Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 1192–1195

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Determination of fatty acid ethyl esters in hair by GC–MS and application in a population of cocaine users

Lucia Politi[a,*], Francesco Mari[a], Sandra Furlanetto[b], Ester Del Bravo[a], Elisabetta Bertol[a]

[a] Division of Forensic Toxicology, Dept Anatomy, Histology, and Legal Medicine, University of Florence, Largo Brambilla 3, 50134 Firenze, Italy
[b] Dept. of Pharmaceutical Sciences, University of Florence, Via U. Schiff 6, 50019 Sesto Fiorentino, Italy

ARTICLE INFO

Article history:
Received 28 October 2010
Received in revised form
18 November 2010
Accepted 19 November 2010
Available online 30 November 2010

Keywords:
FAEE
Hair
GC–MS
Alcohol biomarkers
Cocaethylene

ABSTRACT

A gas chromatography–mass spectrometry method for the determination of ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate in hair samples was developed, validated and applied to real samples. Ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate are fatty acid ethyl esters (FAEE) which are known to be direct biotransformation products of ethanol. Their presence in the body fluids and tissue is therefore indicative of alcohol intake and, in particular, FAEE concentration in hair higher than 0.5 ng/mg is indicative of excessive chronic alcohol consumption. The method was applied to 80 hair samples formerly found positive for cocaine and FAEE analytical results were compared with the presence of cocaethylene, a cocaine metabolite formed only when alcohol and cocaine are used together. According to our data the two biomarkers (FAEE and cocaethylene in hair) are tools of great value in the assessment of the diagnosis of use of cocaine and ethanol. In fact, discrepancies were noted and might be related to various factors including differences in consumption habits and thus permitting to distinguish the use of both substances non-concurrently or concurrently. Also, the determination of both markers may, in some cases, discriminate the use of moderate or heavy alcohol amounts when associated with cocaine. Finally, in a population of non-cocaine-users our results support FAEE as valuable means in the assessment of excessive alcohol chronic use.

1. Introduction

The importance and diffusion of hair analysis in the diagnosis of alcohol heavy use and misuse have recently been establishing in the routine of analytical toxicology laboratories, following, in particular, the development of analytical procedures able to determine alcohol biotransformation products, such as fatty acid ethyl esters (FAEE) and ethyl glucuronide in hair samples. Both FAEE and ethyl glucuronide are recognized by the Society of Hair Testing as biomarkers of chronic excessive alcohol consumption [1].

FAEE have been known as biotransformation products of ethanol since the 1960s [2] and were proposed in the clinical practice as cardiomyopathy markers before [3] and as ethanol use/abuse markers later: in 2001 [4] FAEE started being used as markers of alcohol excessive use. Several different molecules formed from ethanol and fatty acids, phospholipids, or lipoproteins were identified and attributed to FAEE group, but only four of them were found to be correlated with alcohol use when retrieved in the keratinic matrices (ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate) [4,5].

Nearly all methods reported by the literature identify these analytes by head space-solid phase microextraction-gas chromatography–mass spectrometry (HS SPME GC–MS) [4–9] or, more recently, by GC–tandem mass spectrometry [10]. Both techniques performed satisfactorily and, on the basis of the analytical results a cut-off of 0.5 ng/mg for the sum of the 4 analytes was found to best reflect a chronic excessive alcohol consumption. In fact, as alcohol is a legal substance in most countries a cut-off is strictly needed in order to discriminate a moderate and an excessive use, i.e. more than 60 g of pure ethanol per day, according to the World Health Organization Guidelines and corresponding to roughly four alcoholic drinks per day [1].

The present study aimed at developing and validating a GC–MS method for FAEE in hair, which might be useful in laboratories whose instrumental equipment do not comprise HS-SPME or GC–MS–MS. As a matter of fact, a reliable (as ensured by full validation) GC–MS method will permit to extend and diffuse the analysis of excessive chronic alcohol use. Furthermore, the method was applied to hair samples previously analyzed and found positive for cocaine and either positive or negative to cocaethylene, a transesterification product of cocaine. Cocaethylene is a cocaine metabolite formed by carboxylesterases when cocaine and ethanol are consumed simultaneously and, therefore, can be used as ethanol intake indicator in cocaine users populations. For this

* Corresponding author. Tel.: +39 0557947208; fax: +39 0557946171.
E-mail address: lucia.politi@unifi.it (L. Politi).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.11.032

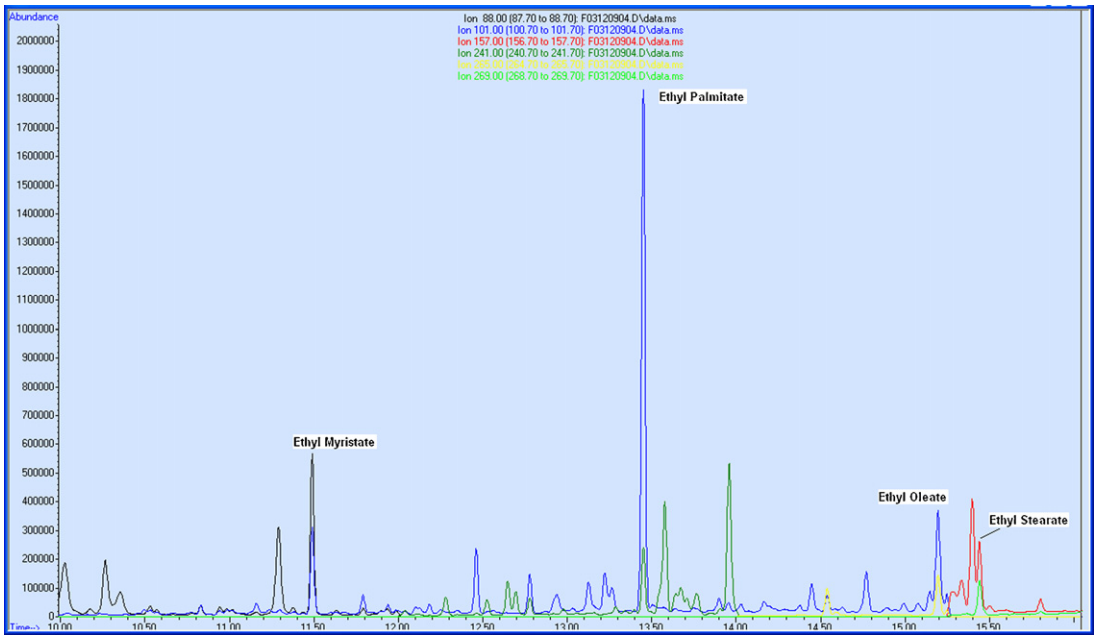

Fig. 1. Gas chromatogram of a positive sample (ethyl myristate: 0.13 ng/mg, ethyl palmitate: 0.61 ng/mg, ethyl oleate: 0.71 ng/mg, ethyl stearate: 0.25 ng/mg, total FAEE: 1.87 ng/mg) acquired in SIM mode.

reason, 80 cocaine positive hair samples were selected and analytical results of FAEE and cocaethylene were compared in order to evaluate their reliability as markers of excessive and/or chronic drinking.

2. Materials and methods

2.1. Hair samples

Samples of head hair were chosen among those found positive to cocaine in individuals controlled for drugs of abuse for regranting driver's license. Samples were admitted to the study after complete anonymization. The same length (between 3 and 5 cm) was used for both cocaine and FAEE analysis.

2.2. Standards and reagents

Ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate were acquired from Sigma–Aldrich (Milan, Italy), cocaine and its main metabolites (benzoylecgonine, ecgonine methylester, and cocaethylene) were purchased from LGC Standards (Milan, Italy). All reagents were of HPLC grade or higher and acquired from Sigma–Aldrich.

2.3. Sample preparation for FAEE determination

All hair samples were accurately double washed with methanol (2 ml for about 2 min each). The four FAEE analytes were extracted from the keratinic matrix finely cut with scissor (about 100 mg, lower amounts were considered acceptable down to 30 mg) by overnight incubation in n-hexane/dimethylsulphoxide (4 and 0.5 ml respectively) with alpha-colestane as internal standard (50 ng, purchased from Sigma–Aldrich). The following morning, the incubation layer of n-hexane was extracted on aminopropyl-NH_2 solid phase extraction (SPE) cartridges (Varian, Harbor City, CA) that were initially conditioned with dichloromethane followed by n-hexane (3 ml each). After elution of the incubation solvent, the analytes were extracted by n-hexane (3 ml) and, subsequently, by dichloromethane (3 ml) and the resulting extract was evaporated under a stream of nitrogen. The residue was dissolved in 50 μl of n-hexane and injected in the gas chromatograph–mass spectrometry (GC–MS) apparatus in selected ion monitoring (SIM) mode.

2.4. Sample preparation for cocaine and its metabolites

Samples were finely cut with scissor, and, after nalorphine addition (50 ng, internal standard), overnight incubated with hydrochloric acid 0.1 N, solid phase extracted (Bond Elut Certify LRC cartridges, Varian, Harbor City, CA) using the method proposed by the manufacturer for basic drugs (cartridges conditioned with methanol and phosphate buffer, addition of the sample, rinsed with water, hydrochloric acid and methanol and eluted with dichloromethane–isopropanol (8:2) with 2% ammonium hydroxide), derivatised with 50 μl of N-methyl-N-(trimethylsilyl)trifluoroacetamide (75 °C for 15 min) and injected in the GC–MS apparatus.

2.5. Gas chromatography–mass spectrometry

The analysis was developed, validated and performed on an Agilent GC–MS with Inert MSD (GC 7890A, MS 5975C equipped with a 7693 autosampler) equipped with a phenylmethylsilicone 5% (HP-5MS) capillary column (30 m × 0.25 mm I.D. × 0.25 μm film thickness). The injector was set at 300 °C and splitless injection was performed. The column oven temperature was programmed at 100 °C for 0.5 min, then increased to 200 °C at 12 °C/min, to 300 °C at 8 °C/min, and held at 300 °C for 3 min. Identification was performed in selected ion monitoring mode with at least three ions for each analyte: *m/z* 88, 213, 256 for ethyl myristate (*m/z* 88 used for quantification); 101, 241, 284 for ethyl palmintate (quantifier: 101); 101, 265, 310 for ethyl oleate (quantifier: 101); 157, 269, 312 (quantifier: 157); and 217, 357, 372 for internal standard (217 used for quantification).

For cocaine analysis the same instrument was used. Main parameters were the same described above except for oven temperature that was maintained at 100 °C for 1 min, increased then to 300 °C at 20 °C/min, and hold at 300 °C for 3 min. For identification purposes, three ions were monitored for each analyte: *m/z*

Table 1
Calibration, accuracy (bias %) and imprecision (RSD%) results at LLOQ ($n = 5$).

	Slope (mean ± SD)	Intercept (mean ± SD)	Correlation coefficient (R^2)	Accuracy (%) at 0.01 ng/mg	Imprecision (%) at 0.01 ng/mg
Ethyl myristate	0.032 ± 0.06	0.0083 ± 0.007	0.9992 ± 0.007	7.9	5.6
Ethyl palmitate	0.045 ± 0.07	0.0083 ± 0.017	0.9989 ± 0.008	12.1	10.7
Ethyl oleate	0.073 ± 0.32	0.0075 ± 0.056	0.9988 ± 0.009	9.7	15.1
Ethyl stearate	0.056 ± 0.14	0.0032 ± 0.032	0.9992 ± 0.009	13.5	16.8

82, 182, 303 for cocaine (m/z 182 used for quantification); 82, 240, 361 for benzoylecgonine (quantifier: 240); 82, 96, 271 for ecgonine methylester (quantifier: 96); 196, 82, 317 for cocaethylene (quantifier: 196); and 324, 414, 440, 455 for nalorphine (455 used for quantification).

2.6. Method validation

No endogenous or exogenous compounds generally retrieved in hair were found to interfere with the analysis. In particular, FAEE that were previously found not to correlate with alcohol use and easily commercially available (ethyl linoleate, linolenate, and arachidonate) were found to be chromatographically resolved from compounds of interest. Also, identification of each molecule was always based on the acquisition of at least 3 ions for each compound, as suggested for GC–MS by the guidelines for analysis involved in the forensic field [11].

A 5-point calibration curve was built using a mixture of the four compounds at 0.01, 0.05, 0.1, 0.2, 0.5 ng/mg each. Controls were prepared by a different operator at 0.01, 0.05 and 0.3 ng/mg.

Extraction recovery was measured by comparing the analytical response of the compounds (at 0.01, 0.05 and 0.3 ng/mg) extracted according to the procedure with the response obtained adding the same amount of FAEE after extracting blank samples.

All analytes were demonstrated to be stable after extraction for at least 24 h. In fact, autosampler stability was assessed for all analytes and internal standard by injecting 3 real samples after 0, 12, and 24 h after sample preparation.

3. Results and discussion

The four analytes (ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate) were chromatographically separated (Fig. 1). Also, the available FAEE not significant in hair analysis were found not to interfere with the compounds of interest. Nevertheless, free fatty acids, phospholipids, lipoproteins and triglycerides are abundantly present in the human body and, virtually, any of them could form FAEE after ethanol intake and, as expected with MS fragmentation of rather similar molecules, same ions are recurrent in the spectra of different FAEE. For these reasons, it is advisable that chromatographic separation is preferred over sample throughput when detection occurs in single MS and it is also essential that ion ratios of samples fall in ±20% of those of the calibrators to avoid improper quantification due to interferences.

The method was validated according to international and national guidelines of the field [12,13]. The lower limit of quantification was found at 0.01 ng/mg for each of the four substances (imprecision and accuracy measure as relative standard deviation (RSD) and bias: 16.8% and 13.5%, respectively). Imprecision and accuracy for the control samples were always better than 13.6% and 9.8%, respectively. Extraction recovery was higher than 76.5% at all concentration levels. More information is reported in Table 1.

The cocaine and metabolites method was formerly validated and routinely used in the laboratory. The LLOQ was 0.05 ng/mg for each substance and LOD (limit of detection) was set at the same value.

For both FAEE and cocaine methods the variations in peak area of the analytes after 12 and 24 h after sample preparation were found to be negligible and, therefore, autosampler stability is not an issue at the working conditions.

The quantitative analysis of the four FAEE molecules ranges from 0.02 to 17.67 ng/mg (median: 0.82, mean: 1.46 ng/mg). Of the 80 samples analyzed, 25 were below the cut-off of 0.05 ng/mg, indicating a moderate alcohol use or abstinence. Hair samples were selected among those positive to cocaine after routine laboratory analysis (from 0.11 to 330.55 ng/mg, median: 2.30 ng/mg, mean: 15.54 ng/mg) and were found either positive or negative to cocaethylene. Cocaethylene was found in the range from <LOD to 12.97 ng/mg (median: 0.07, mean: 0.44 ng/mg). When comparing results obtained for FAEE and cocaethylene, using the suggested cut-off value in both cases (i.e. 0.05 ng/mg for FAEE [1] and 0.05 ng/mg cocaethylene [12–14]), 15 samples were negative to both markers indicating that ethanol was consumed in moderate amounts, if any, and not in association with cocaine. Thirty-eight samples were positive to both FAEE and cocaethylene, thus proving that alcohol was excessively used and was associated with cocaine. Nevertheless, 17 samples were positive to FAEE and negative to cocaethylene, where a non-concomitant use of the two substances can be supposed; 10 samples tested negative for FAEE and positive to cocaethylene. This discrepancy might be related to a different intent in setting the cut-offs, i.e. discriminating an excessive drinking behavior (more than 60 g/day, as stated in reference [1]) in the case of FAEE, or discriminating the use of cocaine from external contamination [14].

Cocaethylene was chosen for comparison with FAEE in this study since it is formed only in the case of simultaneous intake of alcohol and cocaine. Hence, it can be used as an ethanol use indicator in a population of cocaine users. However, its concentration in the blood stream and, consequently, the quantity incorporated into keratinic matrices, is strictly dependent on cocaine concentration, as ethanol can be assumed to be used in largely higher extent. On this rationale, the percentage of cocaethylene with respect to the total amount of cocaine, benzoylecgonine and ecgonine methylester (expressed as cocaethylene equivalents) was calculated. This measure can be a more reliable indication of the amount of ethanol ingested by the user, as it tends to be unaffected by the amount of cocaine consumed. Cocaethylene percentage was calculated between zero and 18.98% (median: 2.97%, mean: 4.73%). The maximum percentage found (18.98%) is likely to be related to a very high ethanol use, as 17 ± 6% cocaine intake was reported to be transformed into cocaethylene [15]. When comparing FAEE concentration with cocaethylene percentage after setting an arbitrary cut-off at 1%, 13 samples tested negative to both (teetotalers or moderate alcohol users) and 46 samples tested positive to both markers (excessive users of alcohol usually associated with cocaine). Still, in 9 cases a non-concurrent use of ethanol and cocaine may be assumed (positive to FAEE, negative to cocaethylene) and 12 cases were negative to FAEE and positive to cocaethylene. In consideration of the samples positive to cocaethylene and negative to FAEE with both cocaethylene taken as absolute concentration and as percentage over cocaine main metabolites, it can be concluded that cocaethylene is, in heavy cocaine users, more sensitive than FAEE to alcohol

use. In fact, cocaethylene positive/FAEE negative samples can be indicative of a severe cocaine use associated with moderate alcohol consumption. Therefore both markers can be valuable tools in the diagnosis of dependence in cocaine users.

It is interesting to note that a certain discrepancy between markers was observed before when comparing ethyl glucuronide with cocaethylene [16], where it was hypothesized that one of the possible reasons (besides non-concurrent use and individual differences in biotransformation pathways) was a different mechanism of incorporation sustained by the differences in physical–chemical properties of the molecules. When excluding the only polar compound (ethyl glucuronide) and comparing cocaethylene with FAEE similar results were obtained, thus suggesting that polarity and incorporation mechanism have only a partial role in explaining the differences in markers concentrations.

4. Conclusions

A GC–MS method for the detection and quantification of FAEE in hair was developed, fully validated and applied on 80 samples where comparison with positivity to cocaethylene was possible. First of all, FAEE analysis was carried out and full validation was accomplished without the need of HS-SPME-GC–MS or GC–MS/MS. Also, FAEE and cocaethylene were found to be useful and complementary tools in the elucidation of a diagnosis of alcohol and cocaine use, permitting to discriminate different habits in the intake habits (non-simultaneous, simultaneous with moderate or non-moderate alcohol amount). Finally, FAEE performance shows again the helpfulness of these analytes as biomarkers of excessive chronic ethanol consumption.

References

[1] P. Kintz, Consensus of the Society of Hair Testing on hair testing for chronic excessive alcohol consumption 2009, Forensic Sci. Int. 196 (2010) 2.
[2] D.S. Goodman, D. Deykin, Fatty acid ethyl ester formation during ethanol metabolism in vivo, Proc. Soc. Exp. Biol. Med. 113 (1963) 65–67.
[3] L.G. Lange, B.E. Sobel, Mitochondrial dysfunction induced by fatty acid ethyl esters, myocardial metabolites of ethanol, J. Clin. Invest. 72 (1983) 724–731.
[4] F. Pragst, V. Auwaerter, F. Sporkert, K. Spiegel, Analysis of fatty acid ethyl esters in hair as possible markers of chronically elevated alcohol consumption by headspace solid-phase microextraction (HS-SPME) and gas chromatography–mass spectrometry (GC–MS), Forensic Sci. Int. 121 (2001) 76–88.
[5] V. Auwärter, F. Sporkert, S. Hartwig, F. Pragst, H. Vater, A. Diefenbacher, Fatty acid ethyl esters in hair as markers of alcohol consumption. Segmental hair analysis of alcoholics, social drinkers, and teetotalers, Clin. Chem. 47 (2001) 2114–21123.
[6] F. Pragst, M. Rothe, B. Moench, M. Hastedt, S. Herre, D. Simmert, Combined use of fatty acid ethyl esters and ethyl glucuronide in hair for diagnosis of alcohol abuse: interpretation and advantages, Forensic Sci. Int. 196 (2010) 101–110.
[7] F. Pragst, M. Yegles, Determination of fatty acid ethyl esters (FAEE) and ethyl glucuronide (EtG) in hair: a promising way for retrospective detection of alcohol abuse during pregnancy? Ther. Drug Monit. 30 (2008) 255–263.
[8] S. Süsse, C.M. Selavka, T. Mieczkowski, F. Pragst, Fatty acid ethyl ester concentrations in hair and self-reported alcohol consumption in 644 cases from different origin, Forensic Sci. Int. 196 (2010) 111–117.
[9] F.M. Wurst, E. Kelso, W. Weinmann, F. Pragst, M. Yegles, I. Sundström Poromaa, Measurement of direct ethanol metabolites suggests higher rate of alcohol use among pregnant women than found with the AUDIT—a pilot study in a population-based sample of Swedish women, Am. J. Obstet. Gynecol. 198 (2008), 407e1–407e5.
[10] C.M. Zimmermann, G.P. Jackson, Gas chromatography–tandem mass spectrometry for biomarkers of alcohol abuse in human hair, Ther. Drug Monit. 32 (2010) 216–223.
[11] L. Rivier, Identification and confirmation criteria for LC–MS, in: A. Polettini (Ed.), Applications of LC–MS in Toxicology, Pharmaceutical Press, London, 2006, pp. 97–110.
[12] GIFT (Group of Italian Forensic Toxicologists), Guide Lines, 2010, http://www.gtfi.it/doc/LG2010.pdf. Accessed on October 14.
[13] R. Agius, P. Kintz, European Workplace Drug Testing Society, Guidelines for European workplace drug and alcohol testing in hair, Drug Test. Anal. 2 (2010) 367–376.
[14] Society of Hair Testing, Recommendations for hair testing in forensic cases, Forensic Sci. Int. 145 (2004) 83–84.
[15] D.S. Harris, E.T. Everhart, J. Mendelson, R.T. Jones, The pharmacology of cocaethylene in humans following cocaine and ethanol administration, Drug Alcohol Depend. 72 (2003) 169–182.
[16] L. Politi, A. Zucchella, L. Morini, C. Stramesi, A. Polettini, Markers of chronic alcohol use in hair: comparison of ethyl glucuronide and cocaethylene in cocaine users, Forensic Sci Int. 172 (2007) 23–27.