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Short communication

Automated tagging of pharmaceutically active thiols under flow conditions using monobromobimane

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ABSTRACT

The thiol-specific derivatization reagent monobromobimane (MBB) is applied – for the first time – under flow conditions. Sequential injection analysis allows the handling of precise volumes of the reagent in the micro-liter range. The effect of the main chemical and instrumental variables was investigated using captopril (CAP), N-acetylcysteine (NAC) and penicillamine (PEN) as representative pharmaceutically active thiols. Previously reported hydrolysis of MBB due to interaction with nucleophilic components of the buffers was avoided kinetically under flow conditions. The proposed analytical scheme is suitable for the fluorimetric determination of thiols at a sampling rate of 36 h^{-1}.

1. Introduction

Thiols are an important group of compounds due to their unique role in biological systems, medicine, food, pharmaceutical and aroma industries. From an analytical chemistry point of view, the continuously growing amount of the published research and review articles on the determination of thiols in various matrices verifies the interest of analytical scientists for this group of analytes [1]. The analytical challenge to developing new methods for thiols can be pointed out characteristically by a very recent special issue of *Journal of Chromatography B* (Elsevier) under the title "*Analysis of thiols*" (Volume 877, Issue 28, 2009).

The majority of the naturally occurring and medically active thiolic compounds lack of chromo- or fluoro-phore groups in their molecule. Due their low detectability their determination in real samples may be problematic. A typical and widely accepted way to overcome this analytical problem is the derivatization of this group of compounds prior to the final measurement using suitable reagents [2]. One of the most widely used fluorescent labeling reagents for thiols over the last thirty years is monobromobimane (MBB) (Fig. 1A). MBB was introduced in 1978 by Kosower et al. [3] and reported applications vary from small molecules to proteins [4]. The most recent methods involving MBB include HPLC separation of its derivatives with cysteine/glutathione [5], captopril [6], bucillamine [7] and 2,3-dimercaptosuccinic acid [8] and capillary electrophoretic (CE)-based separation of thiolic peptides in various biological and food samples [9,10]. The main advantages of MBB can be summarized to the following: (i) it reacts selectively with both small and large thiolic compounds even in biological systems, as it is neutral and penetrates easily into intact cells; (ii) the reactivity of the reagent is high under mild conditions; (iii) MBB is essentially non-fluorescent and is stable when stored in the dark; (iv) as it is a small molecule, it is less likely to change the biological, physical, and chemical properties of the labeled molecule as compared with the unlabeled molecule; (v) it is less reactive towards other nucleophilic compounds (amines, carboxylates); (vi) the MBB-thiols derivatives are stable to air, light, chemical and biochemical procedures, and are resistant to irradiation [11].

The common characteristic of the analytical methods utilizing MBB for the fluorescent tagging of thiols is that the derivatization reactions are carried out exclusively under batch, equilibrium based conditions. To the best of our knowledge there are no reports on the reaction of MBB with thiols under flow conditions (e.g. post-column HPLC or flow injection analysis). A profound reason might be the high cost of the reagent that practically excludes such approaches (119.70€/25 mg, Sigma–Aldrich 2010). The scope of this study was therefore to present data on the reaction of MBB with three selected pharmaceutically active thiols – Captopril (CAP), N-acetylcysteine (NAC) and penicillamine (PEN) – under flow conditions. Sequential injection analysis (SI) was selected for this purpose as it offers the critical advantage of fluidics-manipulation in the microliter level, providing the potential of precise handling of such an expensive reagent [12]. The studied "chemistries" were

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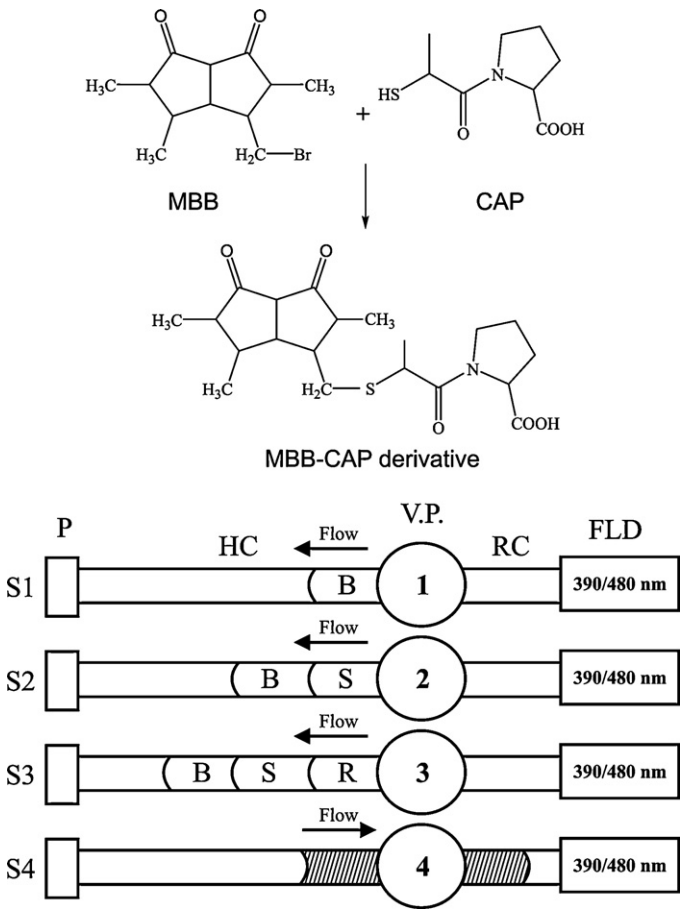

Fig. 1. (A) Scheme of a representative reaction between MBB and a thiol (CAP); (B) Sequential injection protocol for the derivatization of thiols under flow conditions. S1–S4: SI steps; P: peristaltic pump; HC: holding coil (300 cm/0.7 mm i.d.); V.P.: valve position; RC: reaction coil (30 cm/0.5 mm i.d.); FLD: flow-through fluorimetric detector; B: carbonate buffer (50 μL, pH = 11/100 mmol L^{-1}); S: thiol compounds (50 μL, CAP/NAC/PEN); R: MBB (50 μL, 1 mmol L^{-1}); the carrier was de-ionized water in all cases.

the basis for the development of a generic automated fluorimetric method for the quality control of thiols-containing pharmaceutical formulations.

2. Experimental

2.1. Instrumentation

The SI setup was comprised of the following parts: a micro-electrically actuated 10-port valve (Valco); a RF-551 flow-through spectrofluorimetric detector (Shimadzu); a Minipuls3 peristaltic pump (Gilson). The control of the system was performed through a special program developed in LabVIEW 5.1.1 (National Instruments). Data acquisition was carried out using the Clarity® software (DataApex). A FIAStar 5101 thermostat was employed to control the temperature of the reaction coil (Tecator).

2.2. Reagents and solutions

All reagents were of analytical grade, while de-ionized water produced by a Millipore system was used throughout this study. Working solutions of MBB (c = 1.0 mmol L^{-1}) were prepared daily by dissolution of the reagent in ACN/water (4:96, v/v). During experiments the reagent was kept protected from the light. Standard stock solutions of the analytes were prepared at the 500 mg L^{-1} level by dissolving accurately weighed amounts of captopril (CAP, Sigma), N-acetylcysteine (NAC, Merck) and penicillamine (PEN, Sigma) in 50 mL of 5 mmol L^{-1} EDTA. The solutions of CAP and NAC were found to be stable for 1 week if kept refrigerated, while the stock solution of PEN was prepared daily. Working solutions for all thiols were prepared – at the desired concentrations – daily, by appropriate dilution of the respective stocks in water. Borate and carbonate buffers (0.1 mol L^{-1} each) were prepared using suitable amounts of $Na_2B_4O_7{\cdot}10H_2O$ (Merck) and Na_2CO_3 (Merck) respectively. The pH was regulated at the desired values by adding appropriate amounts of 2.0 mol L^{-1} NaOH or HCl solutions.

2.3. SI procedure for aqueous solutions

As can be seen in Fig. 1B, the SI sequence was carried out at three general steps: (i) aspiration and stacking of buffer (V(B) = 50 μL), sample (V(S) = 50 μL and MBB (V(R) = 25 μL) in the holding coil, (ii) derivatization on passage through a suitable reaction coil (q_V = 0.6 mL min^{-1}, l(RC) = 30 cm/0.5 mm i.d.) and (iii) fluorimetric detection of the formed derivatives (λ_{ex} = 390 nm/λ_{em} = 480 nm). The sampling throughput was 36 h^{-1}.

3. Results and discussion

3.1. Study of the MBB-thiols reaction under flow conditions

When developing a new flow method based on the MBB-thiols reaction, there some properties of the reagent have to be taken into account:

(i) The reagent is photosensitive and should be protected from the light during preparation and all experiments. For this reason, apart from keeping the MBB solution in an amber-glass container, all PTFE connection tubing (holding coil, sampling lines etc.) were also wrapped with aluminum foil. Additionally, the study of the reaction kinetics by stopped-flow experiments had to be modified. Preliminary blank experiments showed that due its photo-sensitivity, MBB could not be "trapped" in the flow cell of the detector for real-time monitoring. Stopped-flow experiments were therefore carried out in the reaction coil, prior to the cell. These experiments confirmed that the reaction could proceed rapidly under flow conditions. A representative stopped-flow experiment for PEN can be seen in Fig. 2A. CAP and NAC were found to react more rapidly, leveling-off within the first 30 s.

(ii) Previous batch studies indicate that the reactions of MBB with low molecular weight thiols are strongly pH dependent and that an advantage of MBB over other derivatization reagents is its ability to react even at relatively low pH values of 7–8 [4,11]. We examined the effect of pH in the range of 7–12 using various buffers. In all cases, the on-line derivatization reactions were favored at more alkaline media and were not influenced significantly by the buffer species. A representative set of experiments for CAP can be seen in Fig. 2B. An explanation for the pH values chosen in previous batch reports is the hydrolysis of MBB by the nucleophilic components of the buffers. Although MBB reacts preferentially with thiols, it also reacts slowly with amines, phosphate, carboxylates, and other nucleophiles when these are present at high millimolar or molar concentrations to yield fluorescent by-products which can interfere with the analysis. It is therefore recommended to use non-nucleophilic and dilute buffers. Interestingly, such behavior was not observed under flow conditions. We examined several buffers (Tris, borate, carbonate, phosphate) and

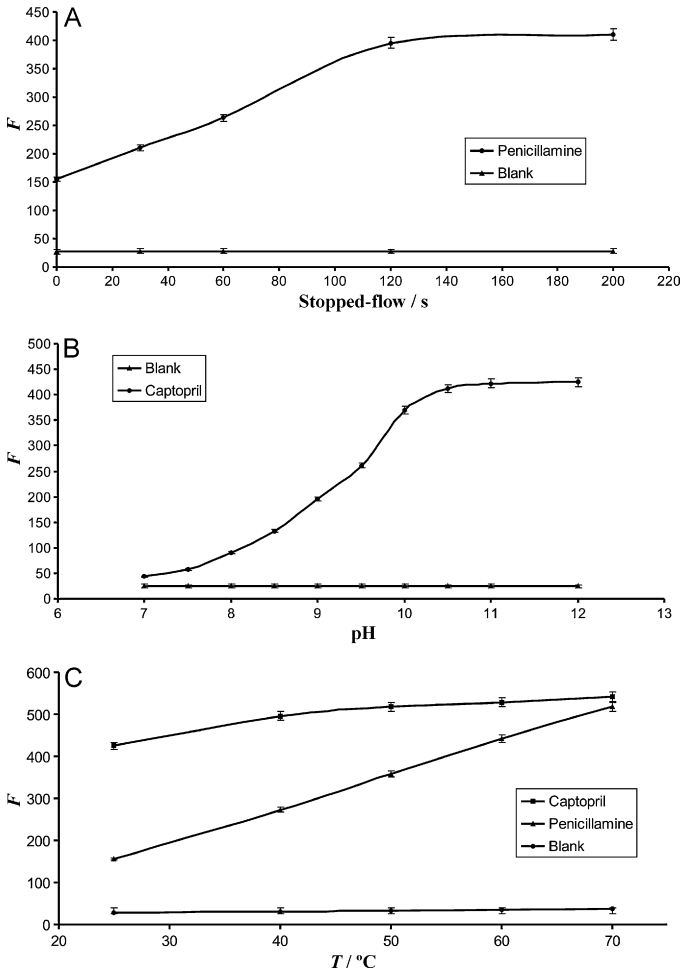

Fig. 2. (A) Reaction of MBB (1 mmol L^{-1}) with PEN using stopped-flow; (B) Effect of the pH on the reaction of MBB (1 mmol L^{-1}) with CAP (100 mmol L^{-1} borate buffer); (C) Effect of the temperature on the reaction of MBB (1 mmol L^{-1}) with CAP and PEN.

even at high alkaline pH values (e.g. 12) and ionic strength (e.g. 100 mmol L^{-1}) the blank was low and constant for stopped flow periods for at least up to 300 s (maximum tested) (Fig. 2A and 2B). Additionally, the selectivity of the reaction towards amines was investigated using glycine (c = 1.0 mmol L^{-1}) as model compound. No reaction was observed under flow conditions even at alkaline medium.

(iii) MBB-based derivatization protocols report various conditions in terms of reaction temperature, ranging from R.T. to even 60 °C. Under flow conditions, the effect of the temperature was investigated by thermostating the reaction coil in the range of 25–70 °C. The reaction of CAP and NAC with MBB was found to be independent of the temperature above 40 °C, while the reaction rate between PEN and MBB increased linearly within the studied range (Fig. 2C). In all cases, the blank values remained unaffected.

3.2. Development and validation of the SI method

The development of the generic SI method for the determination of the selected pharmaceutically active thiols involved the systematic investigation of the effect of several instrumental (geometrical) and chemical variables. These variables, the studied range and the selected values can be found in Table 1. The selection of the "optimal" value for each parameter was mainly based on the goal to develop a generic assay for all analytes. Compromises were therefore made in terms of sensitivity, simplicity (e.g. selection of room temperature) and sampling rate.

Table 1
Study of chemical and instrumental variables.

Parameter	Studied range	Selected value
Instrumental		
q_V(C)/mL min^{-1}	0.45–1.2	0.6
V(sample)/μL	25–100	50
V(buffer)/μL	25–100	50
V(MBB)/μL	25–75	25
l(RC)/cm	30–100	30
Chemical		
T/°C	25–70	25
pH	10.0–12.0	11.0
c(buffer)/mmol L^{-1}	25–100	100
c(MBB)/mmol L^{-1}	0.5–2.0	1.0

Validation was carried out for determination range, limits of detection (LOD) and quantitation (LOQ), precision (repeatability and reproducibility), accuracy and specificity [13] (Table 2). A mass concentration of 50 mg L^{-1} was selected as the 100% level.

The range of the assay for each thiol was validated between 50 and 150% of the above mentioned target concentration. This range is adequate for both assay and content uniformity quality control applications. Six calibration points were used in all cases. The validity of the obtained regression lines was evaluated by the residuals approach. The acceptance limits were set a relative error in the back-calculated concentrations of less than ±3.0% in all cases. The LODs/LOQs were calculated by the following equations:

$$\text{LOD} = \frac{3.3 \times s_b}{m} \text{ and LOQ} = \frac{10 \times s_b}{m}$$

where s_b is the standard deviation of the blank values and m is the slope of the regression calibration graph for each analyte.

The repeatability was validated within the same day by sequentially analyzing eight independently-prepared samples of each analyte at the 100% level. The relative standard deviations should not exceed 3.0% in all cases. On the other hand, reproducibility was examined within six days, by constructing calibration curves in the 50–150% range (n = 6). The relative standard deviations of the slopes should be not more than 10.0%.

The selectivity was evaluated by the placebo approach. Elevated concentrations of the placebo mixture (colloidal silicon dioxide, pre-gelatinized starch, magnesium stearate, titanium dioxide, sodium saccharin, sodium citrate, microcrystalline cellulose, polyvinyl pyrolidone, hydroxypropyl cellulose and gelatin) were spiked with the analytes at the 100% level. The percent recoveries criterion for non-interference was set a range of 95–105%.

The accuracy was validated by preparing synthetic samples of all thiols in the presence of the excipients, at 50, 100 and 150% concentration levels respectively. Three samples were prepared at each level. The percent recoveries acceptance criterion was set at a range of 95–105% in all cases.

The results from the validation experiments are included in Table 2. The acceptance criteria mentioned above were met in all cases.

3.3. Analytical applications

The proposed method was applied to the assay and content uniformity of CAP (Capoten® tabs, 25 mg/tab and Dosturel® tabs, 50 mg/tab) and NAC (Trebon® tabs, 600 mg/tab and Trebon® sach, 200 mg/sach) and PEN (Penicillamine® caps, 250 mg/cap) commercially available formulations.

Sample preparation for assay analysis of the tablets, capsules or sachets (n = 20) involved the typical steps of homogenization, dissolution in water, sonication (15 min) and filtration

Table 2
Validation parameters of the SI method.

Validation parameter	Captopril	N-acetyl cysteine	Penicillamine
100% level/mg L^{-1}	50	50	50
Range (50–150%, $n=6$)/mg L^{-1}	25–75	25–75	25–75
Residuals range[a]/%	−2.2 to + 1.6	−1.9 to + 1.8	−2.5 to + 2.7
LOD/LOQ[b] (mg L^{-1})	0.23/0.70	0.21/0.63	0.81/2.4
Repeatability [c]/RSD (%, $n=8$)	0.8–1.4	1.0–1.5	0.8–1.2
Intermediate precision [d]/RSD ($n=6$)	2.5	3.1	6.1
Selectivity[e]/R (%)	97.2–103.4	97.4–101.2	98.0–102.2
Accuracy[f]/R (%)	98.3–102.1	98.6–102.6	98.4–102.8

[a] Acceptance limits: ±3.0%.
[b] Calculated as LOD (or LOQ) = 3.3 (or 10) × s_b/m, where s_b is the standard deviation of the blank and m is the slope of the regression line for each thiol.
[c] Acceptance limits: RSD not more than 2.0%.
[d] Acceptance limits: RSD not more than 10.0%.
[e] Recoveries (%) for placebo concentrations in the range of 500–2500 mg L^{-1}. Acceptance limits: R = 95.0–105.0%.
[f] Acceptance limits: R = 95.0–105.0%.

Table 3
Analysis of pharmaceutical samples.

Sample	Recovery (%)
Assay analysis[a]	
Capoten® tabs (25 mg CAP/tab)	98.4–103.0
Dosturel® tabs (50 mg CAP/tab)	97.4–103.6
Trebon® tabs (600 mg NAC/tab)	99.2–101.8
Trebon® sach (200 mg NAC/sach)	98.8–101.5
Penicillamine® caps (250 mg PEN/cap)	98.5–101.4
Dosage uniformity analysis[b]	
Capoten® tabs (25 mg CAP/tab)	92.3–106.2
Dosturel® tabs (50 mg CAP/tab)	95.3–105.9
Trebon® tabs (600 mg NAC/tab)	92.1–106.8
Trebon® sach (200 mg NAC/sach)	95.3–104.9
Penicillamine® caps (250 mg PEN/cap)	91.7–104.0

[a] Three independently-prepared sub-samples were analyzed for each formulation.
[b] Ten independently-treated tablets/sachets/capsules were analyzed for each formulation.

(0.45 μm). Dosage uniformity analysis involved application of the above-mentioned procedure to individual tablets/sachets/capsules ($n=10$). The acceptance limits, as set by the US. Pharmacopoeia, were 95–105% of the declared value for the assay analysis and in the case of content uniformity tests 85–115% ($n=10$) [14]. As can be seen from the results of Table 3, all formulations were found to be within specifications.

4. Conclusions

This report presents, to the best of our knowledge, the first data on the reaction of MBB with thiols under flow conditions. Sequential injection analysis played a key role in this attempt since it allowed (i) the automated handling of well defined volumes of the reagent and (ii) reaction with the analytes under flow conditions. The experiments confirmed the ability of MBB to tag thiols under flow conditions, with moderate though sensitivity. One of the important findings of this research may be the absence of formation of hydrolytic by-products of MBB with other nucleophilic species that commonly exist in the buffers, even at highly alkaline pH values. The MBB-thiols reaction was the basis for the development of a generic SI method for the simple and rapid determination of three pharmaceutically active thiols (CAP, NAC & PEN) in various quality control samples.

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