The Use of Stable Isotope-Enriched Standards as a Key Component of the MS/MS Analysis of Metabolites Extracted from Dried Blood Spots

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Isotopes and Clinical Analysis

Accurate quantification of endogenous and exogenous metabolites and biomarkers of disease is essential to laboratory medicine and clinical research. The methods chosen have to meet the analytical criteria of high sensitivity (low detection limits), high selectivity (few interferences from compounds not being measured) and excellent precision (reproducibility). Immunoassays are the foundation of hospital-based clinical analysis and a major part of commercial diagnostic and newborn screening laboratories. These methods have the advantage of being inexpensive, easy to use, supported technically, sensitive and able to offer a wide scope of analytical targets. Their main drawback is poorer selectivity compared to newer technology such as mass spectrometry. Mass spectrometric (MS) based methods are more superior in their selectivity because they are based on the detection of chemical and physical characteristics related to its elemental composition and structure. MS methods have adequate analytical sensitivity for most applications and can be quite precise especially if stable isotopes are utilized as reference standards. Addition of stable isotope-labeled internal standards to a biological specimen is commonly referred to as isotope dilution mass spectrometry (IDMS). The basis for IDMS is that a known amount of the analyte to be measured that has been enriched with one or more isotopes (*i.e.* deuterium, carbon-13) is added to (or diluted with) its unlabeled target analyte. The mass spectrometer measures each analyte separately based on their mass-to-charge ratios, and a concentration can be based on the quantity of the labeled and unlabeled compound detected.

Dried Blood Spots and Metabolic Screening

One area of clinical laboratory science that has gained much attention in recent years is metabolic screening or newborn screening. 2013 marks the 50th anniversary of the introduction of analyzing blood for metabolites that are key markers for inherited disease. In 1960, Dr. Robert Guthrie developed a method for measuring phenylalanine (Phe) in the blood of infants to detect whether an infant was at risk for phenylketonuria (PKU), a disorder of Phe metabolism. Early detection of this disease was shown to reduce the mental retardation in affected infants by enabling early intervention and treatment, often as simple as a dietary change. However, rather than utilize a liquid specimen (whole blood or plasma) which would require expensive shipping from every birthplace, Dr. Guthrie championed the use of collecting blood on a strip of filter paper, drying the specimen and sending by regular mail. In addition to cost savings, blood dried on filter paper has a small footprint for storage, is less infectious and requires smaller volumes of blood collected from infants (~1/10th). During the decades that followed Guthrie's PKU test, newer analytical methods were developed to detect PKU and an array of other disorders of hormone, amino acid and carbohydrate metabolism.

Metabolic screening programs have had one great concern that is always an important topic: accuracy in measuring abnormal concentrations of metabolites in a dried blood spot (DBS) as it relates to blood volume in a sample. Hematocrit, the volume of blood applied to the filter paper, and the absorption characteristics of filter paper are critical in the quantification of metabolites. Although the manufacturing of paper and its absorptive characteristics could be controlled, the hematocrit and manner and volume of blood applied is much less controlled. Add patient metabolic variability to the volume variations and you have an analysis that is less precise than its liquid counterpart.

MS/MS and Stable Isotope Internal Standards

Tandem mass spectrometry (MS/MS) is a specific type of MS method that has two mass analyzers separated by a fragmentation chamber (collision cell) that can break ionized molecules (precursor ions, intact molecular ions) into specific and reproducible and smaller pieces or product ions. Amino acids and acylcarnitines as classes of compounds produce common highly reproducible and charcterizable fragments. Using MS/MS one can selectively detect just acylcarnitines or alpha amino acids in separate scans, simultaneously, without any chromatography yet still maintain high selectivity. Therefore, MS/MS can detect many different compounds in a single analysis in about two minutes per sample. It is for this reason that MS/MS has replaced older methods for amino acid analysis and detection of metabolites such as phenylalanine for PKU and has added a series of metabolites, *i.e.* the acylcarnitines for a series of disorders such as MCAD (medium chain acyl CoA dehydrogenase deficiency). In total, several dozen metabolites are detected in a single analysis. Quantification is critical to MS/MS analysis and therefore requires reference standards to properly measure the concentration of the markers.

The ideal reference standards for mass spectrometry are stable isotope-enriched analogues of the most important screening markers such as phenylalanine (Phe) or octanoylcarnitine (C8).

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The design of internal standards in terms of choice of isotope, the number of isotopes, their position in the molecule, high purity and high enrichment is critical to MS/MS analysis. During a period of two decades, standards have been introduced to meet the quantitative requirements of MS/MS analysis in dried blood spots for amino acids and acylcarnitines. For example, amino acids primarily lose a formic acid molecule (or formic acid butyl ester if derivatized) in the MS/MS analysis. In order to detect and quantify the precursor ion (original ionized molecule) in both labeled and native forms, the carboxyl carbon cannot be enriched with ^{13}C. Further, a minimum of two (preferably three or more) enriched atoms must be achieved in order to shift the mass sufficiently from its non-enriched value. Finally the choice of isotope must be such that if deuterium, it is in a non-exchangeable position, *i.e.* $^{13}C_6$ is used for the isotope of phenylalanine.

Enriched Sets of Common Metabolites Used in DBS Analysis

Historically, just a few key internal standards were produced in a few hundred milligram quantities. Amino acid stable isotope-enriched standards were often readily available, but acylcarnitines generally required special synthesis from specialty labs. Acylcarnitines are also notoriously unstable in the long term, especially in solution, and it has been difficult to always ensure adequate amounts of good-quality standards. Most importantly, due to their relatively high expense and limited quantity, the preparation of standards was a challenge when just a few milligrams were prepared. For all of these reasons and to increase the number and variety of standards to meet the requirements of a complex comprehensive MS/MS profile, more than 20 standards were synthesized. In my experience, weighing out small quantities of standards (2-3 mg or less) was a challenge especially as some standards are hygroscopic. Weighing out 200-300 mg was not practical because those quantities were not available for many standards or were cost-prohibitive. Instability added to problems since solutions of acylcarnitines in methanol hydrolyzed in less than two weeks, producing lower concentrations of short-chain acylcarnitines and higher concentrations of free carnitine.
The solution was obtaining all of the standards required for our MS/MS analytical needs for DBS analysis in two or three sets and individual vials with dry standard such that vials could be reconstituted as needed.

Current State of the Art

There are over 20 acylcarnitines and amino acid stable isotope-enriched standards used in metabolic screening and research in dried blood spots. These standards can be obtained in three sets of standards available from CIL (NSK-A, NSK-B, NSK-B-G). The amount used per day in a laboratory primarily depends on the screening volume (samples/day), size of the dried blood spot sample (1/8th or 3/16th), and the relative amount of standard desired relative to the extracted endogenous metabolite.
The internal standards are reconstituted in pure methanol (acylcarnitines) or a 50/50 methanol/water mix (amino acids). They can then be mixed together and diluted with pure methanol to the concentration needed. In each assay a specific volume of the of the methanol containing the stable isotope internal standards is mixed with the blood spot. Only the extracted metabolites can be quantified with the internal standard in the methanol mixture. However, in every step that follows, the internal standard and its unlabeled isotope analog is carried out in a manner consistent with traditional IDMS methods. For DBS, therefore, we designate it as pseudo IDMS because a quantitative error may be introduced if the extraction efficiency is much less than 100%. Fortunately for most acylcarnitines and amino acids, the extraction efficiency is greater than 90%. The method is validated with various peer-reviewed publications and in fact, a large number of metabolic screening labs have embraced MS/MS technology and have chosen to use pre-prepared sets of standards for improved reliability and more accurate quantification.

Future Applications

The use of stable isotope standards is not limited to the analysis of dried blood spots for newborn and metabolic screening. In fact, the use of isotope standards suitable to the analyte being measured are now being investigated for use in drug metabolism and pharmacokinetic studies. In addition, new dried matrices of biological fluids (*i.e.* plasma, urine) are being investigated with unique applications of stable isotope standards for quantification, *i.e.* fluids or filter paper pre-spiked with stable isotope standards) that better approximate traditional IDMS. Clearly, research using stable isotopes, mass spectrometry and dried blood specimens has much growth ahead, far beyond newborn screening. It's only a matter of time before other industries and scientific fields like environmental science, forensic science and materials science use dried specimens and stable isotope standards in new, unique and cost-effective ways.