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On-line coupling of sequential injection with liquid chromatography for the automated derivatization and determination of γ-aminobutyric acid in human biological fluids

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Abstract

The principle of sequential injection analysis (SIA) was exploited to develop a rapid fully automated and efficient pre-column derivatization procedure coupled on-line to liquid chromatography (HPLC). Using the SIA-HPLC derivatization protocol γ-aminobutyric acid (GABA) was determined fluorimetrically in human biological fluids with *o*-phthaldialdehyde (OPA) as derivatization reagent and minimum sample pretreatment. A lab-built SIA system was used to handle samples, standard solutions and OPA reagent. Appropriate volumes of the reagents were introduced in the holding coil of the SIA system and were mixed on propulsion to the HPLC loop through a suitable reaction coil. The chemical (pH, *c*(OPA), *c*(mercaptoethanol)) and instrumental variables (volumes of sample and reagent, reaction time) of the reaction were studied and optimized in terms of maximum sensitivity. The chromatographic variables (gradient composition of the eluent and flow rate) were studied for optimum selectivity and peak characteristics. The developed experimental configuration facilitated fully-automated operation thus minimizing errors in handling. Additionally the method as a whole provided very satisfactory sensitivity, precision and accuracy. Direct determination of GABA in human urine and cerebrospinal fluid (CSF) at $\mu g\,L^{-1}$ (ppb) levels was accomplished, with minimum sample pretreatment.

Keywords: Sequential injection; Derivatization, LC; γ-Aminobutyric acid

1. Introduction

Pre-column derivatization is one of the most popular approaches today for the determination of amino groups containing compounds because it offers very effective assays in terms of sensitivity, precision and accuracy. One of the most crucial demands in modern derivatization assays is automation. Unattended operation of automated derivatization protocols offer significant advantages in terms of sampling throughput and improved precision and accuracy. On the other hand some other aspects such as cost efficiency, instrument complexity and carry over phenomena must be considered.

Sequential injection analysis (SIA) launched in 1990 has been proposed to satisfy the demands for mechanical simplicity and robustness in automated flow-injection techniques [1]. A typical SIA setup is built around a low-pressure multi-position valve. Typically, zones of reagents and samples are sequentially aspirated into a holding coil, and are propelled to the detector by reversing the flow of the syringe pump. The desired chemical reaction takes place on passage through a reaction coil at the mutually overlapped regions of the sample and reagent zones. SIA has great potential, especially for on-line measurements and sample preparation due to the simplicity, convenience and automation in solutions manipulation. It offers significant advantages, such as low cost and widely available instrumentation, single-lined manifolds, low reagents and sample consumption, while it is not necessary to reconfigure the manifold geometry to apply different chemical systems. In addition, all major parameters such as reagents and sample volumes, flow rates, the order of mixing and reaction time can be easily optimized

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via computer-control [2]. These features make the coupling of SIA to a separation technique such as HPLC very attractive and challenging. Especially based on the very promising results of the first work on this area reported recently by our group, where SIA was coupled on-line to HPLC to automate solid phase extraction sample pretreatment [3].

The present work exploits the on-line coupling of SIA to HPLC for the development of a fully automated sample derivatization protocol. The assay was based on a two-step process. Derivatization is performed automatically in the SIA setup (step I) and next the reaction product is driven in the injection loop of the HPLC, where it is injected into the analytical column (step II). The feasibility of SIA-HPLC coupling is demonstrated by developing an automated method for the sensitive and selective determination of γ-aminobutyric acid (GABA) in human biological fluids, based on its reaction with *o*-phthalaldehyde and fluorimetric detection [4–9]. GABA is the most important inhibitory neurotransmitter in the mammalian central nervous system and is widely distributed throughout in brain. Due to its essential role in numerous neurochemical events, it is among the most frequently monitored amino acids [10]. The developed method enables the direct determination of GABA in human urine and cerebrospinal fluid (CSF) at the $\mu g\,L^{-1}$ level with minimum sample pretreatment, as tedious and time consuming solid phase extraction is not necessary. It combines the above-mentioned advantages of SIA with the high selectivity and separation efficiency of HPLC to yield a very attractive and advantageous alternative to conventional HPLC high-pressure automated schemes.

2. Experimental

2.1. Reagents

GABA and OPA were purchased from Fluka (Buchs, Switzerland). 2-Mercaptoethanol (MCE) (1.114 g/mL) was purchased from Sigma (St. Louis, MO, USA). 1,7-Diaminoheptane (DAH) and all HPLC-grade solvents used (methanol, acetonitrile) were provided by Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade and were also provided by Merck (Darmstadt, Germany). Water was double de-ionized and filtered through a 0.2 μm filter (Schleicher & Schuell, Dassel Germany).

Standard aqueous stock solutions of GABA ($100\,mg\,L^{-1}$) and DAH ($100\,mg\,L^{-1}$)—used as the internal standard—were prepared daily. Borate buffer ($0.05\,mol\,L^{-1}$, pH = 9.4) was prepared by dissolving 19.07 g of borax in water to produce a 1000 mL solution. The pH was adjusted to the appropriate value by addition of small volumes of $1\,mol\,L^{-1}$ NaOH. This buffer can be stored in ambient temperature for 2 weeks.

The OPA buffered reagent was prepared according to Roth's method [11] by mixing 425 μL of an OPA solution ($10{,}000\,mg\,L^{-1}$ OPA in ethanol) with 475 μL of MCE standard solution ($5\,\mu L\,ml^{-1}$ in ethanol) finally diluting to 22.5 mL with the borate buffer. The OPA solution was stable at least 4 days if it was kept refrigerated and protected from the light.

All the mobile phases where filtered through 0.2 μm membrane filters and degassed with helium for 10 min. Helium spargers were employed for continuous degassing of eluents.

2.1.1. Safety considerations

Care should be taken when working with 2-mercaptoethanol (MCE) due to its toxicity and unpleasant odour. Unused serum samples should be treated with Javex before disposal as hazardous waste.

2.2. Instrumentation

A schematic diagram of the SIA-HPLC setup is shown in Fig. 1. The SIA part consists of the following parts: an Intellect 12-port valve (Kloehn Co. Ltd., Las Vegas, NV), and a Versa 6 syringe pump with 10 mL syringe's volume (Kloehn Co. Ltd., Las Vegas, NV). The flow system comprises 0.7 mm i.d. Teflon tubing throughout. The hardware was interfaced to the controlling PC through a serial port. The control of the system was performed through a Kloehn Control BETA 0.81 software program.

The derivative was introduced to the HPLC analytical column through an automated injection valve system from ABI Spectroflow bearing a Rheodyne (Cotati, CA, USA) 7010 six port injection valve. HPLC separations were performed on a 250 mm × 4.6 mm C_{18} Kromasil 4 μm analytical column from MZ-Analysentechnik (Mainz, Germany) protected by a guard column (C_{18} bonded silica). The mobile phase was driven through the column by an LCP 5020 gradient pump (INGOS, Czech Republic). The Shimadzu RF-535 spectrofluorimetric detector was operated at $\lambda_{ex} = 340$ nm and $\lambda_{em} = 455$ nm. The response signal of the detector was acquired digitally and the data were saved in ASCII format for further manipulation (peak height/area measurement, digital filtering, etc.) using a home-made software programmed by Prof. P. Nikitas (Laboratory of Physical Chemistry, Department of Chemistry, Aristotle University Thessaloniki), running in Visual Basic® 6.0.

2.3. SIA-HPLC determination of GABA in aqueous solutions

The protocol of the SIA-HPLC derivatization setup is shown in Table 1. In brief, 100 μL of the sample and the OPA reagent are sequentially aspirated into the holding coil of the SIA system (control blank analyses were performed by aspitating water instead of the aqueous GABA solution). By reversing the pump flow and by choosing the appropriate port of the selection valve, the reaction mixture is propelled to the HPLC injection loop. The derivative is formed on the overlapped regions of the zones on passage through a suit-

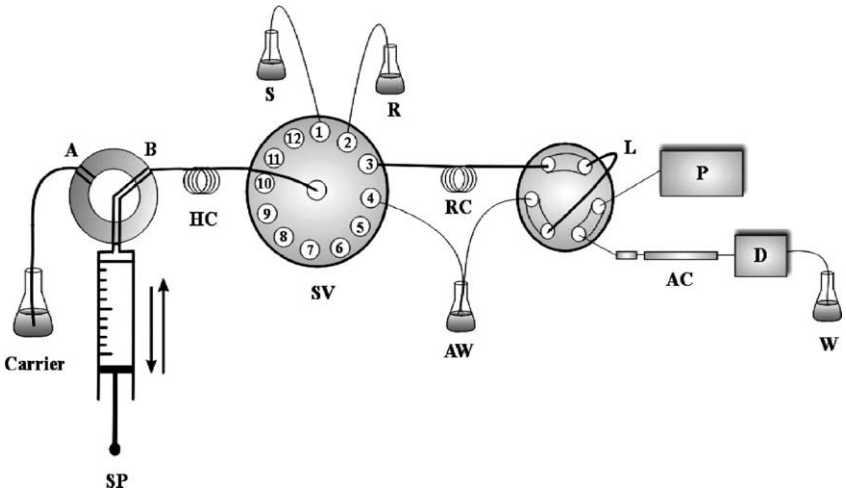

Fig. 1. SIA-HPLC analysis instrumentation set-up, where: carrier, water; SP, syringe pump; SV, selection valve; HC, holding coil (300 cm/0.7 mm i.d.); RC, reaction coil (30 cm/0.7 i.d.); S, sample; R, OPA buffered reagent; AW, auxiliary waste; L, loop (250 μL); P, high pressure pump; AC, analytical column; D, fluorescence detector and W, waste.

able reaction coil. At a pre-determined time—when the major part of the formed derivative was trapped in the HPLC injection loop—the reaction mixture was injected automatically in the HPLC system. Elution and effective separation of the analyte and the internal standard (DAH) was achieved through a simple binary gradient elution program. Solvent A was 0.015 mol L^{-1} aqueous CH_3COONH_4 and solvent B was CH_3OH. Gradient elution started at 40% B for 3 min, was increased to 55% at 6 min and to 100% at 10 min, kept constant at 100% until 16 min and was reduced to 40% at 16.1 min. Three replicates were made in all instances.

2.4. SIA-HPLC determination of GABA in human fluids

Human cerebrospinal fluid (CSF) was ultra-filtered using a Centrex UF-0.5 ultra-filter (10.000 M_r cut-off) obtained from Schleicher & Schuell. The sample was centrifuged at 12,000 rpm (8000 × *g*) for 40 min and the filtrate was 20 fold diluted in the internal standard. CSF samples were stored at −5 °C prior to use.

Blank urine specimen was a pooled mixture of seven healthy individuals. Twenty milliliters of urine were filtered through a 0.45 μm filter (Whatman®). Hundred microlitres aliquots of the filtered urine were spiked with GABA and diluted 50-fold with water to final analyte concentrations in the range 0.10–2.0 mg L^{-1}. The final concentration of the internal standard was 0.5 mg L^{-1}. Additionally, a urine sample was collected from a male member of the laboratory 6 h after oral administration of 6 g of a GABA-containing (99% purity) dietary supplement. Twenty millilitres of the collected urine sample were filtrated. Hundred microlitres aliquots of the filtered urine were diluted 50-fold with the internal standard solution.

All samples were finally analyzed in triplicate using the SIA-HPLC procedure for aqueous solutions described above.

Table 1
Sequence SIA steps of a complete measurement cycle

Time (s)	Pump action	Flow rate (mL min^{-1})	Volume (μL)	Valve position	Action description
0	Off	–	–	1	Selection of OPA reagent port
5	Aspirate	1.2	100	1	Aspiration of reagent in holding coil
1	Off	–	–	2	Selection of sample port
5	Aspirate	1.2	100	2	Aspiration of sample in holding coil
1	Off	–	–	3	Selection of HPLC port
55	Deliver	0.6	550	3	Propulsion of reaction mixture to HPLC loop
0	Off	–	–	3	HPLC analysis

3. Results and discussion

3.1. Study of the chemical and instrumental SIA variables

Preliminary experiments on the derivatization reaction were performed using SIA connected directly to the fluorimetric detector, omitting the HPLC separation step. The main objective was to check whether the derivative could be formed under flow conditions. This was necessary because there are no literature data on the reaction of GABA with OPA using flow or sequential injection analysis. The preliminary experiments indicated that both GABA and the internal standard (DAH) can be readily derivatized by OPA under flow conditions. The starting values of the chemical and SIA variables of these experiments were: γ(GABA)

$= 1\,mg\,L^{-1}$ γ(DAH) $= 0.5\,mg\,L^{-1}$, c(OPA) $= 12.0 \times 10^{-4}\,mol\,L^{-1}$ c(MCE) $= 1.15 \times 10^{-3}\,mol\,L^{-1}$ (pH = 9.4), the aspirated volume of each zone was V(sample) = V(OPA) = 50 μL (5 s aspiration time at 0.6 mL min^{-1}), respectively; the reaction coil (RC) was 15 cm/0.7 mm i.d., while the reaction mixture was propelled to the detector at a flow rate of 1.2 mL min^{-1}.

The order of the aspiration of the sample and the reagent proved not to be critical. The almost negligible differences in the signals were caused by slightly different dispersion effects on the sample zone according to the order of its aspiration. In addition, stopped-flow experiments showed that steady state of the reaction was succeeded within 30 s, indicating fast reaction kinetics. When changing between samples or standards, an additional washing step was performed in order to avoid carryover effects.

The SIA variables studied are the chemical (pH, c(OPA) and c(MCE)) and the instrumental ones: sample volume V(sample), reagent volume V(OPA), length of reaction coil l(RC) and delivery flow rate q_V(deliver). The starting values of these variables were those mentioned in the previous Section. The optimization was performed in favor of sensitivity and precision using the univariate approach. The variables studied, their range and the chosen values are shown in Table 2. The effect of pH value, OPA and MCE concentration and OPA reagent volume on the detector signal is also depicted schematically in Fig. 2. It should be noted that under the selected SIA variables 0.5 mg L^{-1} of DAH produced a sharp and reproducible peak, with adequate intensity to be used as the internal standard.

Table 2
Study of chemical and SIA variables

Variable	Studied range	Optimal value
pH	7.8–12.4	10
c(OPA) (mol L^{-1})	10.45×10^{-4} to 19.84×10^{-4}	13.6×10^{-4}
c(MCE) (mol L^{-1})	1.0×10^{-3} to 1.75×10^{-3}	1.45×10^{-3}
V(OPA) (μL)	50–250	100
V(sample) (μL)	50–250	100
RC (cm)	15–90	30
q_V(del) (mL min^{-1})	0.4–2.0	0.6

3.2. Optimization of the HPLC variables

The optimization of the HPLC variables was performed by off-line derivatization of GABA. The analyte and the internal standard was mixed 1:1 with the optimum OPA reagent found in the SIA experiments; it was left to react for 1 min and was manually injected in the HPLC system. Hundred microlitres of the final mixture were injected in triplicate in all instances.

Several gradient elution protocols were tested to facilitate adequate elution and separation of the analyte and the internal standard along with optimum peak shape and symmetry. Finally, a binary elution system was chosen and applied. The flow rate was maintained at 0.7 mL min^{-1}, while all separations were performed in ambient temperature. Under the selected HPLC conditions each run was completed in less than 20 min. The retention time of GABA was 12.1 min and the DAH 16.4 min. A typical chromatogram of GABA and DAH in aqueous solutions is depicted in Fig. 3.

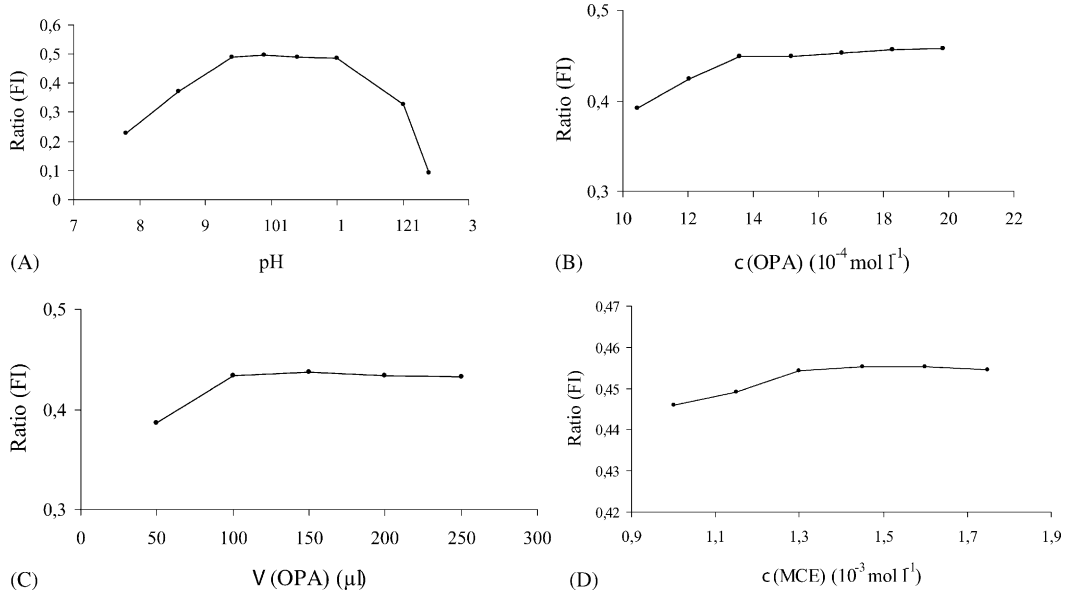

Fig. 2. Optimization of SIA variables: Effect of pH value (A), OPA concentration (B), OPA volume (C) and concentration of MCE (D). Ratio (FI) stands for the ratio of the fluorescence intensity of the GABA derivative vs. the blank (water is aspirated and mixed with OPA buffered reagent instead of aqueous GABA solution as described in Section 2.3).

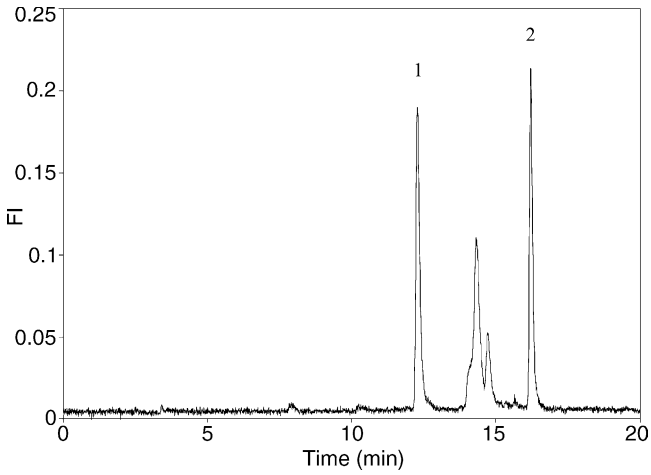

Fig. 3. Typical chromatogram obtained with fluorescence detection for standard solution of 0.5 mg L^{-1} GABA with 0.5 mg L^{-1} DAH. Peaks: (1) GABA, (2) DAH. FI: Fluorescence intensity. Chromatographic conditions are described in the text.

3.3. On-line coupling of SIA to HPLC

The main principle of continuous flow techniques such as flow and sequential injection analysis is the formation of concentration gradients of the injected sample zones due to dispersion effects. The concentration gradient is Gaussian-shaped with the highest analyte concentration being in the "head" of the traveling zone. For this reason it is clear that the major aspect in coupling SIA to HPLC is to achieve the optimum "tuning" of the reaction mixture in the injection loop of the HPLC. In other words, in order to gain maximum sensitivity it must be ensured that the most "concentrated" part of the sample (or derivative) zone will be injected in the HPLC column.

As it is shown in Table 2, the chosen values for the sample and reagent volumes aspirated in the SIA setup were 100 μL each. The "tuning" experiments were performed at three different HPLC injection loop volumes, namely 50, 100 and 250 μL. Under the optimum tuning conditions for each loop volume, the obtained peak area for GABA was directly compared to the value taken under off-line derivatization conditions. The analyte and OPA reagent were mixed at equal volumes, left to react for 1 min and 50, 100 and 250 μL of the final mixtures were injected directly to the HPLC column. In all cases, the SIA-HPLC signals were lower than the ones produced by off-line derivatization at the equal injected volumes. This was expected due to two main reasons. The first is the inevitable loss of derivative due to dispersion effects in the SIA system. The second reason is derived by one of the basic principles of continuous flow injection techniques: under flow injection conditions measurements are done under no physical or chemical equilibrium of the reactions.

Best results were obtained at 250 μL where the SIA-HPLC/HPLC signals ratio was about 0.85, indicating minimum sensitivity loss in favor of the automation. This result was more or less expected, as the larger loop volume ensures minimum derivative loss compared to an off-line equally injected volume.

3.4. Analytical figures of merit

Under the chosen SIA and HPLC variables and using the SIA-HPLC setup depicted in Fig. 1, a calibration graph for GABA in aqueous solutions was recorded. The calibration graph was linear in the range 0.01–3.0 mg L^{-1} GABA and obeyed the equation

$$A = (4.83 \pm 0.09)\gamma[\text{GABA}] + (-0.50 \pm 0.18)$$

where A is the GABA to DAH peak area ratio—γ(DAH) = 0.5 mg L^{-1} in all instances—and γ[GABA] is the mass concentration of the analyte in mg L^{-1}. The detection limit (at the S/N = 3 level) was 1 μg L^{-1}, with an r of 0.9983.

The within-day relative standard deviation varied between 3.2 and 5.0% at 0.5–1.0–2.0 mg L^{-1} GABA (n = 12). The within-day accuracy of the developed protocol also proved to be satisfactory as the recoveries of the above-mentioned GABA concentrations were in the range 96–103%.

The day-to-day reproducibility of the method was validated in a time period of 8 days. Each day calibration graphs were constructed in the range 0.25–2.0 mg L^{-1} GABA (n = 5) and their slope was compared to that of the original curve. The slopes of the calibration curves were in the range 4.58–5.01 which is in less than three times the standard deviation of the slope of the original calibration curve (4.83 ± 0.09). The relative standard deviation of the slopes was 2.9% (n = 8).

3.5. Analysis of real samples

Blank urine was treated as described in the Section 2.4 and was spiked with GABA in the range 0.1–2.0 mg L^{-1}. In all cases the concentration of the internal standard was kept constant at 0.5 mg L^{-1}. The obtained calibration graph was linear and described by the regression equation:

$$A = (4.81 \pm 0.20)\gamma[\text{GABA}] + (0.46 \pm 0.11)$$

where A is the GABA to DAH peak area ratio and γ[GABA] is the mass concentration of the analyte in mg L^{-1}. The relative standard deviation was 3% (at 0.2 mg L^{-1} GABA, n = 8), with an r of 0.9946.

The developed SIA-HPLC assay was applied to the determination of the analyte in a human urine sample collected 6 h after oral administration of a GABA containing dietary supplement (usual dose 3–6 g) and in spiked human cerebrospinal fluid (CSF). The results are shown in Table 3. The accuracy of the method was evaluated by recovery experiments of known amounts of GABA spiked in the real samples. This data also included in Table 3, verified the accuracy of the proposed assay as the recoveries were in the range 90–107% in all cases. Typical urine and CSF chromatograms are shown in Figs. 4 and 5.

No interference was observed from analytes sharing common pharmacological interest such as glycine, glutamine, arginine, aspartate and taurine. The retention times of the above amino acid derivatives were: $R_{t\,(\mathrm{Glycine})} = 14.8\,\mathrm{min}$, $R_{t\,(\mathrm{Glutamine})} = 4.5\,\mathrm{min}$, $R_{t\,(\mathrm{Arginine})} = 11\,\mathrm{min}$, $R_{t\,(\mathrm{Aspartate})} = 7.1\,\mathrm{min}$, $R_{t\,(\mathrm{Taurine})} = 10.4\,\mathrm{min}$.

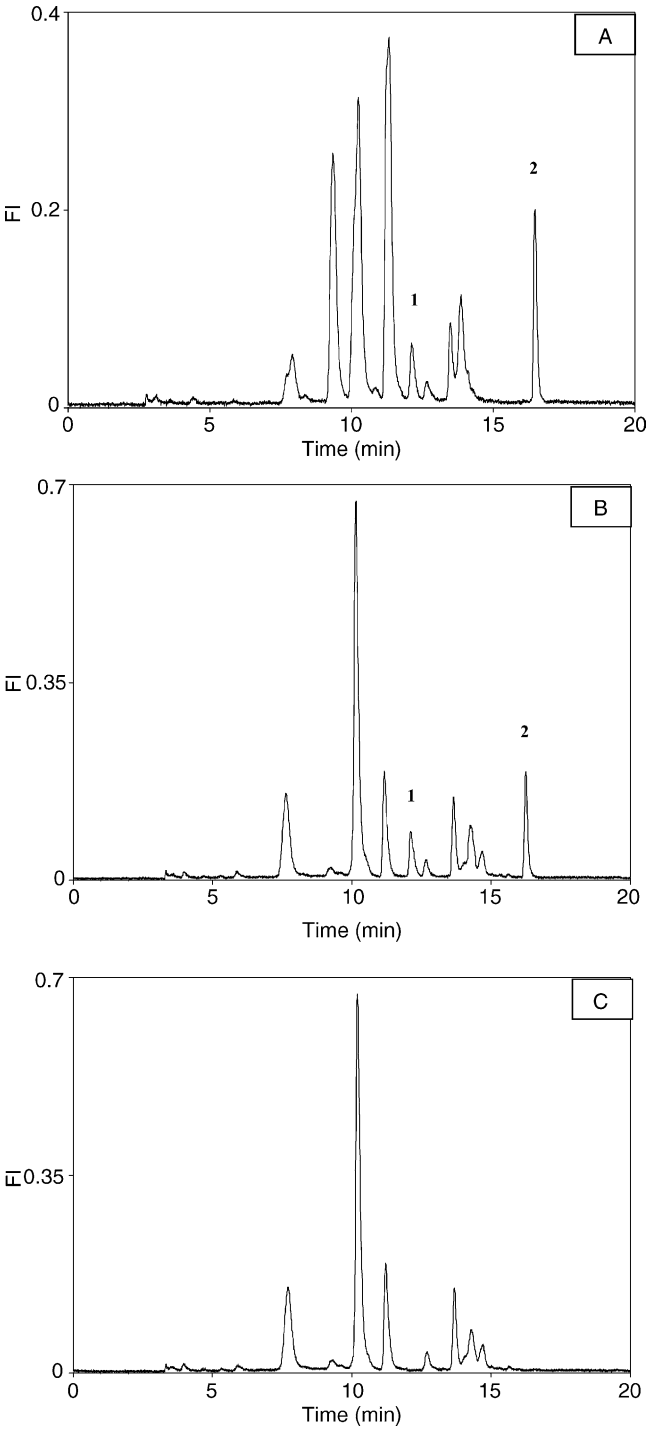

Fig. 4. Chromatographic analysis of urine diluted 50-fold: (A) urine collected 6 h after oral administration of GABA-containing dietary supplement. (B) Spiked with $0.2\,\mathrm{mg\,L^{-1}}$ GABA + $0.5\,\mathrm{mg\,L^{-1}}$ DAH: (1) GABA ($R_t = 12.1\,\mathrm{min}$), (2) DAH ($R_t = 16.4\,\mathrm{min}$) (C) blank. FI: fluorescence intensity. Chromatographic conditions are described in the text.

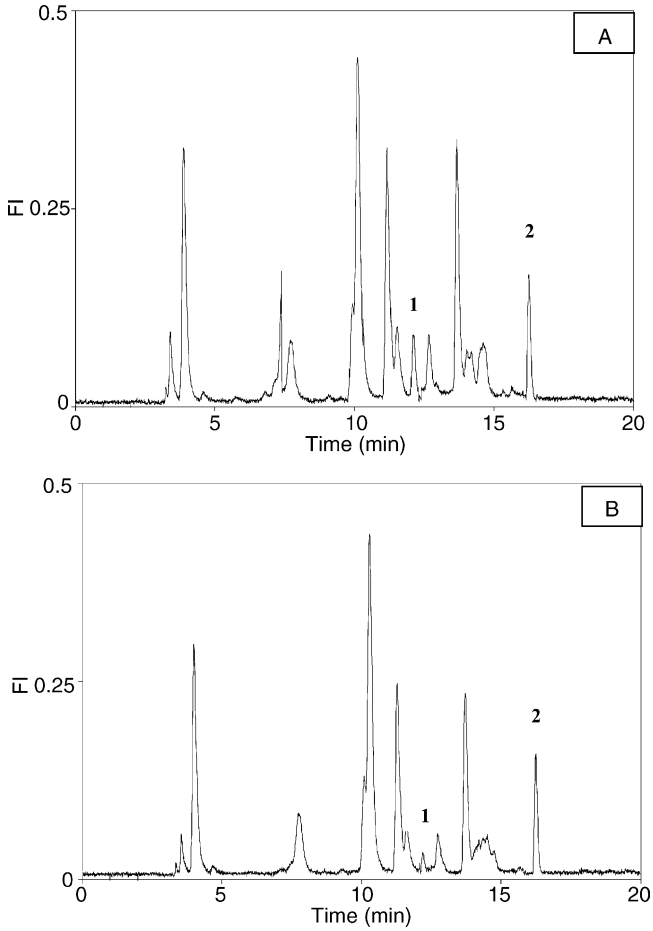

Fig. 5. Chromatographic analysis of CSF diluted 20-fold: (A) spiked with $0.2\,\mathrm{mg\,L^{-1}}$ GABA + $0.5\,\mathrm{mg\,L^{-1}}$ DAH. (1) GABA ($R_t = 12.1\,\mathrm{min}$), (2) DAH ($R_t = 16.4\,\mathrm{min}$), (B) blank. FI: fluorescence intensity. Chromatographic conditions are described in the text.

Table 3
Analysis of biological samples

Sample	Added GABA ($\mu\mathrm{g\,L^{-1}}$)	Found GABA[a] ($\mu\mathrm{g\,L^{-1}}$)	R[b]
CSF	–	26[c]	–
	50	71	90
	100	132	106
	200	236	105
Urine[d]	–	54	–
	100	161	107
	200	244	95
	500	542	97.6

[a] Mean of three results.
[b] Percent recovery.
[c] GABA mass found in 20-fold diluted sample.
[d] GABA mass found in 50-fold diluted urine 6 h after oral administration of GABA-containing dietary supplement.

4. Conclusions

The present work reports the first automated derivatization protocol based on the on-line coupling of sequential injection analysis (SIA) to HPLC. The SIA-HPLC setup is fully automated enabling precise computer-control of

all major reaction parameters such as sample and reagent volumes and reaction time, using cost-effective and simple low-pressure instrumentation. The feasibility of the SIA-HPLC coupling was clearly demonstrated by the development of an assay for the determination of γ-aminobutyric acid (GABA)—an important neurotransmitter—in human biological fluids. The proposed protocol was directly compared to conventional off-line derivatization HPLC with only 15% loss in sensitivity. The method was applied successfully to the analysis of urine and cerebrospinal fluid with minimum sample pretreatment.

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