Amino Acids (2005) 28: 431–433
DOI 10.1007/s00726-005-0195-8

Amino Acids
Printed in Austria

Analysis of saliva for glutathione and metabolically related thiols by liquid chromatography with ultraviolet detection

Short Communication

E. Bald and **R. Głowacki**

Department of Environmental Chemistry, University of Lodz, Lodz, Poland

Received December 5, 2004
Accepted February 16, 2005
Published online May 20, 2005;

Summary. A method for simultaneous determination of glutathione and its precursors cysteine, cysteinylglycine and homocysteine in saliva is presented. The procedure involves reductive conversion of disulfides to thiols, derivatization to their 2-S-quinolinium derivatives with 2-chloro-1-methylquinolinium tetrafluoroborate and separation and quantitation by reversed-phase ion-pairing high performance liquid chromatography with ultraviolet detection at 355 nm. The calibration performed with saliva samples spiked with thiol disulfides, within the practical concentration ranges, showed linear response of the detector. The method applied to the saliva samples donated by volunteers showed mean concentration (SD, n = 8) of cysteine, cysteinylglycine, glutathione and homocysteine: 26.5 (31.6), 6.05 (5.12), 16.97 (7.68), 3.64 (1.34) nmol/ml respectively.

Keywords: Saliva – Cysteine – Cysteinylglycine – Glutathione – Homocysteine – Liquid chromatography

Non standard abbreviations: CSH, cysteine; CGSH, cysteinylglycine; GSH, glutathione; HCSH, homocysteine; CMQT, 2-chloro-1-methylquinolinium tetrafluoroborate; TBP, tri-n-butylphosphine; ABDF, 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide; DTNB, 5,5′dithio-bis-(2-nitrobenzoic acid); DTE, dithioerytritol; PCA, perchloric acid; TCA, trichloroacetic acid; LLQ, lower limit of quantitation

Introduction

Saliva is a complex secretion whose components exert a well documented role in health and disease (Moore et al., 1994) and its diagnostic use is spreading (Streckfus and Bigler, 2002). In addition to its lubricant properties, saliva contains many biochemical systems, known to be involved in tissue repair, and antibacterial components (Tenovuo et al., 1987). Moreover, saliva contains various antioxidants, including uric acid, which contributes more than half of the total radical-trapping capacity (Moore et al., 1994), and glutathione and its precursors (Zappacosta et al., 2003). Glutathione and other aminothiols are also involved in the production of volatile sulphur compounds responsible for the bad breath in periodontophatic patients (Persson et al., 1990).

Little is available in the literature pertaining to the determination of aminothiols in saliva. Tonzetich and Johnson (1977) employed Ellman's (1959) method for determination of thiol groups, and method described by Zahler and Cleland (1968) for determination of disulphide groups in saliva. Both methods are not specific for individual compounds and measure the sum of thiols and disulphides, respectively. The methods consist in reaction of the –SH group with DTNB reagent and subsequent measurement of the absorbance at 412 nm. In the case of disulphides the –S-S-linkages have to be reductively converted to thiols with DTE. Zappacosta et al. (2003) determined cysteine, cysteinylglycine and glutathione by slightly modified method of Araki and Sako (1987) originally developed for homocysteine in plasma. The method takes advantage of derivatization of the –SH groups with fluorescence tag ABDF and separation and quantitation by HPLC. The only mention of homocysteine quantitation in saliva was done by Boulot-Tolle et al. (1992) who applied for this purpose a radioisotopic method developed earlier for plasma by Chadefaux et al. (1989).

In the present communication we report our results on determination of glutathione and three metabolically related aminothiols cysteine, cysteinylglycine and homocysteine in saliva. The elaborated analytical procedure for

saliva constitute extension of our recently published method (Bald et al., 2004) for determination of plasma thiols by HPLC with ultraviolet detection.

Materials and methods

Collection of saliva samples

Saliva samples were collected in the early morning, after fasting and before tooth brushing, by standard method consisted in spitting into a container [Boulot-Tolle et al., 1992]. A 1.5 ml of saliva were collected and analyzed without delay for total CSH, CGSH, GSH and HCSH.

Chemicals, reagents and apparatus

All chemicals and reagents needed, except TBP, were purchased and prepared as was described in previous paper [Bald et al., 2004]. TBP (Fluka, Buchs, Switzerland) was used in the form of 10% solution in methanol. HPLC analysis was performed with the Hewlett-Packard (Waldbronn, Germany) HP 1100 Series System consisted of quaternary pump, an autosampler, vacuum degasser, diode array detector, and controlled by HP ChemStation software.

Analytical procedure

To 200 μl of saliva 50 μl of 0.1 M EDTA, 200 μl of 0.1 M pH 7.4 phosphoric buffer and 25 μl of 10% TBP were added. The mixture was incubated for 30 min at 60°C followed by addition, after cooling, 50 μl of 0.1 M phosphoric buffer and 50 μl of 3 M PCA and centrifuged (10 min, 10000 × g). A 20 μl of the supernatant was transferred into analytical column (ZORBAX SB-C18, 150 × 4.6 mm, 5 μm; Waldbronn, Germany) of the HPLC system. The column oven temperature was 25°C, the flow rate 1.2 ml/min, and the detector wavelength 355 nm. Under gradient elution with profile: 0–8 min 10–30% B, 8–10 min 30–10% B (elution solvent A was 0.07 M pH 1.65 TCA buffer prepared from TCA and lithium hydroxide solutions of the same concentration, and B acetonitrile) CMQT derivatives of GSH, HCSH, CSH and CGSH eluted after 5.1, 5.4, 6.8, and 6.9 min, respectively.

Table 1. Method validation data

Aminothiol	Regression equation	R^2	Linear range [nmol/ml]	RSD [%]		LLQ[a] [nmol/ml]
				Minimum	Maximum	
Cysteine	y = 2.1521x + 2.19	0.9903	0.5–100	1.39	15.56	0.5
Cysteinylglycine	y = 1.4955x − 0.184	0.9979	0.5–30	2.85	12.49	0.5
Glutathione	y = 1.5723x + 5.28	0.9792	0.5–30	1.30	10.29	0.5
Homocysteine	y = 1.558x + 0.761	0.9929	0.5–30	1.38	14.14	0.5

[a] Lower limit of quantitation understood as the lowest measurable concentration at which imprecision and inaccuracy are lower than 25%

Table 2. Total aminothiols content in saliva from apparently healthy donors

Subject	Sex (F, M)	Age (Years)	Total aminothiols [nmol/ml]							
			Cysteine		Cysteinylglycine		Glutathione		Homocysteine	
			Found (SD)	RSD[a] [%]	Found (SD)	RSD[a] [%]	Found (SD)	RSD[a] [%]	Found (SD)	RSD[a] [%]
1	M	24	98.67 (2.62)	3.63	16.84 (0.99)	4.12	20.08 (0.14)	1.06	4.10 (0.28)	4.42
2	F	48	43.97 (2.76)	6.56	10.34 (0.49)	4.14	17.39 (0.42)	2.12	4.89 (0.14)	2.14
3	M	52	13.83 (0.99)	3.31	3.47 (0.42)	8.66	5.43 (0.07)	1.52	2.42 (0.21)	5.98
4	F	24	10.23 (3.82)	7.26	3.63 (0.42)	3.34	21.59 (0.49)	4.29	5.28 (0.64)	8.00
5	F	24	5.91 (0.49)	4.83	1.72 (0.14)	7.07	10.10 (0.49)	4.69	1.64 (0.14)	7.86
6	F	24	9.62 (0.85)	2.81	2.93 (0.00)	0.00	14.93 (0.35)	2.33	4.84 (0.07)	1.03
7	M	24	20.44 (0.49)	1.55	6.25 (0.35)	4.14	15.34 (0.85)	5.08	2.55 (0.21)	4.56
8	F	30	9.41 (0.28)	2.01	3.25 (0.21)	5.81	30.93 (0.14)	1.06	3.43 (0.00)	0.00

[a] n = 3

Calibration

Calibration was performed as described earlier (Bald et al., 2004). In brief, the saliva samples were spiked with growing amounts of the four analytes in their disulfide forms and processed according to the recommended procedure using seven- to nine-point calibration curves, and at each concentration three replicates were assayed.

Results and discussion

The method validation data are summarized in Table 1. The calibration curves were linear within the concentration ranges studied. The recommended HPLC procedure enables measurement of four main saliva aminothiols CSH, CGSH, GSH and HCSH with low imprecission (mean RSD within calibration range, 4.19, 6.34, 4.45 and 4.26%, respectively) and high sensitivity (LLQ, 0.5 nmol/ml saliva).

The developed method was applied to the analysis of saliva samples donated by volunteers, 24–52 years old (3 man and 5 women). The results are placed in Table 2. Concentrations of aminothiols, mostly cysteine and glutathione, vary significantly from person to person, and are in general higher than those measured by Zappacosta et al. (2003). Our volunteers were not checked for their health conditions.

In conclusion, the proposed method easily and reliably measures glutathione and metabolically related cysteine, cysteinylglycine and homocysteine. Moreover, at our best knowledge, this is the first report describing possibility of determination of all these compounds in one analytical run.

Acknowledgement

We are highly indebted to the University of Lodz for grant No. 505/666 supporting this research.

References

Araki A, Sako Y (1987) Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detector. J Chromatogr 422: 43–52

Bald E, Chwatko G, Głowacki R, Kuśmierek K (2004) Analysis of plasma thiols by high-performance liquid chromatography with ultraviolet detection. J Chromatogr 1032: 109–115

Boulot-Tolle M, Chadefaux B, Kamoun P (1992) Salivary homocysteine concentration. Clin Chem 38: 1504–1505

Chadefaux B, Coudac M, Hamet M, Kamoun P (1989) A rapid method for the determination of total homocysteine in plasma. Clin Chem 35: 2002

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82: 70–77

Moore S, Calder KA, Miller NJ, Rice Evans CA (1994) Antioxidant activity of saliva and periodontal disease. Free Radic Res 21: 417–425

Persson S, Edlund MB, Claesson J (1990) The formation of hydrogen sulfide and methylmercaptan by oral bacteria. Oral Microbiol Immunol 5: 195–201

Streckfus CF, Bigler LR (2002) Salivary gland and saliva. No. 3: Saliva as diagnostic fluid. Oral Dis 8: 69–76

Tenovuo J, Grahn E, Lehtonen OP, Hyyppa T, Karhuvaara L, Vilja P (1987) Antimicrobial factors in saliva: ontogeny and relation to oral health. J Dent Res 66: 475–479

Tonzetich J, Johnson PW (1977) Chemical analysis of thiol, disulphide and total sulphur content of human saliva. Arch Oral Biol 22: 125–131

Zahler WL, Cleland WW (1968) A specific and sensitive assay for disulphides. J Biol Chem 243: 716–719

Zappacosta B, Manni A, Persichilli S, Scribano D, Minucci A, Lazzaro D, De Sole P, Giardina B (2003) HPLC analysis of some sulphur compounds in saliva: comparison between healthy subjects and periodontopatic patients. Clin Chim Acta 338: 57–60

Authors' address: Prof. Dr. Edward Bald, Department of Environmental Chemistry, University of Lodz, Pomorska 163, 90-236 Lodz, Poland, E-mail: ebald@uni.lodz.pl