Journal of Chromatography B 1076 (2018) 77–83

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Supercritical fluid extraction (SFE) of ketamine metabolites from dried urine and on-line quantification by supercritical fluid chromatography and single mass detection (on-line SFE–SFC–MS)☆

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ARTICLE INFO

Keywords:
Ketamine
On-line SFE–SFC–MS
Supercritical fluid extraction
Supercritical fluid chromatography
Validation

ABSTRACT

On-line solid-phase supercritical fluid extraction (SFE) and chromatography (SFC) coupled to mass spectrometry (MS) has been evaluated for its usefulness with respect to metabolic profiling and pharmacological investigations of ketamine in humans. The aim of this study was to develop and validate a rapid, highly selective and sensitive SFE–SFC–MS method for the quantification of ketamine and its metabolites in miniature amounts in human urine excluding liquid-liquid extraction (LLE). Several conditions were optimized systematically following the requirements of the European Medicines Agency: selectivity, carry-over, calibration curve parameters (LLOQ, range and linearity), within- and between-run accuracy and precision, dilution integrity, matrix effect, and stability. The method, which required a relatively small volume of human urine (20 µL per sample), was validated for pharmacologically and toxicologically relevant concentrations ranging from 25.0 to 1000 ng/mL ($r^2 > 0.995$). The lower limit of quantification (LLOQ) for all compounds was found to be as low as 0.5 ng. In addition, stability of analytes during removal of water from the urine samples using different conditions (filter paper or ISOLUTE® HM-N) was studied. In conclusion, the method developed in this study can be successfully applied to studies of ketamine metabolites in humans, and may pave the way for routine application of on-line SFE–SFC–MS in clinical investigations.

1. Introduction

Hyphenation of supercritical fluid extraction (SFE) with separation techniques like liquid (LC), gas (GC) or supercritical fluid chromatography (SFC) is not new to analysts' laboratories. Introduced in the 1980s and early 1990s, a considerable number of online hybrid systems were published: SFE–HPLC [1–3], SFE–GC [4], SFE–SFC–GC [5] and SFE–SFC [6–8], to name just a few. The advantages of on-line compared to off-line systems are as obvious as they are operationally simple: the time for sample preparation is reduced to a minimum, as well as the risk of cross contamination due to lower numbers of preparation steps, analytes susceptible to oxidation and/or degradation can be detected in their native forms and total automatization of the analytical system is but a step away [9]. In spite of these advantages, hyphenated SFE–-systems are not routinely used; instead, off-line analytics are still in practice, necessitating conventional sample preparation like solid-phase (SPE) or liquid-liquid extraction (LLE), protein precipitation and filtration or application of dried matrix spot techniques. Although the technical equipment has improved dramatically over the last few years, on-line systems did not disseminate to the same extent, and recent publications such as determination of unstable lycopene in food [10], quantification of complex polymer additives [11] and reactive quinone analysis [12] only hint at the magnitude of possible applications.

The combination of SFE and SFC in particular offers compelling advantages, like mild extraction conditions and short analysis times due to high diffusion properties and low viscosity of the mobile phase. Supercritical carbon dioxide (sCO_2) is used most commonly because it is non-flammable, non-toxic, easily available and combines favorable physical characteristics which enable a convenient conversion to its supercritical state. Regarding polarity, sCO_2 is highly lipophilic and

Abbreviations: CYP, cytochrome P450; DHK, dehydronorketamine; KET, ketamine; NK, norketamine; KET-d_4, ketamine-d_4; HNK, hydroxynorketamine; SFE, supercritical fluid extraction; SFC–MS, supercritical fluid chromatography single quadrupole mass spectrometry; LC–MS/MS, liquid chromatography tandem mass spectrometry; BPR, back pressure regulator; IPA, 2-propanol; MeOH, methanol; LLQC, lower limit of quantification; ULOQ, upper limit of quantification; sCO_2, supercritical carbon dioxide; LLE, liquid-liquid extraction; SPE, solid-phase extraction

☆ In memory of Professor Dr. rer. nat. Dr. h. c. Ernst Günter KLESPER (1927–2017), pioneer in supercritical fluid chromatography, who would have turned 90 on the 6th of December 2017.

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https://doi.org/10.1016/j.jchromb.2018.01.024
Received 29 November 2017; Received in revised form 11 January 2018; Accepted 16 January 2018
1570-0232/

therefore possesses excellent solvation power especially for hydrophobic compounds [13]. Moreover, addition of organic modifiers (mostly aliphatic alcohols like methanol, ethanol or isopropanol) to increase the polarity of the mobile phase, has made extraction of more hydrophilic compounds possible as well, yet SFE–SFC hyphenated systems are still not commonly used. Besides the relatively high procurement costs of such a system, the paramount issue might be the scarceness of reliable and validated methods.

Earlier publications examined the redox status of coenzyme Q10 from in vitro experiments [14] or investigated phospholipids from dried plasma spots [15]. More recently, attention was drawn to disease biomarkers in dried serum spots [16] and carotenoids from red habaneros (*Capsicum chinense* Jacq.) [17]. To the best of our knowledge, however, no method has been described that is fully validated according to internationally accepted guidelines like EMA's [18] and deals with human urine as biological matrix.

Complex matrices like urine pose several difficulties: on the SFE part it is crucial that the sample is loaded onto solid-phase materials which release the compounds of interest completely and reproducibly. While SPE for HPLC applications is typically performed by passing the aqueous sample over an octadecylsilyl extraction cartridge and subsequent elution with organic solvents, it is more convenient in SFE-SFC to dry the aqueous phase prior to extraction with sCO_2. The simplest adsorbent is a piece of filter paper, however, more sophisticated and standardized preparation units such as dried blood spot cards have been developed. Additionally, materials based on silica gel with high surface areas should be considered for their favorable stationary phase properties. In any case, the adsorbent used must be inert towards analytes and should be of high purity to keep background noise at a low level.

Transfer of the extract to the analytical column is also among the technical difficulties linked to hyphenated SFE–SFC systems. The concept of cryogenic focusing or trapping was commonly utilized, as Ashraf-Khorassani et al. among others showed [19,20], but use of the analytical column for both collection and analysis of the extract at the same time has proven to be an elegant approach. However, this can have negative effects on the separation efficiency, since parts of early extracted compounds may start eluting, while the extraction progress is not yet completed. The aim of this process should be to focus the extract on the column head and to start the analysis not before the extraction is completed. The key to this problem is to use only low concentrations of modifier during SFE, since the elution power of pure sCO_2 on normal phase columns is marginal.

Initially designed as an anaesthetic drug substance, ketamine (KET) has meanwhile proven to be an effective option for the treatment of major depressive (MDD) or mood disorder (MD) [21–23]. Although subject to intensive research, it is still not clear whether KET itself or one of its metabolites is causative for the antidepressant effects, owing to the complex metabolism of racemic KET and its chiral degradation products [24,25]. KET is heavily metabolized by cytochrome P450: demethylation yields (*R*)- and (*S*)-norketamine (*R*-NK and *S*-NK), with subsequent oxidation producing dehydrometabolites, such as (*R*)- and (*S*)-dehydronorketamine (*R*-DNK and *S*-DNK). Hydroxylation introduces a second stereogenic center, thus giving rise to at least 12 hydroxynorketamine (HNK) isomers differing in either constitution, stereochemistry or both, among which (2*R*,6*R*)-hydroxynorketamine (*RR*-HNK) has proven particularly promising with regards to antidepressant effects [26].

Therefore, several quantification methods have been devised and optimized to investigate KET metabolites in human matrices (see [27] for an overview). Since then, Toki et al. [28] improved the methodology by decreasing the matrix volume required for analysis to 2.5 µL, but investigated only KET and NK which could be insufficient, if *RR*-HNK is confirmed as the active agent in effective MDD treatment. Similarly, Ramiole et al. [29] shed new light on the importance of sample preparation techniques (yielding superior results with SPE treatment compared to both LLE and acetonitrile precipitation), but neither included HNK into their investigation nor achieved enantioselectivity. This could prove detrimental for future pharmacological studies, since KET and its metabolites exhibit high eudysmic ratios. Our previously described SFC-method enantioselectively separated the relevant metabolites, but necessitated 1 mL of biological matrix and laborious sample preparation by LLE [30]. While procuring this volume from adult patients might be unproblematic, there are cases where every µL is precious. One such instance are critical matrices like spinal fluid, but even plasma can be a restraining factor, for instance in neonates, where retrieval of 1 mL of blood can be equivalent to removing 70 mL from an adult [31], or urine of small rodents used in animal studies [28]. In this work, we describe a fully validated method that uses a hyphenated SFE–SFC system for the enantioselective quantification of ketamine and all relevant metabolites from only 20 µL of an easily accessible matrix such as urine. The automated approach could have a great impact on further pharmacokinetic studies of ketamine with patients suffering from MDD or MD, as well as sample preparation for pharmacokinetic studies in general.

2. Material and methods

2.1. Chemicals

Carbon dioxide (99.995% purity) was obtained from Air Liquide (Duesseldorf, Germany). 2-Propanol (IPA) was provided by Carl Roth (Karlsruhe, Germany), methanol (MeOH) and qualitative “415” filter paper by VWR (Leuven, Belgium), formic acid by Fisher Scientific (Geel, Belgium) and ISOLUTE® HM-N from Biotage Europe (Uppsala, Sweden). All additives were purchased in LC–MS grade purity. Ammonia (25% aqueous solution), acetonitrile (ACN), the internal standard (racemic, tetradeuterated KET-d_4), as well as *R*- and *S*-KET, *rac*-NK and *rac*-DNK were bought from Sigma-Aldrich (Steinheim, Germany). The National Center for Advancing Translational Sciences (Rockville, Maryland, USA) kindly provided (2*S*,6*S*)-HNK and (2*R*,6*R*)-HNK (all analytes were provided as hydrochlorides). Stock solutions were prepared in ACN and stored at −20 °C, while working solutions were prepared weekly in IPA and stored at 4 °C.

2.2. Analytical instruments

Data acquisition was realized using a Nexera SFE-SFC/UHPLC switching system (Shimadzu Corporation, Kyoto, Japan) directly coupled to a Shimadzu LCMS-2020 single quadruple mass spectrometer. The system consisted of two LC-20ADXR pumps for delivering modifier and make-up flow, a LC-30ADSF pump for liquid CO_2, two SFC-30A back pressure regulators (BPR), an SFE-30A auto-extractor equipped with 0.2 mL extraction vessels, a SIL-30 AC autosampler, a CTO-20 AC column oven, a DGU-20A5R degasser and a CBM-20A communication module. The SFC–MS instruments were controlled by Shimadzu's LabSolution Version 5.82 software.

2.3. Sample preparation for online supercritical fluid extraction

SFE samples were prepared by charging extraction vessels with ISOLUTE® HM-N (20 mg), spiked urine (20 µL), and 10 µL of internal standard (KET-d_4, 1 µg/mL). Vessels were placed into a vacuum oven (51 °C, 7 mbar, 1 h), before proceeding with extraction.

2.4. Extraction and analytical conditions

On-line extraction was performed in 97% of CO_2 (A) and 3% modifier (B) (all ratios as v/v). Modifier consisted of IPA augmented by aqueous ammonia solution to a final concentration of 0.075% NH_3. After 3.0 min of static extraction at 25 °C and BPR pressure of 163 bar (0–3 min), dynamic extraction followed for 30 s (3.0–3.5 min). The end of extraction was marked by setting BPR pressure to 400 bar at the