Fluorescence detection of Amino Acids derivatized with o-phthalaldehyde (OPA) based reagents.

What is chemical derivatization?

Amino acids in their native form are generally weak chromophores (do not absorb UV light) and do not possess electrochemical activity. This means that for analytical purposes, they must first be chemically modified (derivatized). These products can be detected at much higher sensitivity by certain types of liquid chromatographic detectors.

The first automated amino acid analyzer, described by Spackman, Stein and Moore in 1958 [1], used ninhydrin as a post-column reagent yielding blue or brown-yellowish derivatives. These analyzers are still popular in clinical chemistry laboratories. However, there have been dramatic improvements in the apparatus involving the following modifications:

- **fluorescence detection** - *improved sensitivity*
- **microparticle materials** - *efficiency, faster analysis*
- **reverse-phase HPLC and**
- **precolumn derivatization** - *easier maintenance, automation, convenient, reproducible analysis at picomolar levels.*

The reactivity of all common derivatization reagents is directed towards the amino group(s) in the amino acid structure. Several fluorogenic reagents are available, among which o-phthalaldehyde (OPA)/thiol [2] based reagents play the most significant role in modern amino acid analysis.

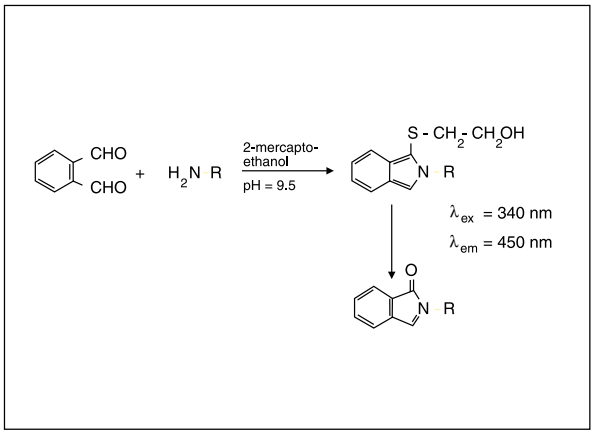


Fig.1. Reaction scheme of OPA/thiol reagent with a primary amine (amino acid) in alkaline medium [3].

The most important advantage of OPA is that it does not fluoresce intrinsically. The relative drawback is the specificity of OPA only towards primary amines and the instability of resulting fluorophores. Fluorescent isoindoles are rapidly formed at room temperature, but subsequently degrade to non-fluorescent derivatives. Fluorescence quantum yield and stability of "active" derivatives is dependent on each particular amine [4], excess of OPA [5], but mostly on the nature of the nucleophile (thiol) [6,7] used in the cyclization reaction. Besides thiols, other reducing agents such as cyanide [8] or sulphite [9] have also been reported.

The following is a comparison of fluorescence yield and stability of two model amino acids (glutamate, GABA) derivatized with different OPA/nucleophile reagents.

RESULTS:

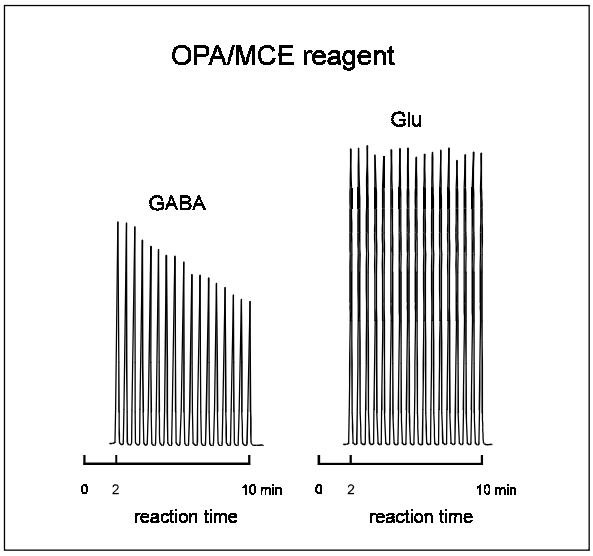


Fig. 2.
Loss of fluorescence for GABA and Glutamate derivatized with OPA/MCE reagent.

The flow-injection analysis (FIA) method was used for the evaluation: 20 µl samples of derivatized GABA or Glu were injected every 30 s into the ***CMA/280 Fluorescence Detector*** *using water at 0.3 ml/min as a carrier. The first peak is 150 s after the start of the reaction.*
After 10 minutes, the decrease in fluorescence intensity was 33% for GABA and 4% for Glutamate.

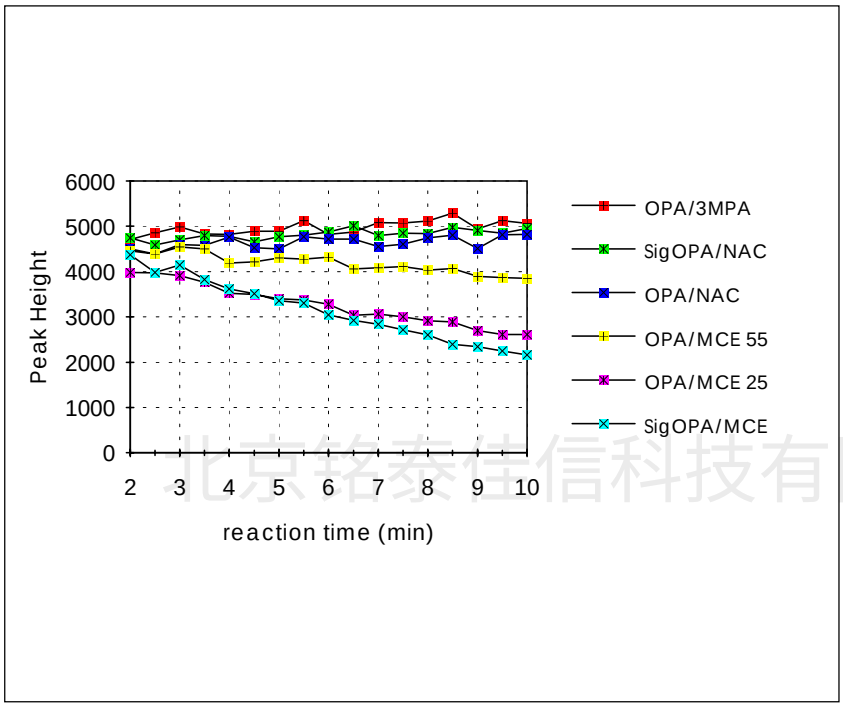
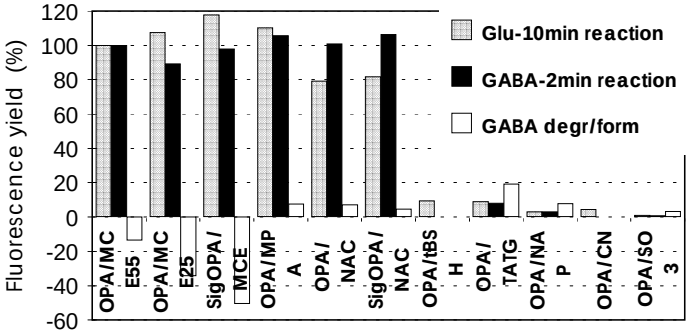


Fig.3.
Fluorescence stability of GABA derivatives depending on the type of OPA/thiol reagent.
An FIA system was used to measure fluorescence signals. Abbreviations: (3MPA) 3-mercaptopropionic acid, (NAC) N-acetyl-L-cysteine, (SigOPA/NAC) Sigma OPA solution + NAC, (MCE 55) - mercaptoethanol, final conc. 55% methanol, (MCE 25) - mercaptoethanol, final conc. 25% methanol, (SigOPA/MCE) Sigma OPA solution + MCE. All reagents (except of Sigma OPA) were prepared in borate buffer, pH 9.5, giving a final concentration of 0.1 M.

Fig. 4.
Fluorescence yields of OPA reagents with Glu (10 min reaction time) and GABA (2 min reaction).
Percentage of decrease and/or increase of fluorescence for GABA-OPA derivatives after 10 min reactions (see also Fig. 2 for illustration).

Fluorescence of OPA/MCE 55 was taken as 100%.
Abbreviations: (tBSH) 2-methyl-2-propanethiol, (NAP) N-acetyl-D-penicillamine, (TATG) 1-thio-D-glucose tetra-acetate, (SO_3) sodium sulphite, (CN) sodium cyanide.



Which is the best reagent?

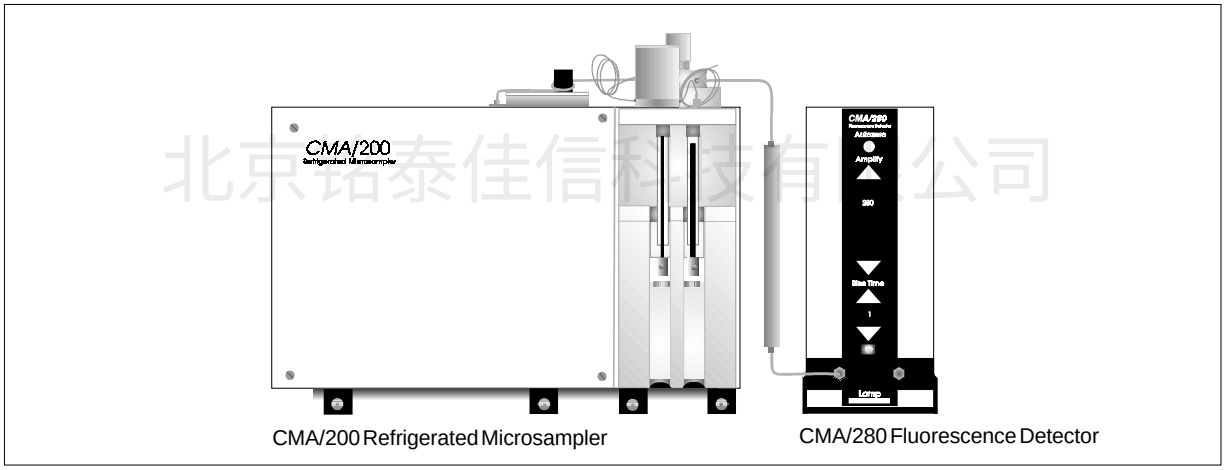
The best derivatization reagent is the one giving the highest signal and which is easiest to prepare, store and handle in the laboratory.

Besides the question of a “lab made” vs. commercially available OPA solution and its concentration, the most important issue is the choice of the nucleophile.
3MPA seems to be the best candidate, since it produces the most stable GABA and other amino acid derivatives. Furthermore, amino acids conjugated with OPA/3MPA are more polar because of an extra carboxyl group in the thiol moiety [10]. This simplifies optimization of gradient elution in HPLC.

However, MCE is still the most common additive, probably due to its low odour among other liquid mercaptans. Other thiol compounds (NAC,NAP,TATG) used for chiral separations of enantiomeric amino acids were also tested. NAC gave stable derivatives with fluorescent yields comparable to OPA/MCE (20% lower for Glu). Other thiols as well as sulphite and cyanide derivatives gave 10-100 times lower fluorescence than OPA/MCE and therefore are not suitable for physiological amino acid analysis.

The practical use of OPA/MCE (and consequently OPA/3MPA and OPA/NAC) reagents for HPLC determination of amino acids in microdialysis samples will be discussed in the following Application Notes.

What are the requirements for a successful analysis?



CMA/200 Refrigerated Microsampler

CMA/280 Fluorescence Detector

The CMA/200 Refrigerated Microsampler.

HPLC analysis of amino acids derivatized with OPA/MCE requires exact control of reaction times, temperature and precision volume pipetting. These criteria are essential to obtain the highest reproducibility (< 3% RSD) of the assay. Accurate sampling of volumes ranging between 0.5-20 µl can hardly be achieved manually.
Furthermore, by having an autosampler working overnight, the throughput of samples is increased dramatically. What the **CMA/200 Refrigerated Microsampler** does in one day would normally require three working days. Standard 10 min fraction collection in microdialysis can generate so many samples that non-automated analysis requiring 30-40 min could impede the whole project.

The CMA/280 Fluorescence Detector.

This detector is especially designed for sub-picomolar determinations of amino acids derivatized with OPA based reagents. It offers maximal (>75%) transmittance in the ranges 330-365 nm and 440-530 nm for ex./em. wavelengths.
For these applications the **CMA/280 Fluorescence Detector** is fully compatible with any of the more advanced fluorescence detectors. However, the total costs per analysis can be significantly reduced.

What has been published on the chemistry of amino acid derivatization?

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If you require further details on Microdialysis procedures, HPLC analysis, instrumentation or bibliography, please do not hesitate to contact:

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