentration. This concentration could not be chosen as the LOQ of the method for this compound. The other RSD values illustrate a relative good precision of the method.

The accuracy profile could be a relative good representation of the values presented in Table III. As an example, the accuracy profile of COC was showed in Figure 3. The LOQ was chosen as the smallest concentration inside the acceptance limits. The LOQ was fixed to 0.05 ng/mg for COC, EME and COET and to 0.1 ng/mg for AEME.

Conclusion

A specific method was developed in GC/MS/MS using an ion-trap detector in positive chemical ionization mode with isobutane as gas reagent to detect and quantify cocaine and its three metabolites, COET, EME and AEME, in hair. The procedure fully validated showed that the SFSTP guide could be applied to hair analysis. The validation of this analytical method allowed to know the performances of the method and its characteristics and therefore to better apprehend its limits.

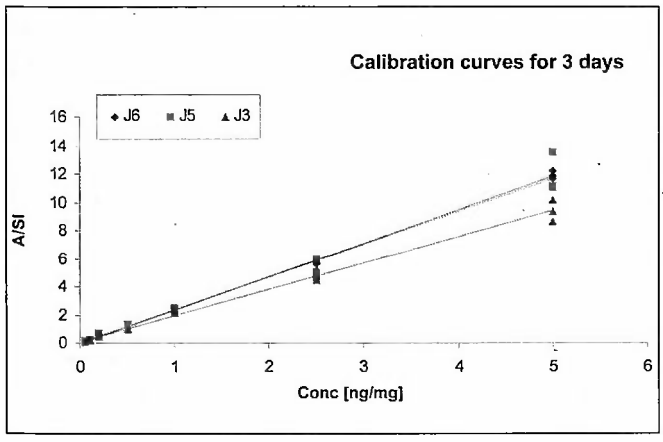

Figure 2 : Response function obtained for cocaine.

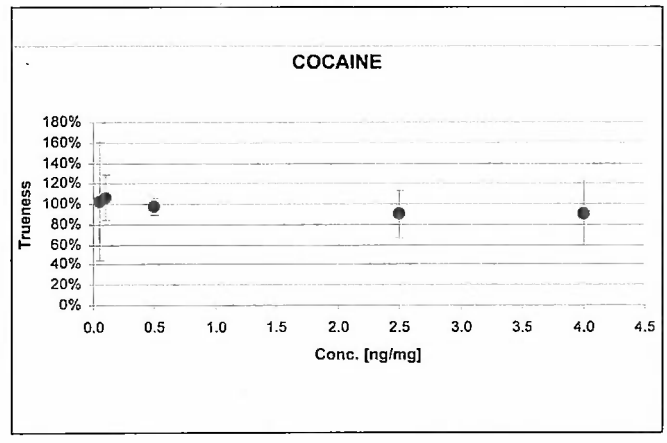

Figure 3 : Accuracy profile of cocaine.

Table III : *Validation of the method for the determination of COC, EME, AEME and COET in hair.*

Response function (0.05-5 ng/mg) (k=3, m=6, n=3)												
	COC			EME			COET			AEME		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Slope	2.3	2.5	2.1	2.73	2.41	1.99	2.45	2.5	2.1	2.37	5.3	0.99
Intercept	0.017	0.036	-0.004	0.023	0.018	0.02	-0.015	-0.0006	0.015	0.089	0.008	0.048
R	0.995	0.985	0.993	0.976	0.984	0.993	0.988	0.986	0.991	0.905	0.986	0.865
Trueness (k=3, n=4) ng/mg relative bias (%)												
0.05	2.4			9.4			-6.3			-50.1		
0.1	6.4			8.8			-0.4			23		
0.5	-2.3			-3.6			-0.7			-11.6		
2.5	-9.8			-8.9			-12.2			-20.2		
4	-9			-2.1			-7.2			-13.7		
Precision (k=3, n=4) ng/mg												
Repeatability (RSD %)												
0.05	11.0%			11.0%			7.0%			57.7%		
0.1	7.5%			15.4%			10.8%			10.5%		
0.5	3.5%			10.7%			4.8%			13.2%		
2.5	5.5%			7.1%			4.5%			16.1%		
4	8.6%			3.2%			6.0%			16.6%		
Intermediate precision (RSd %)												
0.05	22.7%			15.9%			12.3%			96.1%		
0.1	8.3%			15.4%			10.8%			26.4%		
0.5	3.5%			14.5%			4.8%			13.8%		
2.5	10.2%			12.0%			8.8%			16.1%		
4	13.8%			14.2%			7.7%			23.4%		
Linearity (k=3, m=5, n=20) and **limit of quantification** (ng/mg)												
Range	0.05 - 4			0.05 - 4			0.05 - 4			0.15 - 4		
Slope	0.903			0.963			0.916			0.846		
Intercept	0.02			-0.009			0.005			-0.002		
R^2	0.9999			0.9982			0.9992			0.9976		
LOQ	0.05			0.05			0.05			0.1		

References

1. R.C. Baselt, and R.H. Cravey. Disposition of toxic drugs and chemicals in man. Ed. Year Book Medical Publishers, 5th Ed (2000).
2. Clarke's isolation and identification of drugs. The Pharmaceutical Press, London, (2004).
3. P. Jatlow, J.D. Elsworth, C.W. Bradberry, G. Winger, J.R. Taylor, R. Russell, and R.H. Roth. Cocaethylene: a neuropharmacological active metabolite associated with concurrent cocaine-ethanol ingestion. Life Sci. 48: 1787-94 (1991).
4. E.J. Cone, M. Hillsgrave and W.D. Darwin. Simultaneous measurement of cocaine, cocaethylene, their metabolite and "crack" pyrolysis products by gas chromatography-mass spectrometry. Clin. Chem. 40: 1299-1305 (1994)
5. W.J. Wang, W.D. Darwin, and E.J. Cone. Simultaneous assay of cocaine, heroin and metabolites in hair, plasma, saliva and urine by gas chromatography-mass spectrometry. J. Chromatogr. 660: 279-290 (1994).
6. P. Kintz, V. Cirimele, and P. Mangin. Testing human hair and urine for anhydroecgonine methylester, a pyrolysis product of cocaine. J. Anal. Toxicol. 19: 479-482 (1995).
7. E. Cognard, S. Rudaz, S. Bouchonnet, and Ch. Staub. Analysis of cocaine and three of its metabolites in hair by gas chromatography-mass spectrometry using ion-trap detection for CI/MS/MS. J. Chromatogr. B. (2005) in press.
8. Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P.Chiap. N. Cohen, P.-A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, and L. Valat, Validation des procédures analytiques quantitatives. Harmonisation des démarches. STP Pharma Pratiques 13 (3) : 101 (2003)
9. Ch. Girod, and Ch. Staub, Analysis of drugs of abuse in hair by automated solid-phase extraction, GC/EI/MS and GC ion trap/CI/MS. Forensic Sci. Int. 107: 261-271 (2000).

10. E. Chapuzet, N. Mercier, B. Bervoas-Martin, B. Boulanger, P. Chevalier, P. Chiap,D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, and J.C. Nivet, Méthodes chromatographiques de dosage dans les milieux biologiques : stratégie de validation. Rapport d'une commission SFSTP. STP Pharma Pratiques 7: 169-194 (1997)

11. Ph. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, S. Bervoas- Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, and J.C. Nivet, The SFSTP guide on the validation of chromatographic methods for drug bioanalysis: from the Washington conference to the laboratory. Anal. Chim. Acta. 391: 135-148 (1999)

12. J. Caporal -Gautier, J.M. Nivet, P. Algranti, M. Guilloteau, M. Histe, M. Lallier, J.J. N'Guyen-Huu, and R. Russotto. Guide de validation analytique- Rapport d'une commission SFSTP .I . Méthodologie (1992)

13. J. Caporal -Gautier, J.M. Nivet, P. Algranti, M. Guilloteau, M. Histe, M. Lallier, J.J. N'Guyen-Huu, and R. Russotto. Guide de validation analytique- Rapport d'une commission SFSTP .II . Exemples d'application (1992)

14. Ph. Hubert, J.-J. Nguyen-Huu , B. Boulanger, E. Chapuzet, P.Chiap. N. Cohen, P.-A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, and L. Valat, Harmonization of strategies for the validation of quantitative analytical procedures. J. Pharma. Biomed. Anal. 36: 579-586 (2004)

15. P. Chiap, A. Ceccato, B. Miralles Buraglia, B. Boulanger, Ph. Hubert, and J. Crommen, Development and validation of an automated method for the liquid chromatographic determination of sotalol in plasma using dialysis and trace enrichment on a cation- exchange pre-column as on-line sample preparation. J. Pharm. Biomed. Anal. 24: 801-814 (2001)

16. V. P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, and S. Spector. Analytical Methods Validation : Bioavailability, Bioequivalence and Pharmacokinetic Studies. Pharmaceutical Research. 9: 588-592 (1992).

17. B. Boulanger, W. Dewé, P. Chiap, J. Crommen,and Ph. Hubert, An analysis of the SFSTP guide on validation of chromatographic bioanalytical methods: progress and limitations. J. Pharma. Biomed. Anal. 32: 753 (2003)

18. ISO 5725-1, Application of the statistics-accuracy (trueness and precision) of the results and methods of measurement. Part 1: General principles and definitions, International Organization for Standardization (ISO), Geneva, Switzerland.

19. B. Streel, A. Ceccato, R. Klinkenberg, and Ph. Hubert. Validation of a liquid chromatographic-tandem mass spectrometric method for the determination of loperamide in human plasma, J. Chromatogr. B, 814: 263-273 (2005).