Journal of Pharmaceutical and Biomedical Analysis 117 (2016) 155–162

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Methods for differential and quantitative analyses of brain neurosteroid levels by LC/MS/MS with ESI-enhancing and isotope-coded derivatization

Tatsuya Higashi*, Naoto Aiba, Tomoya Tanaka, Kazumi Yoshizawa, Shoujiro Ogawa

Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

ARTICLE INFO

Article history:
Received 22 July 2015
Received in revised form 26 August 2015
Accepted 28 August 2015
Available online 29 August 2015

Keywords:
Neurosteroid
Rat brain
LC/ESI–MS/MS
Isotope-coded derivatization
Antipsychotic drug

ABSTRACT

The analysis of changes in the brain neurosteroid (NS) levels due to various stimuli can contribute to the elucidation of their physiological roles, and the discovery and development of new antipsychotic agents targeting neurosteroidogenesis. We developed methods for the differential and quantitative analyses of the brain levels of allopregnanolene (AP) and its precursor, pregnenolone (PREG), using liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI–MS/MS) combined with derivatization using 2-hydrazino-1-methylpyridine (HMP) and its isotope-coded analogue, $^{2}H_{3}$–HMP (*d*-HMP). For the differential analysis, the brain sample of an untreated rat was derivatized with HMP, while the brain sample of a treated (stressed or drug-administered) rat was derivatized with *d*-HMP. The two derivatives were mixed and then subjected to LC/ESI–MS/MS. The stress- and drug (clozapine and fluoxetine)-evoked increases in the brain AP and PREG levels were accurately analyzed by the developed method. It was also possible to determine the absolute concentrations of the brain steroids when a deuterium-coded moiety was introduced to the standard steroids of known amounts by the derivatization and the resulting derivatives were used as internal standards. The HMP-derivatization enabled the highly sensitive detection and the use of *d*-HMP significantly improved the assay precision [the intra- ($n = 5$) and inter-assay ($n = 5$) relative standard deviations did not exceed 13.7%] and accuracy (analytical recovery ranged from 98.7 to 106.7%).

1. Introduction

The term "neurosteroid (NS)" was introduced to name steroids that have an effect on the central nervous system (CNS) through action at the membrane ion-gated and other neurotransmitter receptors. 3α-Hydroxy-5α-pregnan-20-one (allopregnanolone, AP), one of the most important endogenous NSs, binds to γ-aminobutyric acid type A ($GABA_A$) receptors with high affinity and positively modulates the GABA action at these receptors [1–3] (Fig. 1). It has also been demonstrated that the endogenous AP in the brain is rapidly elevated to a nanomolar concentration by several acute stress paradigms, such as immobilization, in animal models [4–6], which is proposed to reflect a homeostatic mechanism for raising the threshold of brain excitability during the response to stressful stimuli [1,3]. Furthermore, abundant evidence also suggests that AP is relevant to the antipsychotic drug action and pathophysiology of depression and anxiety disorder. For example, Pinna et al. demonstrated that fluoxetine (FLX), which is a typical selective serotonin reuptake inhibitor (SSRI) and is clinically used for the treatment of depression, increases the brain AP concentration (de novo AP biosynthesis in the brain) and subsequent activation of $GABA_A$ receptors at doses that are inactive toward the serotonin reuptake [7,8]. It has also been reported that clozapine (CLZ), an atypical antipsychotic drug, elevates the brain AP concentration by its action on the peripheral-type benzodiazepine receptor (18 kDa translocator protein) and has anxiolytic effects in behavioral models [9].

A method for the analysis of changes in the brain NS levels can contribute to the elucidation of their physiological roles, and the discovery and development of new antipsychotic agents targeting neurosteroidogenesis. Recently, liquid chromatography (LC) coupled with electrospray ionization (ESI)–tandem mass spectrometry (MS/MS) has been used for the determination of NSs due to its specificity and versatility [5,6,10,11]. However, the ionization efficiency of some NSs is not high in ESI, and therefore, this insufficient sensitivity might become a major problem in the analysis of trace NSs.

* Corresponding author. Fax: +81 4 7121 3660.
E-mail address: higashi@rs.tus.ac.jp (T. Higashi).

http://dx.doi.org/10.1016/j.jpba.2015.08.040
0731-7085/

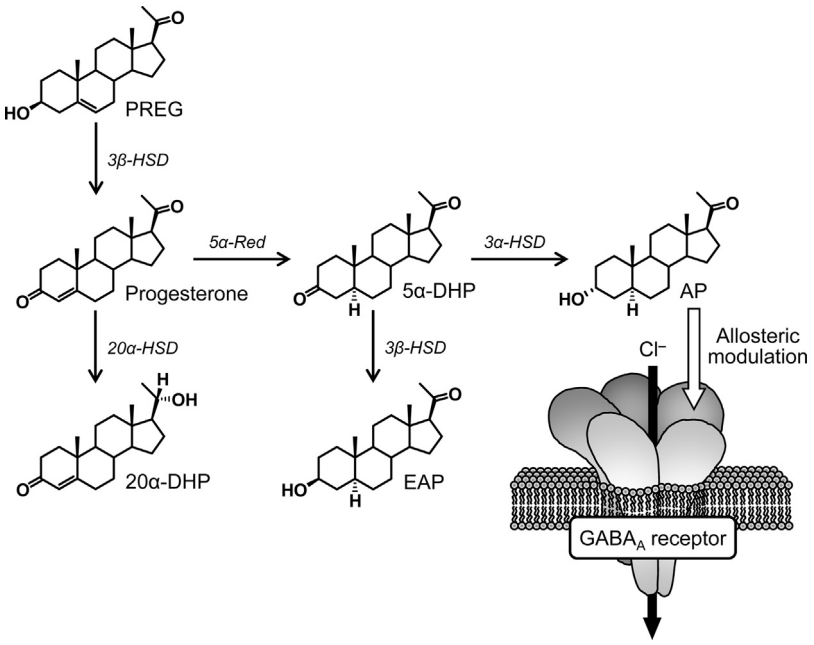

Fig. 1. Biosynthetic pathway and action mechanism of AP. HSD; hydroxysteroid dehydrogenase and Red; reductase.

The use of 2-hydrazino-1-methylpyridine (HMP), a derivatization reagent that we developed (Fig. 2a) [12], will probably be able to solve this problem; the HMP-derivatization improved the detection limit of AP to 0.31 fmol (equivalent to 0.1 pg of intact AP) as will be described later.

The ESI efficiency is sometimes different between two single runs even for the same analyte. The stable isotope-coded derivatization (ICD) has been recently used as a method to correct the run-to-run ionization differences, including matrix effects, for precise analysis [13–16]. The differential analysis (relative quantification) of NSs in two comparative samples can also be done by application of the ICD technique. For example, the brain sample of an untreated rat is derivatized with the H-coded reagent (HMP), while the brain sample of a treated (stressed or drug-administered) rat is separately derivatized with the isotope (^{2}H)-coded reagent (*d*-HMP) (Fig. 2b). The two derivatives are mixed and then subjected to LC/MS/MS. Since the isotopic pairs of the derivatives elute at almost the same time in a single run, the ionization process for the HMP-derivatized NSs are expected to be identical with the *d*-HMP-derivatized NSs. By comparing the peak areas of the HMP- and *d*-HMP-derivatives, the differential analysis of NSs in two comparative samples can be accurately performed. Furthermore, it is possible to determine the absolute concentrations of the NSs in the brain when a stable isotope-coded moiety is introduced to the standard NSs of known amounts by the derivatization and the resulting derivatives are used as internal standards (ISs). Thus, the ICD can improve the analytical performance and precision.

In this study, we developed methods for the analyses of NSs in the rat brain by LC/ESI–MS/MS combined with the ICD. The application of the methods to the analyses of changes in the NSs levels by immobilization stress and drug administration is also described.

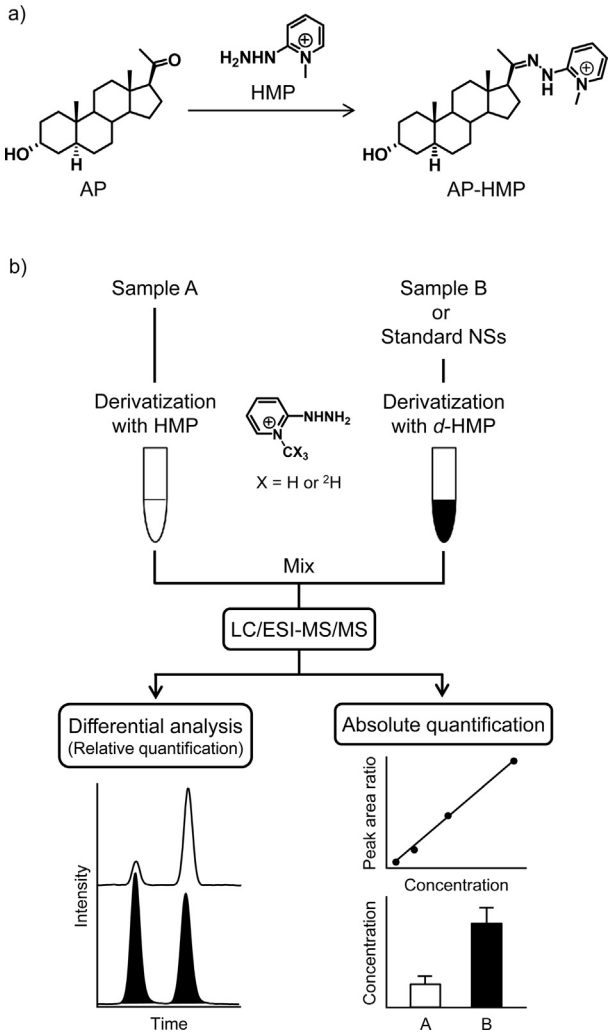

Fig. 2. (a) Derivatization scheme of AP with HMP and (b) scheme of differential and quantitative analysis procedures of brain steroid levels based on ICD technique.

2. Experimental

2.1. Material and reagents

AP, 3β-hydroxy-5-pregnen-20-one (pregnenolone, PREG), 3β-hydroxy-5α-pregnan-20-one (epiallopregnanolone, EAP), 5α-pregnan-3,20-dione (5α-dihydroprogesterone, 5α-DHP) and 20*R*-hydroxy-4-pregnen-3-one (20α-dihydroprogesterone, 20α-