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• 综述 •

液相色谱-质谱法分析生物体内低丰度蛋白多肽的研究进展

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[摘要] 蛋白多肽是一类具有生物活性的物质, 对维持机体各项功能的稳定发挥着至关重要的作用, 快速、准确地检测其水平有助于疾病的诊断、药物治疗效果的监测及药物的研发等, 在临床医学、生物学及药学等领域具有重要意义。传统的蛋白多肽检测方法(如蛋白质印迹法和 ELISA 等)存在灵敏度低、难以同时测定多个待测物等问题。液相色谱-质谱法(LC-MS)具有专属、灵敏、高通量等优势, 但蛋白多肽大分子的低离子化效率及强基质效应等限制了其直接检测蛋白多肽的可行性, 基于 LC-MS 的信号转化与放大策略应运而生。本文针对近年来基于 LC-MS 分析生物体内蛋白多肽的样品前处理方法和信号转化与放大策略的研究进展进行归纳总结, 为基于 LC-MS 开发复杂样品中低丰度蛋白多肽的专属高敏检测方法提供参考。

[关键词] 蛋白多肽; 液相色谱-质谱法; 样品前处理; 信号转化与放大

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Liquid chromatography-mass spectrometry analysis for low-abundance protein polypeptides *in vivo*: research progress

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[Abstract] Protein polypeptides are a class of bioactive substances that play a crucial role in maintaining the stability of various functions of the organism. Rapid and accurate detection of their levels could help in the diagnosis of diseases, the monitoring of drug therapy and the research and development of medicines, which is of great significance in the fields of clinical medicine, biology and pharmacy. Conventional protein polypeptides detection methods, such as Western blotting and enzyme-linked immunosorbent assay, still have problems like low sensitivity or difficulty in determining more than 1 analyte simultaneously. Liquid chromatography-mass spectrometry (LC-MS) has the advantages of specificity, sensitivity and high throughput. However, low ionization efficiency of macromolecules and strong biological matrix effects limit the feasibility of direct detection of protein polypeptides by LC-MS, which has led to the development of signal conversion and amplification strategies based on LC-MS technology. In this review, we summarized the research progress of sample pretreatment methods and signal conversion and amplification strategies for quantification of protein polypeptides *in vivo* based on LC-MS in recent years, providing a reference for developing specific high-sensitivity detection methods for low-abundance protein polypeptides in complex samples based on LC-MS technology.

[Key words] protein polypeptides; liquid chromatography-mass spectrometry; sample pretreatment; signal conversion and amplification

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生物体内低丰度蛋白多肽作为生命活动的重要调控因子,其精确检测与分析对于揭示生物过程机制及疾病诊断治疗至关重要^[1-2]。然而,低丰度特性使得其检测面临巨大挑战。近年来,随着分析技术的不断进步,特别是液相色谱-质谱法(liquid chromatography-mass spectrometry, LC-MS)为低丰度蛋白多肽的分析提供了新的可能性^[3-5]。样品前处理方法的优化及基于 LC-MS 的信号转化与放大策略的应用,为低丰度蛋白多肽的专属灵敏检测提供了有力支持^[6-8]。本文旨在全面梳理生物体内低丰度蛋白多肽的 LC-MS 分析方法研究进展,重点关注样品前处理方法和基于 LC-MS 的信号转化与放大策略,以期对相关领域的研究提供有益的参考,推动低丰度蛋白多肽分析技术的发展。

1 蛋白多肽体内分析的意义

蛋白质和多肽同属于具有生物活性的氨基酸聚合物^[9]。蛋白质由 1 个或多个氨基酸残基长链组成,是较大的多肽结构;而多肽由较短的氨基酸链组成,是相对较小的分子,可以通过蛋白水解产生^[9-10]。蛋白多肽在维持机体组织功能^[11]、运输和储存物质分子^[12]、调节激素^[13-14]及免疫反应^[15]等生物活动中发挥着至关重要的作用。

蛋白多肽类物质包括蛋白多肽类生物标志物、靶标蛋白多肽及蛋白多肽类药物等^[16-18]。其中,蛋白质和多肽作为生物标志物可用于诊断疾病、监测治疗效果及评估疾病进展。研究表明,组织或体液中蛋白多肽浓度的变化及异常表达与一些重大疾病的发生、发展密切相关,如动脉粥样硬化、帕金森病、阿尔茨海默病、乳腺癌、宫颈癌等^[19-21]。生物样本中特定蛋白质或多肽,可作为疾病诊断或预后的潜在指标。蛋白多肽类药物具有特异性强、免疫原性低、不良反应少、疗效显著等特点,在新药研发领域越来越受重视^[22]。随着生物技术和医学的发展,已有上百种蛋白多肽类药物被开发上市,应用范围涉及肿瘤、过敏反应、代谢疾病及心血管疾病等^[23]。然而,蛋白多肽通常分子量较大、结构复杂、体内浓度低且存在严重的内源性干扰,体内定量非常困难,相关定量研究对检测方法的通量、灵敏度、特异性及准确度等有较高要求^[24]。

2 蛋白多肽体内分析的常规方法

监测、分析生物体内蛋白多肽类物质的水平变

化以及选择一种可靠的检测手段,无论在临床医学还是在生物药物分析领域,都具有重要意义。目前已报道的蛋白多肽体内检测方法有比色法^[25]、分光光度法^[26]、荧光法^[27]、电化学法^[28]、免疫分析法^[29]及质谱法(mass spectrometry, MS)^[30]等。

免疫分析法(如蛋白质印迹法和 ELISA)是实验室中应用最广泛的体内蛋白多肽测定法^[29,31]。蛋白质印迹法前处理过程简单,可以直接从粗细胞或组织提取物中对蛋白质进行分析,但方法执行过程中引入的各种因素变化都会影响结果的稳定性和重现性,该方法在蛋白多肽准确定量时较少使用,多用于蛋白质的鉴定或半定量估测^[32-34]。ELISA 凭借操作流程简单、对目标蛋白多肽选择性高的优势,成为免疫测定的“金标准”,被广泛用于生物医学研究、疾病诊断及药物开发等,是复杂样品中目标蛋白多肽准确定量的常用方法之一^[35-36]。然而,ELISA 法存在一些固有局限性,最显著的问题是 ELISA 法难以实现对同一生物样本中的多个目标蛋白多肽同时测定,这意味着检测多种蛋白多肽时需要进行多次独立的 ELISA,这不仅增加了实验操作的复杂性,还可能引入更多的误差^[37]。此外,ELISA 法的检测灵敏度受多种因素影响。标志酶稳定性较差、存储时间短、负载能力有限等都可能导致检测结果的偏差或不稳定^[38]。因此免疫分析法虽因操作简便在实验室中广为使用,但往往难以满足对于准确度和灵敏度有要求且需同时测定多组分的实验需求^[39]。越来越多的研究者开始探索新的检测方法,如 LC-MS^[40],以期能够更精确、更高效地检测生物体内的低丰度蛋白多肽。

3 基于 LC-MS 分析体内蛋白多肽

LC-MS 作为一种在小分子检测领域具有绝对优势的灵敏、准确的分析方法,在分析化学领域得到广泛应用^[41-42]。然而,受离子化效率的影响,LC-MS 在蛋白多肽等大分子化合物分析中的应用有限^[43]。近年来,随着信号转化与放大策略的发展,通过将不易被直接检测到的待测物信号转化为易于检测或检测灵敏度高的化合物响应信号,可以克服待测物不易被直接检测的困难,进一步增强检测灵敏度,因此基于 LC-MS 检测蛋白多肽的信号转化与放大策略应运而生^[44-45]。血浆或组织等生物样品中包含有数千种不同的蛋白质或多肽,在

LC-MS 分析之前往往需要对样品进行前处理, 样品前处理结束后, 再通过一定的技术手段如构建探针、标记标签等, 建立靶蛋白多肽和便于检测的高敏分子之间的量比关系^[46-47], 进一步提高生物体

内低丰度靶蛋白多肽的检测专属性、灵敏度、通用性以及多组分同时检测的能力。有关基于 LC-MS 对蛋白多肽体内分析的样品前处理方法及信号转化与放大策略的流程图见图 1。

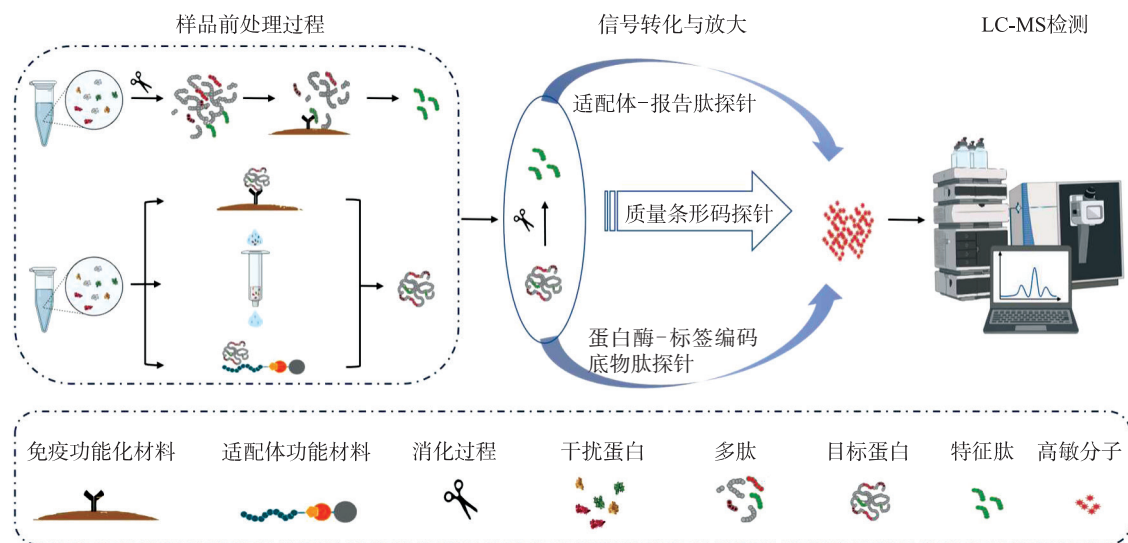


图 1 基于 LC-MS 检测体内蛋白多肽的总流程图

LC-MS:液相色谱-质谱法。

3.1 样品前处理方法 在生物样品中, 多数目标蛋白多肽类物质相对丰度低, 对其进行体内分析易受其他内源性物质或性质接近的高丰度非目标蛋白等基质的干扰, 所以用 LC-MS 定量分析蛋白多肽类物质之前需借助一定的纯化或富集处理手段来提高定量灵敏度和选择性。已报道的蛋白多肽富集方法根据作用原理可分为基于抗原-抗体反应的免疫亲和富集 (immunoaffinity enrichment, IAE) 及不基于抗体的富集方法^[48-49]。

3.1.1 IAE IAE 利用抗体与靶蛋白或多肽之间的高特异性以及高亲和力相互作用原理, 将目标分析物从复杂的样品基质中纯化提取, 便于 LC-MS 检测。IAE 可以在蛋白质或肽水平进行, 目标蛋白被固定在免疫功能化材料上的抗蛋白-抗体捕获, 通过洗涤去除未结合的其他物质后, 再将目标蛋白质消化成蛋白水解肽, 这种富集方法称为抗蛋白-IAE。先将样品整体消化成蛋白水解肽, 特征肽再被固定在免疫功能化材料上的抗肽-抗体捕获, 洗涤去除其他杂质, 这种富集方法称为抗肽-IAE^[50-51]。

IAE-LC-MS 具有高特异性、高灵敏度, 常用于定量复杂生物样本中低丰度的内源性蛋白多肽类物质, 2 种富集方法中抗肽-IAE 更受研究者青睐。Collins 等^[52] 采用抗肽-抗体制备免疫磁珠并特异

性富集威尔逊氏症患者干血斑中极低浓度 ATP7B 蛋白的 2 个特征肽 ATP7B 887 和 ATP7B 1056, LC-MS 高灵敏定量检测结果显示 ATP7B 887 拥有比 ATP7B 1056 更低的检测下限和定量下限 (分别为 2.17、7.14 pmol/L), 分析灵敏度和特异度分别可达到 91.2% 和 98.1%, 阳性预测值为 98.0%, 阴性预测值为 91.5%, 表明该方法的应用减少了铜蓝蛋白和遗传分析导致的歧义, 提高了威尔逊氏症的诊断准确率。抗肽-IAE 比抗蛋白-IAE 更适用于组织和血浆样品中目标蛋白多肽的富集, 原因是影响 IAE 的有害成分如变性剂在富集反应前已在消化过程中被去除^[49,51]。IAE-LC-MS 尚有不足之处, 定量低浓度外源性蛋白多肽类药物时可能受药物本身的耐受性或药代动力学样品中的抗药物抗体干扰 (主要是抗蛋白-IAE), 另外抗体尤其是抗肽-抗体不易获得^[53-54]。

3.1.2 无抗体富集 鉴于开发特异性抗体仍具有挑战性, 研究人员不断尝试探索无抗体富集与 LC-MS 结合的方法, 有研究证明无抗体富集的蛋白多肽前处理方法联合液相色谱串联质谱法 (liquid chromatography-tandem mass spectrometry, LC-MS/MS) 对蛋白多肽类物质定量也可以达到 10~1 000 ng/mL 水平^[55]。无抗体富集是根据目标蛋白或特征肽的一些特殊性

质开发的一种可以使其与复杂基质中大多数其他蛋白或内源性物质分离的方法^[56], 如实验室中常用的固相萃取 (solid phase extraction, SPE)、强阴/阳离子交换或反相萃取富集方法等。采用混合阴离子交换萃取可以富集含有大量酸性氨基酸的蛋白质, 定量线性范围广, 这已在 Weber 等^[57]的研究中证实。另外, An 课题组基于混合阳离子交换和反相吸附剂的 SPE 新型无抗体富集方法, 使用正交及多种机制控制洗脱液 pH、有机溶剂和盐离子的浓度进行研究, 以实现从复杂基质中选择性分离目标肽、降低内源性干扰、提高 LC-MS 检测专属性的目的^[54,58], 为基于无抗体富集结合 LC-MS 开发提高蛋白多肽富集率及提高检测专属性和灵敏度的方法提供了另一种可能。但这种不基于抗体富集蛋白多肽的样品前处理方法的特异性较基于抗体-抗原反应富集的方法低, 适于没有抗体或抗体不易制备的、含量较高的蛋白多肽类物质的富集纯化。

功能类似于抗体但较抗体具有更多优异特征 (易制备、可重复合成、亲和力强及分子特异性高等) 的适配体也越来越受到研究者关注^[59-60]。适配体是通过指数富集系统演化且由人工体外合成的 DNA 或 RNA 序列, 可以代替抗体高选择性、高效地富集生物样本中低丰度的蛋白多肽类物质。例如, 采用一种基于 RNA 的新型适配体高效富集人细胞和组织中神经退行性疾病的内源性生物标志物——43 000 TAR DNA 结合蛋白 (transactive response DNA binding protein of 43 000, TDP-43)^[61]。

样品前处理将目标待测成分从复杂的样品基质中尽可能地富集纯化, 为消除杂质干扰、改善分析方法的特异性提供可能。若想进一步扩大 LC-MS 技术在蛋白多肽等大分子测定领域的应用, 或更大程度提高 LC-MS 对低丰度蛋白多肽定量的灵敏度和准确度, 可以考虑借助信号转化与放大策略, 充分利用 LC-MS 检测优势, 构建更为精准的蛋白多肽分析方法。

3.2 信号转化与放大策略 信号转化与放大策略即将不易被 LC-MS 直接测定的大分子蛋白多肽信息转化为质谱响应信号高的化合物 (高敏分子) 信息实现信号放大, 并根据目标蛋白多肽与高敏分子之间的量比关系提高检测灵敏度的策略^[62]。信号转化和放大策略与探针紧密相关, 探针中主要包含目标蛋白多肽的特异性识别元件、高敏分子及刺激

响应元件等, 笔者将基于 LC-MS 高灵敏检测蛋白多肽的信号转化与放大策略根据探针的构建方式归为 3 类: 适配体-报告肽探针、质量条形码探针及蛋白酶-标签编码底物肽探针。

3.2.1 适配体-报告肽探针 适配体与报告肽构建探针, 适配体高效富集目标蛋白后将待测物的浓度信息转化为报告肽的质谱响应信号, 可以提高检测专属性、增强检测灵敏度^[63]。Zhou 等^[64]设计了一种由适配体和报告肽 (ALVLGVDPFR) 组成的适配体-报告肽探针, 探针适配体部分特异性识别乳腺癌细胞中的人表皮生长因子受体 2 (human epidermal growth factor receptor 2, HER2), 经过水解与消化过程, 报告肽释放并由 LC-MS/MS 检测。该方法得到较低的定量限 (25 pmol/L) 和较宽的线性范围 (25~2 500 pmol/L), 与传统检测方法相比临床试验假阳性/阴性结果也减少。另有文献报道, 采用此类方法测定 HER2, 经过转化与放大后的信号强度约为无信号放大时的 5 倍^[63]。若使适配体-报告肽探针与荧光染料结合, 结果可视化效果更好, 检测效率也更高^[65-66]。由此可见, 适配体-报告肽探针为基于 LC-MS/MS 靶向定量蛋白多肽提供了一种专属、灵敏的可替代方法。

3.2.2 质量条形码探针 LC-MS 的灵敏度通常随待测物的分子量而变, 其对小分子化合物更为灵敏。因此, 质量条形码常设计为小分子化合物或分子量较小的生物分子^[67]。质量条形码介导的探针中条形码的数量远远超过目标物, LC-MS 检测时可产生更强的质谱信号。Li 等^[47]基于高效、高选择性的免疫磁珠富集方法和质量条形码的信号转化与放大策略, 将 2-氨基-3-吡啶甲醛 (2-amino-3-pyridinecarboxaldehyde, APA) 作为质量条形码, 以 LC-MS/MS 为检测手段, 构建了凝血酶的高灵敏检测方法。该方法将核酸适配体 29 (aptamer 29, apt 29) 修饰在磁珠 (magnetic bead) 表面得到复合物 MB-apt 29, 用于富集凝血酶; 将核酸适配体 15 (aptamer 15, apt 15) 修饰在金纳米颗粒 (gold nanoparticles, AuNP) 表面, 再通过键合反应使 APA 对 AuNP 进一步修饰, 得到复合物 apt 15-AuNP-TAPA, 作为质量条形码的载体; MB-apt 29 和 apt 15-AuNP-TAPA 均可特异性识别凝血酶并与之结合, 形成免疫“夹心”复合物即 MB-apt 29/凝血酶/apt 15-AuNP-TAPA 探针。探针利用磁场分

离,并在氧化剂的作用下释放出 APA,被 LC-MS/MS 检测到。结果显示,APA 的质谱响应强度与凝血酶的浓度成正比,且具有较低的检测下限(0.007 nmol/L)。此外,选择腺苷^[68]或小分子含氮化合物^[62]作为质量条形码测定凝血酶的含量,定量灵敏度也可大幅度提高。转化后质量条形码的信号强度远大于未转化之前目标蛋白多肽的质谱响应强度,因此,目标蛋白多肽与质量条形码之间的信号转化有助于靶蛋白或多肽的高效、精准检测。

3.2.3 蛋白酶-标签编码底物肽探针 蛋白酶是一种特殊的蛋白质,具有专一剪切底物肽的特性,研究者基于该特点构建了用于检测蛋白酶活性或浓度的专属灵敏探针。往往一种疾病不会只有一种生物标志物,同时测定待测样品中多个标志物可以大大提高疾病诊断的准确性和可靠性。锌依赖性内肽酶家族的基质金属蛋白酶(matrix metalloproteinase, MMP)^[69-70]在体内的表达水平和活性上调与肿瘤血管生成、侵袭和转移等病理进展密切相关,如 MMP-2 和 MMP-7 与结直肠癌进展有关^[71-72]。Hu 等^[66]利用 LC-MS 和生物传感技术,基于肽序列可设计性、蛋白酶可选择性剪切底物肽、磁性高效分离及等压标签标记的相对和绝对定量(isobaric tags for relative and absolute quantitation, iTRAQ)多重编码的原理构建了能将不同 MMP 活性或浓度信息转化为不同质量标签质谱信号的多重传感探针。该探针由底物肽-iTRAQ 偶联物与 Ni-NTA 磁珠磁性材料制备获得, iTRAQ 中质量标签的相对峰面积与待测样品中靶 MMP 的活性之间呈良好的线性关系,通过设计不同的 iTRAQ 可同时特异性测定 MMP-2 和 MMP-7,提高疾病诊断的准确率。该类方法根据蛋白酶剪切探针上底物肽的原理,建立蛋白酶与底物肽探针中高敏分子之间的量比关系,并利用等压标签多路复用的特性同时检测多个待测物,为构建专属灵敏的蛋白酶检测方法提供了新思路,也为构建多通道检测蛋白多肽的分析方法提供了启示和借鉴。

4 小结和展望

精准测定蛋白多肽在生物体内的水平有助于疾病的诊断、药物治疗效果的监测及药物的研发等,在临床医学、生物学及药学等领域具有重要意义。由于生物样本成分复杂、靶蛋白多肽类物质含

量低等因素给生物体内蛋白多肽的定量带来极大的挑战。LC-MS 以其卓越的分离效率和检测灵敏度在分析领域广受欢迎。但该技术对大分子化合物离子化效率低,使其在蛋白多肽分析领域受到限制,如何克服这一问题是基于 LC-MS 的体内蛋白多肽分析方法发展的重心。

采用 LC-MS 测定目标蛋白多肽前选择合适的样品前处理方法很重要,这可以为仪器分析过程减少许多麻烦。

除此之外,随着信号转化与放大策略的提出,基于 LC-MS 高通量、高灵敏、准确定量生物体内的蛋白多肽成为可能。基于 LC-MS 的信号转化与放大策略,在筛选出 LC-MS 灵敏且与待测蛋白多肽互不干扰的“信号”分子如报告肽、质量条形码或同位素标签之后,再通过构建专属探针使目标蛋白多肽的信息转化为“信号”分子的信息并建立两者之间的量比关系,可以增强 LC-MS 响应信号。

另外,构建探针的过程中通过选择不同的“信号”分子有望实现多组分样品的高通量同时检测。多项研究已经证实,通过信号转化与放大策略不仅可以解决采用 LC-MS 检测生物样品中蛋白多肽时离子化效率低的问题,还可以提高检测灵敏度和通量。

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