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SAMPLE PREPARATION FOR DETERMINATION OF BIOLOGICAL THIOLS BY LIQUID CHROMATOGRAPHY AND ELECTROMIGRATION TECHNIQUES

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Majority of the bioanalytical or environmental methods do not use just one chromatografic or electrophoretic step, but rather involve several sample pretreatment steps which simplfy the matrix, and often preconcentrate and chemically modify the analytes. This work surveys typical procedures for sample preparation for most commonly analyzed biofluids with particular emphasis placed on chemical derivatization of sulfur amino acids for their determination by liquid phase separation techniques. Recent author's laboratory contribution to the development of sample preparation procedures is merked.

Key words: sample preparation, liquid chromatografy, capillary electrophoresis, chemical derivatization, biological thiols.

1. Introduction

The analysis of biological samples presents a variety of problems which are common to high performance capillary electrophoresis (HPCE) and high performance liquid chromatography (HPLC). These encompass: (1) large number of individual compounds in the sample, leading to difficulty in resolving the analytes of interest, (2) the presence of components, such as proteins, that can modify the chromatographic or electrophoretic column, (3) low concentrations of exo- or endogenous compounds of interest, leading to detection difficulties, and (4) conjugation of analytes to proteins and low molecular components of the analysed mixture. Taking under consideration the above, it is not surprising that the majority of bioanalytical (environmental) methods do not use just one simple HPCE or HPLC separation step, but rather involve several sample pretreatment steps which simplify the matrix, and often preconcentrate and chemically modify the analytes [1-3]. Such approach leads to

a relatively purified material being introduced to the final separation unit, making the separation simpler and reliable. There are also several disadvantages of this approach namely, multiple steps usually require more lengthy and more refined sample handling and there is more chance for errors.

The biological fluids that are most commonly analyzed are plasma and urine. Whole blood is less often analyzed, with exception in the case of small animals and forensic toxicology where this may be necessary. Bile, sweat, tears, milk and saliva can also be analyzed. The ease with which samples can be analyzed increases with the degree of fluidity, bone being the most difficult to handle and cerebrospinal fluid the easiest.

2. Direct Injection of Biological Fluids

In HPCE there has been considerable interest in performing single-step analysis, with direct injection of body fluids on-capillary. This is quite often possible because open bare capillaries are less prone to irreversible modification by sample matrix components than a packed HPLC column, and even if adsorption of some compounds does occur, there are few limits on the use of aggressive cleaning procedures. There is very important argument for the development of HPCE methodology for direct injection analyses. With direct injection from microcompartments within living systems this microanalytical technique offers new possibilities for understanding of a variety of life processes that could not be studied previously.

Urine and plasma or serum, the most commonly analyzed biofluids, present different problems. Urine is generally free of proteins and lipids but contains very many components, concentration of which depends largely on the diet, and this can account for pushing the resolving power of the separation unit to the limit. Plasma/serum usually contains fewer individual components at high concentration, but the presence of a large amounts of proteins and lipids constitutes serious challenges. Besides modification of the capillary walls (in HPCE) or packing material (in HPLC), there is a strong affinity between proteins and analytes, leading to conjugates and direct measurement can miss the total content of the analyte and measure only its free fraction. Thus, chemical or enzymatic cleavage of conjugated analytes is still necessary in many cases. The advice to filter the sample, if possible, through a 0,45 μm membrane to remove any particulate capable of blocking the capillary or column is always valid.

One problem with the urine HPCE analysis is the variable, but generally high, concentration of electrolytes. If limits of detection are adequate, the urine can be diluted first and then analyzed in moderately concentrated buffer [4]. Another means to minimize effects of matrix composition on resolution and

sensitivity is to use high concentration background buffer [5]. This requires very good cooling system or limited electric field strength leading to longer time of analysis without the loss of sensitivity. Both capillary zone electrophoresis (CZE) and electrokinetic chromatography (EKC) have been used for direct analysis of urine, provided that the analyte concentration is high enough for quantitation by detection system. The main problem is to apply a robust method with adequate resolution of the analyte from other sample components. In EKC with micellar (MEKC) or other pseudostationary phases sample matrix effects on the results of analysis seem to be relatively minor [6].

The problems that the high concentration of proteins in plasma (about 70 g/l) causes in HPCE are double. They adhere to the wall of capillary leading to variability of electroosmotic flow (EOF) and peak-broadening. Secondly, the broad protein peaks may interfere with analyte peaks. However, several attempts of direct injection of plasma were made. It can be done after several fold dilution of plasma, provided the background electrolyte is of high concentration [7]. This approach makes sense only if concentration of the analyte is high. If undiluted plasma is injected, vigorous washings with strong base or acid between runs are needed followed by reequilibration in order to achieve good EOF repeatibility [8]. Addition of sodium dodecyl sulfate to the washing solution can accelerate removal of proteins and reequilibration [9]. Since a surfactant is present in MEKC by definition, the results of analysis of directly injected plasma with this HPCE mode are better than with CZE. In a typical MEKC analysis of plasma, the surfactant is strongly bound to the protein, and gives it an overall strong negative charge, which reduces interactions with the capillary surface and causes strong migration against EOF.

Direct injection of biological samples into the HPLC system causes column blockage due to adsorption or precipitation of proteins. Plasma proteins are known to precipitate readily particularly in the pH range 4-8. The signs of column deterioration could be such as an increase in column back-pressure, and peak splitting or tailing. Columns designed to exclude proteins but allow smaller molecules to interact with the stationary phase are available, although their use is limited.

3. Traditional Sample Preparation Techniques

Conventional sample preparation techniques, typical scheme of which is shown in Fig.1., are well established and account for the vast majority of HPLC procedures for analysis of biological samples. They also feature widely in the bioanalytical HPCE literature. A relatively large biofluid sample is needed, ranging from a several dozen to a few milliliters. After preparation, a few

microliters to a few milliliters are available for a final analysis. The final analysis could be done by HPCE or HPLC. Considering small volume sample requirements of HPCE its microanalytical abilities are not utilized. The justification for using capillary electrophoresis as the final step of the analytical procedure may be its excellent resolving capabilities for analytes which could be difficult to separate by HPLC, e.g. diastereoisomers. As shown in Fig. 1 the principal steps in sample preparation are extraction, deproteinization, and chemical modification.

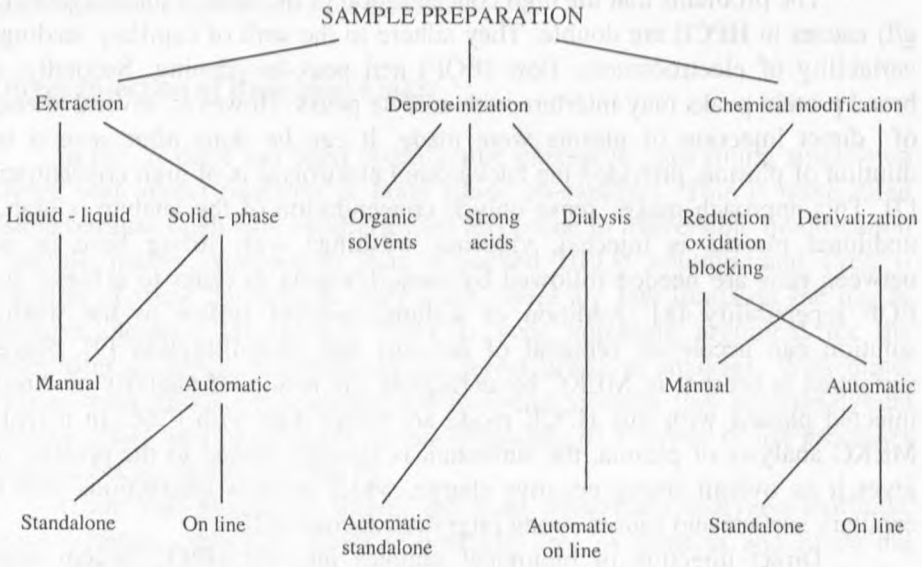

Fig. 1. Main steps in the conventional sample preparation procedure

Both liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are in use. In LLE, an immiscible organic phase is added to the biological fluid and these are then shaken together, resulting in the more hydrophobic sample components being extracted into the organic phase. In SPE, the sample is passed over a particulate material packed into small disposable columns. Some components are retained on the column, and weakly sorbed components may be removed by rinsing, followed by elution of strongly retained compounds. In both LLE and SPE the analyte concentration is possible by evaporation of solvent and reconstitution of the residue with small volume of desired liquid. This liquid could be the HPCE background buffer or HPLC mobile phase. Extraction can be done with some degree of specificity.

Many deproteinization methods have been described [10] and the more commonly employed are (1) precipitation by addition of organic solvents, (2)

precipitation by formation of insoluble salts, (3) ultrafiltration, and (4) dialysis. Ultrafiltration has an advantage over protein precipitation and dialysis in that it is applicable to small volumes and no dilution occurs.

Since there are opportunities for loss of analyte at each step in an off-line procedure several workers have been looking for clean-up and concentration procedures for HPCE either on-capillary, or using a miniaturized system coupled to the capillary. A variety of techniques can be used including liquid chromatography, electrophoresis and microdialysis or ultrafiltration [11].

Chemical modification encompasses chiefly derivatization of the analyte and decomposition of its conjugates with proteins and low molecular endo- or exogenous compounds.

4. Thiols as an Analytical Objects

Thiols play a specific role among reduced sulfur compounds; they are chemically and biochemically very active components of the sulfur cycle of the natural environment and have been extensively studied in various biological systems. Many biological phenomena, among others, redox-, methyl transfer-, and carbon dioxide-fixation reactions, are believed to be dependent on the presence of a thiol group. The determination of thiol containing compounds is important for biochemical research, in pharmacodynamic studies of the thiol drugs, or in the diagnosis of several diseases, e.g. cystinuria and homocystinuria. The homocysteine assay is a sensitive tool for early diagnosis of disturbed remetylation and transsulfuration leading to hyperhomocysteinemia, which in turn is associated with an increased risk of atherothrombotic vascular events [12].

The analysis of thiols can be quite perplexing. Aside from the great susceptibility to oxidation, which can occur before or during analytical process, most thiols lack the structural properties necessary for the production of signals compatible with common HPLC or HPCE detectors, such as UV absorbance and fluorescence. Therefore, the analyst must resort to derivatization for signal enhancement and labile sulfhydryl group blocking if fluorescence or UV-Vis detection methods are employed. Numerous reagent are available for the derivatization via-SH group and subsequent HPLC or HPCE analysis. A majority of the reagents can be classified by type of the reactive moiety into three categories: activated halogen compounds, disulfides, and compounds possessing maleimide moiety, and are reviewed with some experimental details in some excellent works [2, 13]. However, increased demand for measuring thiols, mostly biological ones in clinical practice, raises the issue of developing

new methods better suited to accommodate high testing volumes and faster turnaround time.

5. Chemical Derivatization of Thiols for HPCE and HPLC

We have developed in our laboratory several thiol specific derivatization reagents belonging to the class of azaaromatic compounds with an active halogen atom. These are 2-halopyridinium [14] and 2-haloquinolinium salts [15], which react rapidly and quantitatively with hydrophilic thiols in slightly alkaline water solution to form stable thioethers, S-pyridinium and S-quinolinium derivatives, respectively. Fig. 2. Shows the derivatization reaction scheme for thiols represented by homocysteine [16] with the use of 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT).

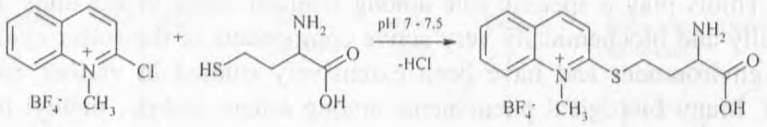

Fig. 2. Chemical derivatization reaction equation of homocysteine with 2-chloro-1-methylquinolinium tetrafluoroborate

This derivatization scheme takes advantage of great susceptibility of the quinolinium molecule at 2 position to nucleophilic displacement, and the high nucleophilicity of the thiol group. The reaction is accompanied by an analytically advantageous bathochromic shift from reagent maximum (328 nm) to the maximum of the derivative (348 nm). This phenomena is displayed in Fig. 3. Of different functionalities (e.g. -COOH, -NH_2. -SH) of hydrophilic thiols potentially able to undergo nucleophilic attack at the 2-position of quinoline (pyridine) molecule, only the sulfhydryl group reacts. This means that no multiple derivatives are formed. Under the recommended derivatization conditions, formation of the 2-S-quinolinium derivatives is over within 1 min, that is virtually just after mixing substrates.

Based on 2-halopyridinium and 2-haloquinolinium salts as derivatization reagents we were able to elaborate several methods for determining endo- [14,16-21] and exogenous [20-25] thiols in plasma and urine by manual [14,16-25] as well as, fully automatic [26] manner.

The bulk of plasma thiols occur in the disulfide forms rendering them inaccessible to derivatization reagent, and in order to determine their total contents disulfide bonds must be cleaved with suitable reducing reagent to liberate a free thiol. For this purpose sodium borohydride [16],

tri-n-butylphosphine [18,24] or 2-mercaptoethanol [19] was used. Total content of thiol is understood as the sum of reduced thiol, free oxidized (dimer and mixed disulfides with other endo- and exogenous thiols) and protein bound thiol.

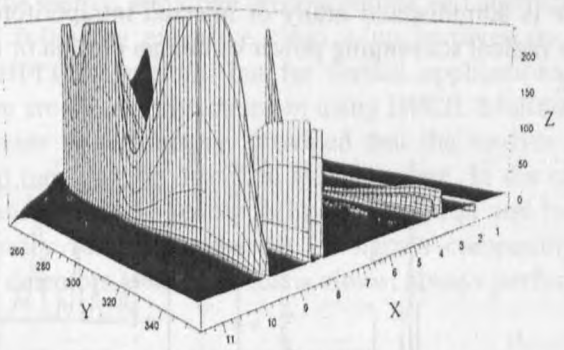

Fig. 3. Three-dimensional chromatogram of the derivatization mixture made with continuous spectral scanning during the elution. Peaks from right: glutathione – CMQT, homocysteine – CMQT, cysteine – CMQT, cysteinylglycine – CMQT derivative and CMQT excess. Axis titles: X – time, Y – wavenlenght and Z – absorbance

Among plasma thiols, homocysteine is the most often target of analysis. Elevated concentration of total homocysteine in plasma (hiperhomocysteinemia, concentration above 15 nmol/ml) is recognized as an independent risk factor for premature development of cardiovascular diseases. However, there is an evident need to measure different species of thiols in order to fully understand the dynamic relationship between homocysteine and other biologically important thiols and disulfides in plasma. Alteration of the redox status of homocysteine rapidly affects and is related to the total thiol redox status in plasma. This observations can be explained by continuos redox and disulfide exchange reactions in plasma. The comprehensive measurement of the reduced thiol fraction has proven to be problematic because of low concentration and susceptibility to oxidation. This promted us to develop a new HPLC methods with UV detection for determining different species of thiols in plasma [16,17] and urine [27]. The methods rely on transformation of thiols to stable 2-S-quinolinium derivatives in the reaction with CMQT reagent, and separation and quantitation by ion-pairing reversed-phase HPLC. Oxidized species are converted to their thiol forms by reduction before derivatization. To circumvent the loss of reduced form ex vivo due to oxidation, the CMQT reagent is added to whole blood immediately after collection and prior to separation of plasma from erythrocytes in order to block the reactive -SH groups. Typical chromatograms

for standard mixture and reduced fraction of main plasma thiols are shown in Fig. 4.

Another new HPLC method (Fig. 5) enables simultaneous determination of N-acetylcysteine and four main endogenous plasma thiols [25]. N-acetylcysteine is administered orally or injected intraperitoneally in order to increase the free radical scavenging power of human plasma or in the capacity of mucolytic agent.

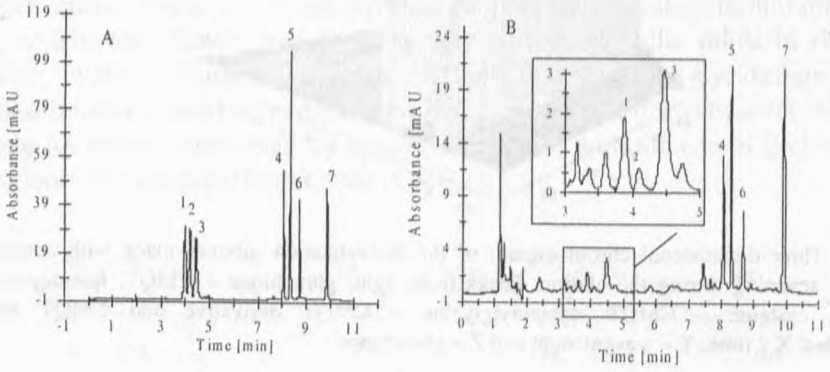

Fig. 4. Typical chromatogram of main plasma thiols. (A) water standard solution, concentration of analytes and internal standards in final analytical solution 20 nmol/ml in respect to each; (B) reduced thiols in plasma. Peaks: 1, glutathione – CMQT 2.51 nmol/ml; 2, homocysteine – CMQT 0.31 nmol/ml; 3, 2-mercaptopropionic acid – CMQT (internal standard) 3.33 nmol/ml; 4, 3-mercaptopropionic acid – CMQT (internal standard) 3.33 nmol/ml; 5, cysteine – CMQT 12.1 nmol/ml; 6, cysteinylglycine – CMQT derivative 2.36 nmol/ml; 7, excess of CMQT

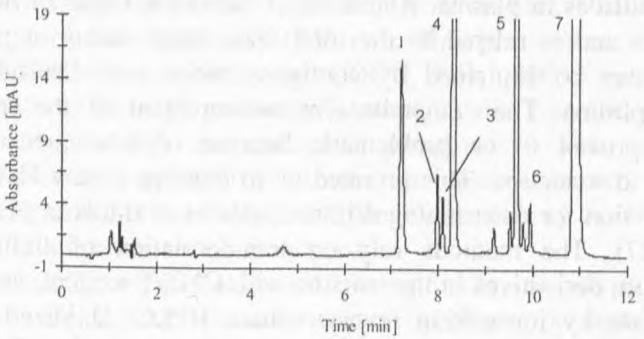

Fig. 5. Typical HPLC chromatogram of human plasma spiked with 40 nmol/ml of N-acetylcysteine and 400 nmol/ml 3,3'-dithiodipropionic acid (internal standard) and derivatized with 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT). Peaks: 1, S-quinolinium derivative of N-acetylcysteine; 2, S-quinolinium derivative of glutathione; 3, S-quinolinium derivative of homocysteine; 4, S-quinolinium derivative of 3-mercaptopropionic acid; 5, S-quinolinium derivative of cysteine; 6, S-quinolinium derivative of cysteinylglycine; 7, excess of CMQT

6. Conclusions

The majority of bioanalytical methods do not use just one simple HPCE or HPLC separation step, but rather involve several sample pretreatment steps which simplify the matrix, and often preconcentrate and chemically modifity the analytes. HPCE following extensive clean – up involves no less effort than performing an HPLC separation, but for certain applications, such as chiral separations, there are clearly attractions in using HPCE. Minimal preparation by removal of proteins is satisfactory, provided that the analyte concentration is within the useful range of the detection system in use. In the case of biological thiols, for the reason of their susceptibility to oxidation and lack the structural properties necessary for the production of signals compatible with common HPCE or HPLC detectors, derivatization is almost always performed.

REFERENCES

[1] G.E. Bainlescu, P. Dumitrescu, P. Gh. Zugrăvescu, Sampling, M. Masson, J.F. Tyson, (Eds), Ellis Horwood Ltd, New York, 1991.

[2] G. Lunn, L.C. Hellwig (Eds), *Handbook of Derivatization Reactions for HPLC*, John Wiley and Sons Inc., New York, 1998.

[3] E. Bald, *Wykrywanie i oznaczanie tioli w próbkach biologicznych.* [in] *Biotiole w fizjologii, patologii i terapii*, L. Włodek, Ed. Wydawnictwo Uniwersytetu Jagiellońskiego, Kraków, 2003, p. 333 – 354.

[4] M. Chicherro, Z. Zapardiel, E. Bermejo, J.A. Perez. L. Hernández, *J. Chromatogr.*, 622 (1993) 103.

[5] S. Li, K. Fried, J.W. Wainer, D.K. Lloyd, *Chromatographia*, 35 (1993) 216.

[6] Z. K. Shihabi. M.E. Hinsdale, *J. Chromatogr. B*, 669 (1995) 75.

[7] Z.K. Shihabi, *J. Chromatogr. A*, 652 (1993) 471.

[8] D.K. Lloyd, *Biofluid and Tissue Analysis of Drugs: Methodological Surveys in Bioanalysis of Drugs*, Vol. 23, Royal Society of Chemistry, Cambridge, 1944.

[9] D.K. Lloyd, H. Wätzig, *J. Chromatogr. B*, 663 (1995) 400.

[10] C.K. Lim, *Liquid Chromatography in Biomedical Analysis: Basic approach*, [in] *Liquid Chromatography in Biomedical Analysis*, T. Hanai, Ed, Elsevier Science Publishing Company Inc. Amsterdam, 1991, p. 1 – 18.

[11] D.K. Lloyd, *J. Chromatogr. A*, 735 (1996) 29.

[12] G.J. Hankey, J.W. Eikelboom, *Lancet*, 354 (1999) 407.

[13] K. Shimada, K. Mitamura, *J. Chromatogr. B*, 659 (1994) 227.

[14] E. Kaniowska, R. Głowacki, G. Chwatko, P. Kubalczyk, E. Bald, *Determination of Reduced Sulfur Compounds in the Aquatic Environment by High-Performance Liquid Chromatography and Capillary Electrophoresis*, [in] *Chemistry for the Protection of the Environment 3*, L. Pawłowski, M.A. Gonzales, M.R. Dudziñska, W. J. Lacy, Eds. Plenum Publishing Corporation. New York, 1998, p. 9-22, and papers cited herein.

[15] E. Bald, R. Głowacki, *J. Liq. Chromatogr.*, 24 (2001) 1323.

[16] G. Chwatko, E. Bald, *J. Chromatogr. A*, 949 (2002) 141.

[17] E. Bald, G. Chwatko, R. Głowacki, K. Kuśmierek, *J. Chromatogr. A*, 1032 (2004) 109.
[18] E. Kaniowska, G. Chwatko, R. Głowacki, P. Kubalczyk, E. Bald, *J. Chromatogr. A*, 798 (1998) 27.
[19] E. Bald, G. Kaniowska, G. Chwatko, R. Głowacki, *Talanta*, 50 (2000) 1233.
[20] G. Chwatko, E. Bald,*Talanta*, 52 (2000) 509.
[21] E. Bald, R. Głowacki, J. Drzewoski, *J. Chromatogr. A*, 913 (2001) 29.
[22] S. Sypniewski, E. Bald, *J. Chromatogr. A*, 729 (1996) 335.
[23] E. Bald, S. Sypniewski, J. Drzewoski, M. Stępień, *J. Chromatogr. B*, 681 (1996) 283.
[24] R. Głowacki, K. Wójcik, E. Bald, *J. Chromatogr. A*, 914 (2001) 29.
[25] R. Głowacki, E. Bald, *Determination of N-acetylcysteine and Main Endogenous Thiols in Human Plasma by HPLC with Ultra Violet Detection in the Form of Their S-quinolinium Derivatives*, Seventh International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers, Brugge, Belgium, February 6-8 , 2002, Book of Abstracts p. 53.
[26] E. Bald, *Fully automated HPLC based analysis of cysteine and related compounds in plasma using on line microdialysis as sample preparation*, 7th International Congress on Amino Acids and Proteins, August 6-10, 2001,Vienna, Austria, Amino Acids, 21 (2001) 3.
[27] R. Głowacki, A. Kropiwnicka, J. Drzewoski, E. Bald, *Determination of Reduced and Oxidized Species of Cysteine and Homocysteine in Human Urine by HPLC with Ultraviolet Detection*, Homocysteine Metabolism, 3rd International Conference, Sorrento, 1-5 July 2001, Abstracts Book, 128.