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Quantification of total content of non-esterified fatty acids bound to human serum albumin

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ABSTRACT

Non-esterified fatty acids bound to the human serum albumin (HSA) contribute to several HSAs properties of special concern in pathologies, for instance to the reactivity of the free HSA-Cys34 thiol group (important antioxidative thiol pool in plasma), and to the affinity for binding of molecules and ions (for example cobalt as a prominent biomarker in heart ischemia). Therefore, the method for determination of FAs bound to HSA was developed. FAs were released from HSA (previously isolated from serum by ammonium sulfate precipitation) using acidic copper(II) sulfate in phosphoric acid, extracted by *n*-heptane-chloroform (4:1, v/v) mixture, spotted on TL silica-gel and then developed with *n*-heptane-chloroform-acetic acid (5:3:0.3, v/v/v). Common office flatbed scanner and software solution for densitometric image analysis, developed in R, were used. The linearity of calibration curve in concentration range from 0.1 to 5.0 mmol/L stearic acid was achieved. The method was proved to be precise (with RSD of 1.4-4.7%) and accurate. Accuracy was examined by standard addition method (recoveries 97.2-102.5%) and by comparison to results of GC. The method is sample saving, technically less demanding, and cheap, and therefore suitable for determination of FAs/HSA ratio when elevated concentrations of free FAs are reliable diagnostic/risk parameter of pathological states.

1. Introduction

Human serum albumin (HSA) is the most abundant protein of human plasma (approx 50–60% of total plasma proteins) with many physiological functions [1]. Although considered mainly as blood plasma protein, much of the HSA is localized extravascularly, almost double of the intravascular pool [1,2]. HSA is a transporter of many endogenous substances including non-esterified fatty acids (FAs), bilirubin, bile salts, steroid hormones, hematin, tryptophan, tyroxine, some vitamins, several metal ions and drugs [1,2]. The important is FAs transporting function, because: FAs are present in enough quantity comparable with HSA abundance, FAs are amphipathic molecules almost exclusively circulating carried by HSA, and bound FAs reflect on the HSA tertiary structure and HSA molecule properties [3]. HSA has seven well defined, and crystallographically characterized, binding pockets for long-chain fatty acids [3], but additional binding places emerge for some FAs under *in vitro* experimental conditions. Fig. 1 shows HSA molecule with stearic acid (STE), constructed using crystallographic data pdb id 1e7i.

It was shown recently that HSA free cysteine-34 thiol contributes in the scavenging of reactive oxygen, nitrogen and carbonyl species [4,5]. Kinetics studies of the reactivity of HSA free Cys34 thiol with methylglyoxal, as potent representative of the carbonyl stress, showed that bound FAs molecules modify reaction kinetics, changing pseudo-first order kinetics constant [6], thus change the course of that scavenging process. In addition, according to epidemiological studies, elevated fasting circulating non-esterified FAs were an independent risk factor for sudden death in middle-aged men free of known cardiovascular disease

Abbreviations: CRAN, a network of web servers around the world that store identical, up-to-date versions of code and documentation for R; FA, fatty acid; FAMEs, fatty acids methyl esters; GNU license, the GNU General Public License (GNU GPL or GPL) is a free software license which guarantees end users (individuals organizations companies) the freedoms to run study share (copy) and modify the software; HPTLC, High performance thin-layer chromatography; qTLC, quantitative thin-layer chromatography; R, programming language and environment for statistical computing and graphics supported by the R Foundation for Statistical Computing; STE, Stearic acid.

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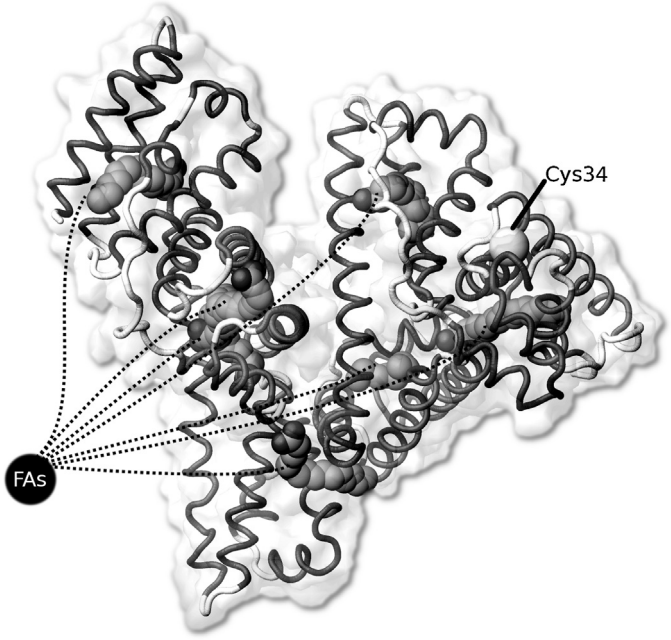

Fig. 1. HSA with STE. The image was constructed using crystallographic data from pdb id 1e7i using Jmol program. Seven STE molecules were bound to the well-defined FAs binding pockets.

[7]. High concentrations of free FAs are associated with insulin resistance (risk factors for the development of type 2 diabetes) [8,9], fatty liver disease [10,11], atherosclerosis [12] and myocardial dysfunction [7,13]. Furthermore, an intriguing FDA approved biomarker termed ischemia-modified albumin (IMA) was defined as early marker for diagnosis of myocardial ischemia [14,15]. However, recent studies suggests that IMA cobalt-binding HSA assay probably depends on elevated free FAs during ischemia, and their binding to HSA could induce significantly reduction of Co^{2+} binding to HSA [16]. That conclusion opens debate whether cobalt binding assay is indirect measurement of FAs bound to HSA, and could it be replaced with direct quantification of bound FAs and FAs/HSA ratio. Based on all of this and on the fact that FAs are predominantly bound to HSA, the quantification of FAs bound to HSA could be very important.

Several methods for determination of non-esterified fatty acids in different biological samples were described. Gas chromatography (GC) quantification of FAs is very accurate and reproductive method [17], but it is time consuming, requires specific instrumentation, and if the analysis was performed from ~0.5 mmol/L HSA solution, it requires significant amount of starting solution for FAs extraction, and consequent fatty acids methyl esters (FAMEs) preparation. Furthermore, precise FAs extract composition in term of quantification of various fatty acids, was not important in every examination of physiological samples, because of HSA binding discrepancy among fatty acids [3]. Thus, single GC analysis could be valuable as FAs profile for many samples, and much cheaper, less time consuming, and sample-saving method should be used for every-day quantification of FAs. Several methods with these criteria were described in literature, mostly spectrophotometric methods based on the FA-transition metal salts formation (often copper, and cobalt salts) in aqueous phase, their extraction in chloroform, then complexing of transition metal ions with corresponding compound to form a color product, and then measuring absorbance at defined wavelength, or using colorimetric studies with appropriate light filter [18,19].

The spectrophotometric methods, proposed in the literature for determination of FAs, were tested, but neither of them could be used for determination of FAs bound to HSA, probably due to high interferences of copper/cobalt ions with protein, and buffer components, but also because of nanomole quantities of FAs, around lower detection limits of those methods. Extraction of FA-metal salts with organic solvent was crucial step in all FA-metal salt methods, and it was merely impossible to achieve identical extraction and separation of phases with each repetition, so this step was major source of experimental error. Other spectrophotometric methods utilizing enzyme kinetics of certain enzymes of fatty acids metabolism [20], coupled NAD-NADH cofactors for standard 339 nm quantification, often were not acceptable because of high costs of those assays, comparable, or even bigger than commercial GC analysis, which was in contrast with the goal of this paper to establish a quantification method suitable for everyday laboratory practice (accurate and precise, but simple, technically less demanding and cheap).

High quality commercial HPTLC plates with silica-gel sorbent coated onto aluminium sheet, makes TLC analysis faster, more reliable in terms of reproducibility, and easier for borrowing digital image using flatbed scanner and computer. Densitometric analysis of digital image versus TLC densitometer has many advantages, does not require expensive instrumentation, but gives comparable results [21]. After initial screening of FAs content during kinetics studies of HSA Cys34 thiol group [6], and taking into account the importance of determination of FAs bound to HSA in clinical practice, the high performance TLC method for this purpose was developed. Using the small serum volumes, HSA was isolated by ammonium sulfate precipitation. The conditions for FAs extraction from HSA were optimized, and the aliquots of extracts were directly applied to the TLC plate. Also, the specific statistical software solution in R language (freely available under GNU license, and as R library from the CRAN repository https://cran.r-project.org/package=qtlc) for image processing and densitometric analysis of scanned qTLC plates was developed.

2. Materials and methods

2.1. Chemicals

Chemicals were purchased from Sigma (Steinheim, Germany) unless otherwise noted. Solution of 20% HSA was purchased from Baxter (Vienna, Austria). All chemicals used were analytical grade. Deionized water was used for all experiments. Professional laboratory glassware and plastics (sample tubes, Pasteur pipettes, and pipette tips, Brand Wertheim, Germany) were used. Commercial silica gel plates for quantitative TLC (TLC Silica gel 60 F_{254}, lot HX265727) were purchased from Merck (Darmstadt, Germany).

2.2. HSA sample preparation

Blood samples of 5 healthy individuals and 7 patients with metabolic syndrome were collected. Informed consent was sought from all participants. Blood was allowed to clot at room temperature, and serum was separated by centrifugation (2500 rpm, 5 min).

Isolation of HSA from serum was performed by ammonium sulfate (AS) precipitation in two steps [22]. The first step was performed using saturated AS (pH adjusted to 7.4 using 0.1 M sodium hydroxide) and adding to 1 mL of the serum sample with gently stirring until the concentration of AS was 54% (1.174 mL of saturated AS). Precipitated proteins (mostly globulins) were then removed after centrifugation at 5000 rpm for 10 min (MiniSpin centrifuge, Eppendorf, Hamburg, Germany) and supernatant decanted. The second step was performed in the same manner by adding saturated AS to 70% final concentration in the supernatant, collected in the first step (1.159 mL of saturated AS). Following 10 min centrifuged at 5000 rpm and separating supernatant from