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Packed capillary liquid chromatography coupled to fluorescence detection: application to human blood samples for the determination of glutathione*

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Abstract: The present investigation analyses the potentials of capillary chromatography using packed fused silica capillaries filled with 5 μm RP-18 for the fluorescence determination of glutathione in human blood samples. Adaptation of conventional HPLC equipment for miniaturized chromatographic assays proved successful. Sample preparation was relatively simple, though care should be taken in sample handling. The thiolic compound mercaptoethanol was used as internal standard. Qualitative determinations were based on standard addition providing increased peak heights at identical retention times. Quantitative determinations gave linear calibration curves, with a standard glutathione recovery of 98.9% and an intra-assay reproducibility of 3.3%. The glutathione values measured appeared within the normal range of 0.9–1.7 mmol glutathione per litre of blood.

Keywords: *Packed capillary liquid chromatography; micro-LC; fluorescence detection; glutathione; human blood; thiols.*

Introduction

Packed capillary chromatography is presently an established technique due to its well-known advantages with respect to conventional columns [1, 2]. The possibilities of adapting conventional HPLC equipment to micro-scale analysis has further increased the potential of this type of separation system. In addition, the development of various stationary phases [3] and of more sensitive detection systems, especially when micro-LC is directly interfaced with GC or MS instruments [4, 5], has made it a very popular method.

The importance of glutathione in the maintenance of normal cellular metabolic processes is widely known. Its presence in red blood cells at its normal physiologic range is essential, as haemolytic anaemia or other serious dysfunctions may be the consequence of erythrocyte glutathione disorders [6]. Several previous studies focused on the fluorescence determination of thiols in general, and more specifically of glutathione in human blood samples by various high-performance chromatographic and electrophoretic techniques [7–9]. The application of micro-LC using fused silica capillaries packed with reversed-phase C_{18} for these same purposes is presented in this paper. Previous optimization of the chromatographic system for the qualitative and quantitative determination of glutathione with regard to different mobile phases (using isocratic and gradient elution), columns (packed with different reversed-phase materials), injection volumes and detection systems (UV absorption and fluorescence detection) have been evaluated and described elsewhere [8, 10]. The optimized micro-LC conditions for the analysis of glutathione are specified in the Experimental section.

Experimental

Chemicals

The fluorogenic reagent SBD-F (ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate) [11] was purchased from Wako Chemicals (Neuss, Germany). Glutathione and mercaptoethanol were from Merck

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(Darmstadt, Germany). High purity water (Alltech Associates, Inc., IL, USA) was used throughout. Acetonitrile for the mobile phase was of HPLC grade (Alltech Associates, Inc., IL, USA). Trichloroacetic acid was obtained from Janssen Chimica (Beerse, Belgium). All other chemicals used were of analytical grade from Merck (Darmstadt, Germany) and UCB (Leuven, Belgium). The human blood samples and the standard cysteinylglycine were kindly provided by Dr E. De Potter (Blood Bank, Ghent University Hospital, Belgium) and by Prof. K. Imai (Branch Hospital Pharmacy, University of Tokyo, Japan), respectively.

Apparatus

The micro-LC instrumentation consisted of a Varian Vista 5560 HPLC pump (Varian, Walnut Creek, CA, USA) equipped with a 'T-piece' split-flow system (Valco, Houston, TX, USA) connected to the following parts: (a) a conventional HPLC column used as a solvent by-pass (150 × 4.6 mm i.d. 5 μm RoSiL C_{18}, Bio-Rad RSL, Nazareth, Belgium); (b) the previously mentioned HPLC pump; and (c) a 60-nl internal volume Valco injector (Valco C14W, VICI, Houston, TX, USA) that was directly connected to the capillary column (a 250 × 0.32 mm i.d. fused silica capillary packed with 5 μm RoSiL C_{18}, Bio-Rad RSL, Nazareth, Belgium). The gradient elution mobile phase was composed of 0.15 M phosphoric acid (H_3PO_4)–acetonitrile (CH_3CN), that changed from 95:5 (v/v) to 65:35 (v/v) over 15 min, followed by an isocratic elution of 0.15 M H_3PO_4–CH_3CN (65:35, v/v) for 10 min at a flow rate of 4.0 μl min^{-1}. For the detection of the separated bioanalytes, a Shimadzu RF-535 fluorescence–HPLC detector (Pleuger, Wijnegem, Belgium) was used, into which a laboratory-made (Bio-Rad RSL, Nazareth, Belgium) detection cell was inserted (excitation wavelength = 380 nm, emission wavelength = 510 nm). This cell was built with a square quartz detection cell originally provided by Hellma Benelux (Edegem, Belgium) of outer dimensions 4.0 × 4.0 × 20 mm, through which two connecting capillaries of 100 μm i.d., 250 μm o.d. were inserted to provide a micro-LC cell of inner dimensions 0.25 × 0.25 × 5.0 mm and with a cell volume of 313 nl. A Linear recorder model 2020-000 (Reno, NV, USA) and a Chromatopac C-R3A integrator computer (Shimadzu, Kyoto, Japan) were used to record and integrate the signals, respectively.

Sample preparation

To a 1-ml sample of fresh blood (EDTA treated), collected in chilled blood-collection disposable tubes, was added an equal volume of a chilled solution of 10% (w/v) trichloroacetic acid (TCA) containing 1 mM disodium EDTA. The sample was vortex mixed for about 10 s, followed by centrifugation at 0°C and 3500 rpm (1850*g*) for 5 min. A 40-μl volume of the supernatant was then added to 220 μl of a borate buffer solution (pH 9.5; 2.5 M, containing 4 mM dissolved EDTA). A 40 μl aliquot of the internal standard (50 μg ml^{-1} mercaptoethanol dissolved in the same borate buffer solution) was added, followed by 100 μl of SBD-F (2 mg ml^{-1} dissolved in the same borate buffer solution). The reaction mixture was then vigorously mixed, heated in a water-bath at 60°C for 60 min followed by ice-water colling. Disposable 4 mm syringe filters (pore size = 0.45 μm, Alltech Associates, Inc., Deerfield, IL, USA) were used to filter the derivatized samples prior to injection into the chromatographic system. Blood standard-addition samples were similarly prepared by adding 40 μl of a 75 μg ml^{-1} standard glutathione solution (i.e. glutathione dissolved in the above-mentioned borate buffer solution followed immediately by a 1:1 (v/v) dilution with 10% (w/v) trichloroacetic acid) to the supernatant prior to derivatization. Aqueous standard glutathione calibration curves were likewise prepared following the described method with the addition of an equal volume (40 μl) of the TCA-treated standard glutathione solution (at different concentrations) prior to derivatization. The human blood sample pretreatment procedure was originally based on a pre-chromatographic fluorescence derivatization method first suggested by Toyo'oka and Imai [11] but to which certain modifications were introduced such as the addition of an internal standard compound (mercaptoethanol) to the derivatization mixture and the inclusion of an increased volume of supernatant containing the bioanalyte of interest (glutathione) for increased sensitivity.

Results and Discussion

Qualitative determination

The chromatograms of Fig. 1 show the

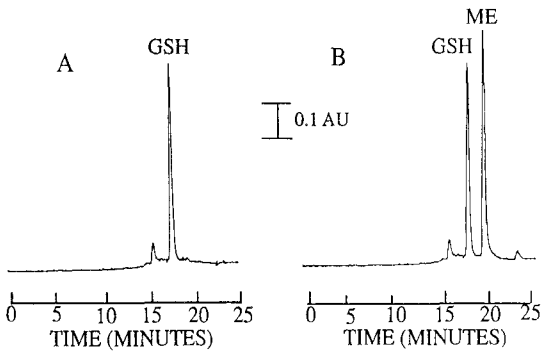

Figure 1
Micro-LC analysis with fluorescence detection of derivatized human blood (A) without and (B) with the addition of the internal standard (mercaptoethanol) to the supernatant prior to derivatization. GSH, SBD-glutathione; ME, SBD-mercaptoethanol. See text for specific experimental conditions.

results obtained (A) without and (B) with the internal standard in the derivatized blood samples. The three main peaks of the derivatized blood sample [Fig. 1(B)] could be identified as SBD-cysteinylglycine (mean retention time = 15.2 min), SBD-glutathione (mean retention time = 17.7 min) and SBD-mercaptoethanol (mean retention time = 19.4 min). Peak identification was based on standard addition to obtain increased peaks at identical retention times under the same experimental conditions. In this case, the chromatograms of derivatized blood samples, to which TCA-treated solutions of (a) standard glutathione, (b) mercaptoethanol and (c) cysteinylglycine had been added to the supernatant, resulted in the increase of the corresponding peaks at the corresponding retention times when analysed under the described micro-LC conditions. Moreover, the absorption spectra of the derivatized TCA-treated aqueous standard solutions (SBD-glutathione, -mercaptoethanol and -cysteinylglycine) were obtained with an HPLC–UV diode array detector. These spectra proved to be similar to those obtained from the derivatized blood sample peaks chromatographed under the same experimental conditions. The addition of non TCA-treated standard glutathione to the supernatant gave glutathione peaks at a different retention time than TCA-treated glutathione samples. This was due to the salt formation of glutathione with the strong trichloroacetic acid, resulting in a different chromatographic behaviour.

Cysteinylglycine is the main thiol-containing hydrolysis dipeptide from glutathione. For determining the presence of this and other hydrolysis products, the acidic and basic degradation of SBD-glutathione were performed by the addition of 6 N HCl and 5 N NaOH, respectively (both containing disodium EDTA), to the derivatized solution (1:1, v/v) followed by 120°C heating for 4 h. The basic hydrolysis of SBD-glutathione did not provide any additional significant peaks when chromatographed under the described experimental conditions, whilst the results of the acidic hydrolysis are shown in Fig. 2(A). By standard addition that resulted in peaks at identical retention times (t_R) SBD-cysteine (peak 1, t_R = 13.4 min) and SBD-cysteinylglycine (peak 2, t_R = 15.1 min) were identified as the main peaks of the chromatogram obtained from the acidic hydrolysis of SBD-

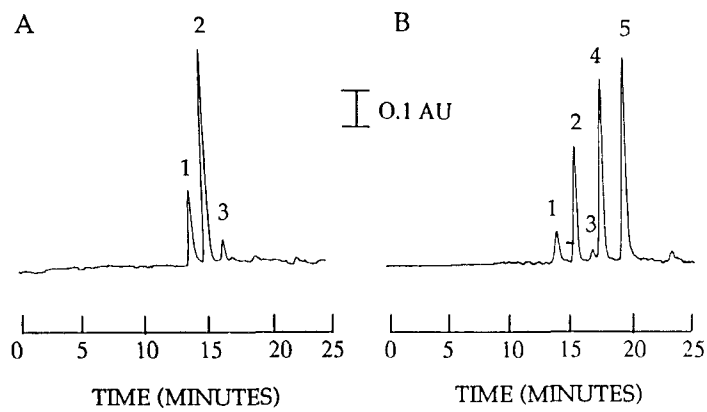

Figure 2
Micro-LC analysis with fluorescence detection of (A) SBD-glutathione after acid hydrolysis and (B) human blood derivatized following the described method and added to (A). 1, SBD-cysteine; 2, SBD-cysteinylglycine; 3, SBD-glutamylcysteine; 4, SBD-glutathione; and 5, SBD-mercaptoethanol. See text for specific experimental conditions.

glutathione [Fig. 2(A)]. The third hydrolysis peak (t_R = 16.9 min) is most probably SBD-glutamylcysteine, the second major thiol dipeptide that resulted from glutathione hydrolysis. A mixture of the acidic hydrolysis products and a derivatized blood sample further confirmed the presence of SBD-cysteinylglycine in the derivative blood [Fig. 2(B)] as the corresponding peak [first small, unlabelled peak at t_R = 15.2 min in Fig. 1(B)] considerably increased when compared to the derivatized blood sample shown in Fig. 1(B).

Quantitative determination

The detection limit ($S/N > 2$) achieved was 150 pg of glutathione per 60-nl injection. Repeated assays of 10 aliquots from one blood sample gave a standard deviation of 0.05 mM (RSD = 3.3%). Repeated analysis of glutathione from the same blood sample during four consecutive days gave a standard deviation of 0.10 mM (RSD = 6.6%). Therefore, the analysis should be performed immediately after blood collection. In fact, the glutathione concentration in erythrocytes decreases *in vitro* with time, even in the presence of blood preservers [12]. Standard glutathione calibration curves (n = 10) were obtained by plotting the ratio between the relative fluorescence intensities (peak heights) of SBD-glutathione and SBD-mercaptoethanol against the ratio between the glutathione concentration (in aqueous solution or in the blood supernatant, added in the 0–3000 pg per injection range) and the mercaptoethanol concentration. Linear and parallel graphs were obtained in both cases (i.e. in blood supernatant, $y = 7.848 + 2.670x$, $r > 0.99$ and in aqueous solution, $y = 0.211 + 2.149x$, $r > 0.99$). To determine the analytical recovery of this micro-LC method, standard glutathione was added at a concentration of 75 $\mu g\ ml^{-1}$ to the TCA solution used to hydrolyse blood and subjected to the described sample pretreatment and micro-LC procedure. The mean recovery of glutathione from 10 determinations was 98.9% with a RSD of 2.9%.

Glutathione in blood appears mainly in its reduced form, mostly concentrated in erythrocytes [13–14]. Normal values range from 0.9 to 1.7 mM glutathione [10, 15]. A mean value of 1.2 ± 0.14 mM (n = 10 different blood samples) from normal volunteers was obtained, with all measured values appearing within the normal range.

Conclusions

The proposed micro-LC method using packed fused silica capillaries and fluorescence detection allowed the selective and sensitive determination of glutathione in human blood, following a rather simple but also accurate and reproducible experimental method. Conventional HPLC equipment could be successfully adapted to micro-LC assays, though the use of specific miniaturized instrumentation could further improve the resolution of these determinations. The optimized system could be similarly applied to other thiolic compounds in blood or in other biological or industrial samples.

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