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Novel zwitterionic HILIC stationary phase for the determination of ethyl glucuronide in human hair by LC-MS/MS

Diego Palumbo[a], Paolo Fais[b], Anna Calì[c], Monica Lusardi[c], Elisabetta Bertol[a], Jennifer P. Pascali[a,*]

[a] Forensic Toxicology Division, Department of Health Sciences, University of Florence, Largo Brambilla 3, 50134 Florence, Italy
[b] University of Bologna, Department of Medical and Surgical Sciences, Unit of Legal Medicine, Via Irnerio 49, Bologna, Italy
[c] Agilent Technologies, Via V. Lamaro, Rome, Italy

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ABSTRACT

Some recent studies have described a shift from traditional reversed-phase to more hydrophilic LC chemistry for EtG determination in hair (hEtG). The reason relies on the poor retention of C8– and C18-based columns for polar compounds, even in presence of great amount of aqueous phase. This work presents the development, validation and application of a new hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-LC-MS/MS) method based on a novel zwitterionic stationary phase for the analysis of hEtG. The linearity was assessed in the range of 5–100 pg/mg hair, with a correlation coefficient of > 0.99. The method was selective and sensitive, with a limit of detection (LOD) and limit of quantitation (LOQ) of 1.4 pg/mg and 4.5 pg/mg in hair, respectively. Suitable diagnostic sensitivity was achieved without the introduction of a sample purification step, or a post column solvent addition. The method was successfully applied to real hair samples after full validation. This method, based on a separation at neutral conditions, confirmed the optimum retention and thus selectivity for weak acids in zwitterionic HILIC columns.

1. Introduction

Alcohol is the most widely consumed psychoactive substance and is becoming a problematic addiction issue in millions of people worldwide. In fact, unhealthy alcohol use can be either a primary or a secondary cause of liver disease and besides medical complications its abuse can be responsible of severe social problems [1,2]. Additionally, from the forensic point of view, it is of extreme importance to monitor alcohol abstinence in patients undergoing an alcohol withdrawal treatment when a legal cause is undergoing. Laboratory testing traditionally based diagnosis on the assessment of biomarkers such as mean corpuscular volume (MCV), carbohydrate deficient transferrin (CDT) and liver enzymes (gamma glutamyl-transferase, aspartate aminotransferase and alanine aminotransferase, aspartate-amine-transferase and alanine-amine-transferase). In the last few years, increased attention has been paid to ethyl glucuronide (EtG), a new specific alcohol intake marker [3]. It is a direct metabolite of non-oxidative breakdown of ethanol accounting for < 0.1% of total ethanol elimination. Through the measurement of EtG in hair (hEtG), it is possible to assess both the chronic alcohol abuse over time and to document the treatment efficacy of patients in a withdrawal program. However, due to its high polarity, EtG is incorporated into hair only in very small amounts and thus the reliable detection of EtG in hair requires sensitive techniques in reason of the low concentrations (pg/mg range) finally present in the matrix, even in presence of abuse behaviours. Previous researches have shown that analytical methods based on gas chromatography (GC) with mass spectrometry (MS) and liquid chromatography (LC) with MS are the techniques of choice and, to reach appropriate sensitivity, sample preparation based on solid-phase extraction (SPE) is, in most cases, applied to obtain cleaner extracts. The recommended cut-off levels for hEtG diagnostic purposes, suggested by the Society of Hair Testing (SoHT), are substantially two values: 30 pg/mg to distinguish from moderate and heavy alcohol consumption and below 7 pg/mg to pass the medical assessment of alcohol abstinence [4]. A small overview on analytical methods developed for hEtG determination published between years 2000 and 2017 is summarized in Tables 1a and 1b, where data on sample amount, details on analytical method and achieved sensitivity are reported for clarity [5–25,35,37–39]. Although GC–MS technique usually allows to reach lower LOQs also in presence of minor quantity of starting material, GC analysis requires a derivatization step, which contributes to increase complexity and renders the procedure time consuming. For this reason, probably, in recent years most of the

* Corresponding author.
E-mail address: jennifer.pascali@gmail.com (J.P. Pascali).

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Table 1a
Short overview of the available GC–MS methods for the determination of hEtG.

Hair amount (mg)	Cut or ground	Clean up	Limit of quantification (pg/mg)	Technique	References
50	Ground	None	5000	GC–MS EI	[5]
30	Ground	Isolute NH2 SPE	6	GC–MS NICI	[6]
30	Ground	Oasis MAX SPE	2.3	GC–MS NICI	[7]
50	Cut	None	2.4	GC–MS NICI	[8]
20	Cut	Oasis MAX SPE	10	GC–MS/MS EI	[9]
20	Cut	Protein precipitation plate	10	GC–MS/MS EI	[10]
30	Ground	Oasis MAX SPE	8.4	GC–MS/MS NICI	[11]
10–50	Ground	Cleanscreen EtG SPE	2.8	GC–MS/MS NICI	[12]
30	Ground	SPE	0.2	GC–MS/MS NICI	[13]

Table 1b
Short overview of the available LC-MS methods for the determination of hEtG.

Hair amount (mg)	Cut or ground	Clean up	LC column chemistry	Limit of quantification (pg/mg)	Linearity (pg/mg)	References
100	Cut	Isolute NH2 SPE	phenyl-hexyl Synergy Polar-RP	102	25–2000	[14]
100	Cut	None	Chrompack Inertsil ODS-3	3	3–2000	[15]
50	Cut	Oasis MAX SPE	Acquity BEH HILIC	10	20–2000	[16]
100	Ground	None	Hypercarb	50	50–5000	[17]
30	Cut	Cleanscreen EtG SPE	Uptisphere-3SI	10	10–3000	[18]
25	Cut	None	Luna HILIC	20	20–1000	[19]
100	Cut	None	Sinergy Polar RP	4	2–400	[20]
50	Cut	None	Acquity BEH C18	1	1–10	[21]
100	Cut	Oasis MAX SPE	Inertsil ODS-3	20	20–2500	[22]
50	Cut	Isolute NH2 SPE	Zorbax Eclipse XDB-C8	2.6	2–200	[23]
30	Cut	Cleanscreen EtG SPE	Acquity UPLC HSS T3	2	2–330	[24]
25	Ground	Oasis MAX SPE	Hypercarb	2	2–100	[25]
50	Ground	BondElut SAX	Acquity UPLC HSS T3	10	10–500	[37]
75	Ground	None	Hypercarb	2.3	4–400	[35]
30	Cut	Oasis MAX SPE	Synergi 4u fusion RP	3	3–500	[38]
50	Ground	None	Acquity UPLC HSS T3	4.7	n.d.	[39]

attention has been focused on LC-MS technique, which is easily prone to the analysis of more polar compounds in aqueous suspensions. However, many LC-MS methods exploit columns based on reversed-phase chemistry, requiring high amounts of aqueous mobile phase to retain polar compounds such as EtG, but strongly negatively affecting the ionization at MS source level. To overcome such limitation, post-column addition of acetonitrile is often used. In this frame, the introduction of more hydrophilic stationary phases for hEtG analysis have revealed successful since the work by Kintz and colleagues already in 2008 [16]. The term “hydrophilic interaction chromatography” was introduced by Alpert over 25 years ago to describe a liquid chromatography technique where polar or ionised solutes can be separated on a polar stationary phase with polar solvents containing water as a minor constituent of the mobile phase [26]. HILIC separations have undergone an upsurge in interest due to its numerous applications for the analysis of solutes of pharmaceutical, biomedical and clinical analysis, for which the technique is often suitable [27]. The advantages of HILIC include the ability to retain polar and ionic solutes that elute too readily in reversed-phase (RP) chromatography, offering an orthogonality in method development. For these reasons, applications of HILIC separation have been reviewed also recently in many scientific fields, such as pharmaceutical analysis [28], amino acids, peptides and proteins [29], proteomics [30] and metabolomics [31]. The broad range of applications is explained by the great variety of available bonded phases. In fact, HILIC phases can be divided into groups based on their chemical structure, which include neutral (e.g. amide, cyano, diol), positively charged (e.g. amino, imidazole, triazole), negatively charged (poly-aspartic acid, bare silica) and zwitterionic (e.g. sulfobetaine or peptide) [32].

In this work, a proprietary zwitterionic stationary phase bonded on a sub 3 µm superficially porous particles has been adopted for the selective retention of ETG in hair matrix. The purpose of this study was to develop and validate an easy but sensitive method for the analysis of hEtG, exploiting this novel HILIC stationary phase and to discuss the obtained results in terms of sensitivity and sample pre-treatment convenience. The final method has been applied to real hair samples.

2. Materials and methods

2.1. Materials

EtG and the deuterated internal standard (d5-EtG) were obtained from Sigma Aldrich – Saint Louis, USA. Stock solution of EtG (1 mg/ml) was diluted in methanol to working standard solutions at concentrations 0,025–0.05–0,15–0,5 ng/µl. Diluted deuterated internal standard (d5-EtG) solution was prepared in methanol from the 0.1 mg/ml stock solution at the concentration of 0,250 ng/µl. All solutions were stored at − 20 °C and left at room temperature at least 2 h for equilibration prior use. Methanol, acetonitrile and water for mobile phases preparation (all LC–MS grade) were purchased from Biosolve Chemie SARL – (Dieuze, France). Ammonium acetate was acquired from Carlo Erba Reagents (Milan, Italy).

2.2. Hair samples preparation

All hair samples for analysis were collected from the vertex posterior region. From these samples a proximal 3 cm segment was used. For validation and analysis, 50 mg of hair were used and kept stored under dry conditions at room temperature until analysis. The selected hair segment was washed three times by shaking the sample with 20 ml of methanol for 5 min. The hair samples were subsequently left to dry at room temperature and then cut into small pieces. 50 mg of hair were weighted and 2500 pg d5-EtG as internal standard and 350 µl of water were added carefully to soak all the material. Samples were incubated overnight and then ultrasound extraction at 50 °C was applied for 2 h. The extract was collected, taken to dryness at 50 °C using a metal