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Review

Determination of endogenous thiols and thiol drugs in urine by HPLC with ultraviolet detection☆

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ABSTRACT

Analysis of urine for endogenous thiols and thiol drugs content by HPLC with ultraviolet detection is addressed. Other methodologies for detection and determination of thiols in urine are only mentioned. Outline of metabolism, role of main biological thiols in physiological and pathological processes and their reference concentrations in urine are presented. In particular, urine sample preparation procedures, including reduction of thiol disulfides, chemical derivatization and reversed-phase HPLC separation steps are discussed. Some experimental details of analytical procedures for determination of endogenous thiols cysteine, cysteinylglycine, homocysteine, *N*-acetylcysteine, thioglycolic acid; and thiol drugs cysteamine, tiopronin, D-penicillamine, captopril, mesna, methimazole, propylthiouracil and thioguanine are reviewed.

Contents

Abbreviations: BCPB, 1-benzyl-2-chloropyridinium bromide; CGSH, cysteinylglycine; CMPI, 2-chloro-1-methylpyridinium iodide; CMQT, 2-chloro-1-methylquinolinium tetrafluoroborate; Cys, cysteine; CysGly, cysteinylglycine; GSH, glutathione; Hcy, homocysteine; MeTTCA, 4-methyl-2-thioxothiazolidine-4-carboxylic acid; NAC, *N*-acetylcysteine; T_3CA, 2-thioxotetrahydro-1,3-thiazine-4-carboxylic acid; TCDI, 1,1′-thiocarbonyldiimidazole; TCEP, tris-(2-carboxyethyl)phosphine; TTCA, 2-thioxothiazolidine-4-carboxylic acid; TTCG, 2-thioxothiazolidine-4-carbonylglycine.

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1. Introduction

Thiols are chemically and biochemically very active components of the sulfur cycle of the natural environment. Low molecular-mass thiols, such as homocysteine (Hcy), cysteine (Cys), cysteinylglycine (CysGly) and glutathione (GSH) are critical cellular components that play numerous roles in metabolism and homeostasis, and are important in a variety of physiological and pathological processes. Biological thiols are metabolically related in the methionine metabolic pathway and occur widely in human tissues and fluids.

Methionine taken with diet is converted intracellularly, via *S*-adenosylmethionine and *S*-adenosylhomocysteine, to homocysteine. Homocysteine can be metabolized back to methionine by the remethylation pathway or converted by the transsulfuration pathway to cystathionine and cysteine, a fundamental substrate for glutathione biosynthesis [1,2]. Glutathione is synthesized in cells by the sequential actions of γ-glutamylcysteine synthetase and glutathione synthetase in a series of reactions, which have been termed as the γ-glutamyl cycle. Cysteinylglycine is formed by the breakdown of glutathione by γ-glutamyl transpeptidase [3].

Kidneys play a major role in the synthesis and interorgan exchange of several amino acids. The quantitative importance of renal amino acid metabolism in the body is not, however, clear. It is well established that, under physiologic circumstances, only minimal amounts of amino acids are excreted into human urine. Approximately 450 mmol of amino acids are filtered daily at the glomerulus [4,5]. More than 99% of filtered amino acids are reabsorbed in the proximal tubule with only approximately 5 mmol being ultimately excreted in the urine [4–6]. Fractional excretions of most amino acids are between 0.2% and 2.5% [4,7]. For example, only 1% of the homocysteine filtered to the glomerulus is normally found in urine, although these proportions may increase in various pathologic conditions.

For all their similarities in structure and common chemistry, the functions of thiols in organism are remarkably different. Glutathione plays an essential role in protecting cells from toxic species, such as free radicals and reactive oxygen intermediates [8]. It has been documented that a decrease of glutathione concentration may be associated with aging [9] and the pathogenesis of many diseases, including AIDS [10], Alzheimer's disease [11], alcoholic liver disease [12] and pulmonary diseases [13,14], e.g. chronic obstructive pulmonary disease and asthma. Homozygous homocystinuria, a rare genetic disorder usually caused by cystathionine β-synthase deficiency, leads to severe increase of plasma homocysteine to concentrations higher than 100 μM [15] and is associated with venous thrombosis and premature atherosclerosis. Mildly elevated plasma homocysteine levels have been associated with an increased risk for cardiovascular and cerebrovascular diseases in men [16], and highly elevated plasma and urine levels are a clinically relevant indicator of well known, but fortunately rare, group of inborn errors of metabolism called homocystinuria [2,17]. Altered levels of homocysteine have been implicated in hyperhomocysteinemia [18,19] and in a number of pathological conditions including Alzheimer's and Parkinson's disease [20], as well as autoimmune deficiency syndrome [21]. Disorders of cysteine metabolism include cystinosis, an autosomal recessive disease caused by a defect in lysosomal transport, and cystinuria, an inherited disorder of transport of the amino acids cystine, ornithine, lysine and arginine leading to a high concentration of cystine in urine [2,22]. Elevated cystine concentration in the urinary tract is responsible for the formation of kidney stones. Elevated levels of cysteinylglycine in plasma or urine are documented in patients with rheumatoid arthritis [23,24] and may be associated with the extent of the inflammation.

2. Thiols as analytical objects

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, homocystinuria.

The analysis of thiols can be quite perplexing. The main challenges in the assay of thiols lie in their unfavorable physicochemical properties. These compounds are highly polar and water soluble, which makes their extraction from biological matrices without derivatization almost impossible. As free amino acids, thiols exist in either their reduced or oxidized forms. The redox chemistry of thiols is dominated by the sulfhydryl group (–SH), which enables them to undergo oxidation in the presence of an electron acceptor to form the disulfides (–S–S–). The absence of chromophore presents the next problem. Thiols lack the structural properties necessary for the production of signals compatible with common chromatographic detectors such as UV–vis absorbance and fluorescence. Therefore, the analyst must resort to derivatization for signal enhancement and labile sulfhydryl group blocking if UV–vis or fluorescence detection methods are employed.

3. Sample collection, storage and preparation

The biological fluid most commonly analyzed is plasma. Urine is usually free from proteins and lipids but contains many other components, the concentrations of which depend largely on diet and lifestyle. Detection and determination of urinary components, including thiols, is therefore very difficult, and so far urine has rarely been analyzed for these compounds. In analysis of urinary thiols it is often difficult to obtain 24-h urine collections. Therefore, random urine samples frequently are collected for analysis of thiols, and the thiol values are normalized against creatinine content to compensate for fluctuation of the concentration of solutes owing to variability in urine volume. The excretion rates of thiols may vary independently of creatinine over a 24-h period. Nevertheless, creatinine has been shown to be the reference compound with the lowest variance both within and between individuals [25,26]. The non-invasive nature of urine collection and the fact that many drugs and metabolites are concentrated in the urine make this physiological fluid very attractive as an object of analysis in clinical practice.

The analysis of biological samples including urine presents a variety of problems. These encompass: (1) large number of individual compounds in the sample, leading to difficulty in resolving the analytes of interest, (2) low concentrations of exogenous or endogenous compounds of interest, leading to detection difficulties, and (3) conjugation of analytes to protein and/or low-molecular-mass components of the analyzed mixture.

Taking under consideration the above-mentioned problems, it is not surprising that the majority of bioanalytical methods do not use just one simple chromatographic separation step, but rather involve several sample pretreatment steps which simplify the matrix, and often preconcentrate and chemically modify the analytes. In general, the procedures recommended for determination of urinary total thiols encompass three steps: (1) generation of free thiols from disulfides in order to make them accessible to the derivatization reagent; (2) derivatization in order to block the labile thiol groups and to enhance the detector signal, and (3) separation and quantitation (Fig. 1). The term total thiol, as applied to urine samples, refers to the sum of concentrations of reduced and oxidized (symmetrical and unsymmetrical disulfides) forms. Normal urine is generally free of proteins and accordingly it does not contain protein-bound thiols.

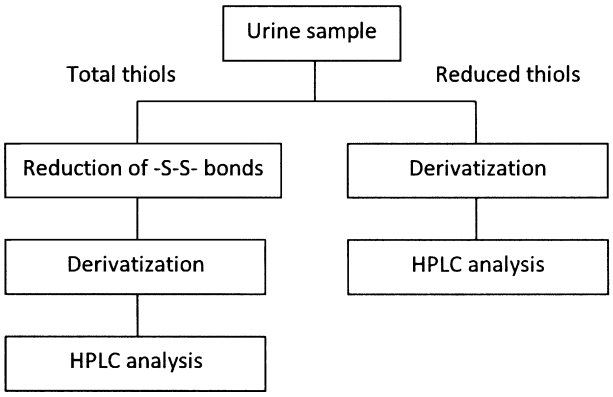

Fig. 1. Scheme of the analytical procedure for determination of total and reduced urinary thiols.

In most cases the first morning urine samples are collected from apparently healthy donors or patients. Samples can be analyzed without delay or kept in frozen state at −20 °C. As was recently found [27], main endogenous urinary thiols ex vivo are fairy stable during the early hours after urine collection. This means that urine, kept at ambient temperature, with no stabilizer added, can be safely analyzed for reduced thiols (and redox status) within 4–5 h. Total loss of reduced cysteine in urine was observed after 14 days at room temperature and after 21 days in the refrigerator (4 °C). A decrease of 96% of reduced cysteine was observed within 3 months in urine stored at −20 °C [27].

3.1. Reduction

Determination of the hydrophilic thiols is complicated by their occurrence in multiple forms, since their free sulfhydryl group is prone to oxidation. Consequently, the bulk of urinary thiols occurs in the disulfide form, rendering them inaccessible to derivatization reagent. A total pool of cysteine in human urine consists of 6.1% in reduced and 93.9% in oxidized form (children 6.0% and 94.0%, and adults 6.3% and 93.7%, respectively) [27]. In children about 9.6% of the total cysteinylglycine is reduced and 90.4% oxidized, whereas in adults 13.9% is reduced and 86.1% is present in the oxidized form [27]. The determination of total amount of a particular thiol must account for both reduced and oxidized forms. For this purpose, a reductive cleavage of the disulfides is made before the derivatization and instrumental final analysis steps. In urine samples, for reduction dithiotreitol [28,29], sodium borohydride ($NaBH_4$) [30–34], tri-*n*-butylphosphine [35,36] or tris-(2-carboxyethyl)phosphine (TCEP) [37–39] are used.

Reducing reagent must be compatible with the specific thiol derivatization agent. Thiol reductants such as 2-mercaptoethanol and dithiothreitol are not suitable for this purpose when highly reactive derivatization reagent, e.g. 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) [34,35,39], is to be used. These reductants consume CMQT and produce additional derivatives finally interfering with chromatogram. The use of sodium borohydride is inconvenient, may lead to problems with derivatization reaction because of pH control difficulties and significant sample foaming. Recently TCEP continues to receive increased popularity for the reason of the user friendliness.

3.2. Derivatization

All methods, except those based on electrochemical and tandem-mass spectrometry detection, depend on pre- or post-column derivatization of thiols. Useful reagents must form thiol derivatives with sufficient absorption or fluorescent yield to measure thiols at trace concentrations. Furthermore, the ideal reagent should show no absorption and should be non-fluorescent and react rapidly and specifically with thiols to form stable products. Numerous reagents are available for the thiol derivatization. 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 excellent works [40–42].

3.3. Chromatographic separation

Large number of individual components in the urine sample and sometimes low concentration of compounds of interest constitute a challenging problem to the analyst. To resolve the analytes of interest separation techniques have to be used and so far HPLC in the reversed-phase mode is the method of choice. Reversed-phase separation is based upon the non-polar, or hydrophobic, interaction between non-polar sample molecules and the non-polar stationary phase. Urinary thiols or their derivatization reaction products, often containing ionizable or ionic moieties, can be too polar to be retained by the reversed-phase mode. In such situation, when mobile phase pH manipulation does not help, addition of ion-pairing reagent can prove to be useful. HPLC can be easily coupled with various detection methodologies, but in this survey only ultraviolet detection will be considered. UV detector, known for its stability, universality and low demand in terms of maintenance, is the most common among HPLC detectors. Moreover, it belongs to the standard instrumentation in hospital laboratories and staff is usually well experienced in its use.

4. Determination of endogenous thiols

The biological fluid most commonly analyzed is plasma. Therefore, plasma thiols are well recognized, but the presence and concentration of thiols in urine remains a matter of debate. The presence of cysteine, cysteinylglycine, homocysteine and *N*-acetylcysteine in urine has been confirmed. Literature values of these endogenous thiols in human urine are presented in Table 1. Thioglycolic acid (mean 0.68 ± 0.16 [43] or 0.81 ± 0.26 mmol per mol of creatinine [39]) has also been detected in urine. Hannestad and Sorbo [43] and Wroński [44,45] reported that 3-mercaptolactic acid, 2-mercaptopropionic acid, and 2-mercaptoethanol may also be assumed to be endogenous constituents of human urine. Nevertheless, not all of them have been confirmed by other researchers. Presence of glutathione in normal human urine is also questionable. It was stated that glutathione is not present in urine [32,34,39,46,47]. On the contrary, Lochman et al. [38] found 0.5 μM glutathione in urine, and Seiwert and Karst [48] determined 0.2–0.5 mmol of glutathione per mol of creatinine.

Several methods have been reported for the determination of urinary thiols, including radioenzymic analysis [49], flow-injection analysis with biamperometric detection [50], isotachophoresis [44,45], CE [38], GC–MS [43], capillary-column liquid chromatography with amperometric detection [46], and, first of all, liquid chromatography with tandem mass spectrometry [29,51,48], fluorescent [28,30–33,52,47] and UV–vis [34–37,39] detection. But in this review only liquid chromatography methods with ultraviolet detection will be reviewed. Detection and quantitation by spectrophotometry can be beneficial considering the fact that UV–vis detector is the most common among HPLC detectors.

In the vast majority of cases HPLC-UV methods for urinary thiols take advantage of derivatization with pyridinium [35] or quinolinium salts [27,34,36,53] possessing an active halogen atom as reactive moiety, followed by separation by ion-pair reversed-phase HPLC. Ion-pairing agent (trichloroacetate) is always added to the

Table 1
Literature values of total and reduced thiols in human urine expressed in mmol per mol of creatinine.

Cys	Hcys	CysGly	NAC	*n*	Method	Ref.
–	–	–	2.2 ± 0.8	22	GC–MS	[43]
30.9 ± 12.3	1.0 ± 0.4	–	–	39	CE-FL	[38]
1.8–20.5	–	–	–	60	HPLC-FL	[31]
33.4 ± 15.5	1.9 ± 1.5	–	–	20[a]	HPLC-FL	[32]
28.3 ± 7.0	1.3 ± 0.4	–	–	24[b]		
31.6 ± 9.6	–	–	–	100	HPLC-FL	[33]
18.7 ± 5.8	1.5 ± 5.8	–	0.4 ± 0.2	10	HPLC-FL	[52]
9.5 ± 4.5[c]	0.4 ± 0.1[c]	–	–			
30.7 ± 4.3	1.1 ± 0.4	1.6 ± 0.3	1.7 ± 0.7	8	LC-MS/MS	[48]
15.8[c,d]	16.3[c,d]	51.3[c,d]	13.5[c,d]			
31.5 ± 14.5	2.0 ± 0.9	1.7 ± 0.8	–	38[a]	HPLC-UV	[34]
31.3 ± 13.2	2.2 ± 0.9	1.9 ± 0.9	–	53[b]		
29.0 ± 11.4	–	1.7 ± 0.9	1.6 ± 0.6	29	HPLC-UV	[39]
30.1 ± 10.5	–	1.7 ± 0.8	–	45	HPLC-UV	[27]
1.5 ± 0.6[c]	–	0.1 ± 0.1[c]	–	45		
130.4 ± 58.8[e]	–	–	–	5	HPLC-UV	[36]
9.8 ± 8.0[c,e]	–	–	–	5		

n: number of donors.
[a] Male.
[b] Female.
[c] Reduced form of thiol.
[d] % of total.
[e] Expressed in μM.

mobile phase in order to enhance chromatographic retention of thiol 2-*S*-pyridinium or 2-*S*-quinolinium derivatives resulted from the derivatization reaction. These cations (for example see Fig. 2) are too polar to be retained by non-polar column stationary phases without ion-paring agent.

4.1. Cysteine, cysteinylglycine and homocysteine

The procedure for simultaneous determination of the three main urinary thiols – cysteine, cysteinylglycine and homocysteine – has been reported [34]. Urine sample was reduced with sodium borohydride, to convert disulfides to thiols which were then derivatized with CMQT, and base-line separated by ion-paring RP-HPLC. The calibration graphs for each analyte, obtained by use of normal urine spiked with increasing amounts of cysteine, cysteinylglycine, and homocysteine, were linear over the ranges covering most practical situations that is 50–500, 2.5–25 and 2–14 μM in urine for cysteine, cysteinylglycine and homocysteine, respectively. The recovery for the assay of each target analyte ranged from 98% to 100%, and lower limit of detection was between 0.12 and 0.25 μM. The method was applied to urine donated by 91 apparently healthy volunteers in order to establish the reference values in humans (Table 1). The urine donors were distributed for sex (38 men and 53 women) and the thiols concentrations are expressed in mmol/mol creatinine. The CMQT derivatization reagent was also applied for determination of reduced and total cysteine in human urine [36].

2-Chloro-1-methylpyridinium iodide (CMPI) has been reported as derivatization agent for urinary excretion measurement of cysteine and homocysteine [35]. Total urine cysteine and homocysteine were determined in urine by reduction of disulfide bonds with tri-*n*-butylphosphine, conjugation of the thiols with CMPI, separation of 2-*S*-pyridinium derivatives by reversed-phase ion pair liquid chromatography, and detection and quantitation by spectrophotometry at 312 nm. The LOD was 2 μM (4 pmol on column) and intra- and inter-day imprecision was below 5% (RSD).

Determination of cysteine and cysteinylglycine in urine with the use of 1-benzyl-2-chloropyridynium bromide (BCPB) as thiol derivatization reagent following reduction of the –S–S– bonds by TCEP has also been performed [54]. The BCPB reacts in slightly alkaline water environment yielding stable 2-*S*-pyridinium derivatives possessing the absorption maximum at 315 nm. Bathochromic shift from the reagent maximum (275 nm) to that of derivative (315 nm) is analytically advantageous. Thanks to this phenomenon, it is possible to apply a large excess of BCPB in order to drive the derivatization reaction to completion (in real world sample) and avoid appearance of peak of unreacted BCPB on the chromatogram. The validation protocol has shown that the method is linear in the ranges of concentrations (50–300 μM for cysteine and 5–50 μM for cysteinylglycine) covering the most practical situation. Limit of detection for both analytes was 0.2 μM urine [54].

Amarnath et al. [37] reported a specific HPLC-UV method for determination of cysteine, cysteinylglycine, homocysteine and D-penicillamine. The key step in the analysis is treatment with 1,1′-thiocarbonyldiimidazole (TCDI) that rapidly and quantitatively reacts with both amino and thiol groups to form stable cyclic dithiocarbamates with intense UV absorption in the wavelength range of 250–300 nm (Fig. 3). The analysis of urine for total thiols had four steps: (1) reduction of disulfides with tris-(2-carboxyethyl)phosphine, (2) cyclization with TCDI, (3) partial purification and (4) separation and quantitation by liquid

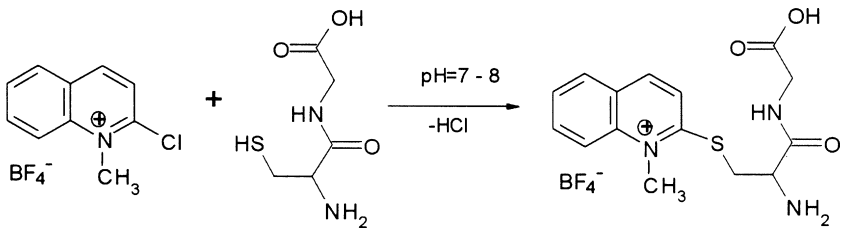

Fig. 2. Equation for the derivatization reaction of thiols, represented by cysteinylglycine, with 2-chloro-1-methylquinolinium tetrafluoroborate.

Table 2
Some details of the analytical procedures for determination of thiol drugs in urine by HPLC with UV detection.

Analyte	Derivatization	Column	Analytical wavelength	Linearity range	Detection limit	Ref.
Mesna	CMQT	Waters Nova-Pak C18 (150 × 3.9 mm, 5 μm)	350 nm	0.2–800 nmol/ml (total) 0.2–30 nmol/ml (reduced)	0.1 nmol/ml	[90]
Captopril	NEM	μBondapak C18 (300 × 4 mm, 10 μm)	254 nm	0.5–11.5 nmol/ml	0.2 nmol/ml	[77]
	p-Bromophenacyl bromide	YWG-C18 (150 × 5 mm, 10 μm)	260 nm	0.05–1 nmol/ml	0.05 nmol/ml	[78]
	BCBP	Separon SGX C18 (150 × 3.3 mm, 7 μm)	314 nm	0.2–13.8 nmol/ml (disulfide) 0.2–5.5 nmol/ml (reduced)	0.01 nmol/ml	[79]
	BMP	Du Pont Zorbax C8 (250 × 4.6 mm, 6 μm)	254 nm	3.6–72.6 nmol per injection	1.4 nmol per injection	[80]
	CMQT	Zorbax C18 (150 × 4.6 mm, 5 μm)	355 nm	0.1–200 nmol/ml	0.05 nmol/ml	[81]
Cysteamine	CMQT	Zorbax C18 (150 × 4.6 mm, 5 μm)	355 nm	0.1–50 nmol/ml (reduced) 0.4–400 nmol/ml (total)	0.05 nmol/ml 0.2 nmol/ml	[61]
Tiopronin	CMQT	Zorbax C18 (150 × 4.6 mm, 5 μm)	355 nm	1–200 nmol/ml	0.5 nmol/ml	[68]
D-Penicillamine	CMQT	Zorbax C18 (150 × 4.6 mm, 5 μm)	355 nm	1–200 nmol/ml	0.5 nmol/ml	[68]
Methimazole	CMQT	Zorbax C18 (150 × 4.6 mm, 5 μm)	345 nm	2–440 nmol/ml	1 nmol/ml	[99]
	Post-column reaction	Symmetry C18 (150 × 4.6 mm, 5 μm)	350 nm	2–10 nmol/ml	1 nmol/ml	[100]
Propylthiouracil	Post-column reaction	Symmetry C18 (150 × 3.9 mm, 5 μm)	350 nm	0.4–1 nmol/ml	0.3 nmol/ml	[101]
Thioguanine	Post-column reaction	Symmetry C18 (150 × 3.9 mm, 5 μm)	350 nm	0.8–1.7 nmol/ml	0.4 nmol/ml	[110]

chromatography with ultraviolet detection. The method is repeatable (variation of less than 5.0%) and sensitive; the limit of detection was 2 pmol per injection. Because the chemical modification is specific for compounds with amino and thiol groups, the resulting chromatograms are transparent and clean. Moreover, TCDI excess was hydrolyzed completely to imidazole that did not have significant UV absorption above 240 nm. The overall analysis time by a HPLC-UV method using derivatization with TCDI [37] amounts to 1 h 20 min, which is longer than that in the case of the procedure using CMPI [35] (1 h 15 min), BCPB [54] (30 min) or CMQT [34] (27 min) as a derivatization reagent.

4.2. N-Acetylcysteine and thioglycolic acid

Urine endogenous *N*-acetylcysteine and thioglycolic acid were determined, along with cysteine and cysteinylglycine, in one analytical run by RP-HPLC-UV in the form of their 2-*S*-quinolinium

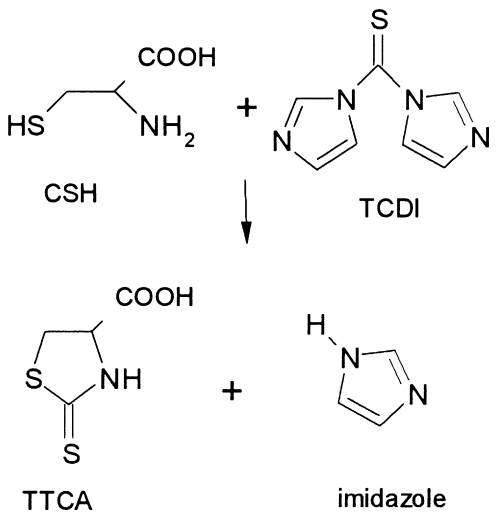

Fig. 3. The cyclization of cysteine to 2-thioxothiazolidine-4-carboxylic acid with 1,1′-thiocarbonyldiimidazole.

derivatives [39]. For total content of the analytes urine sample was reacted with CMQT derivatization reagent following reduction with TCEP. Validation procedure has proved that the method is linear in the concentration ranges studied of 2.5–20 μM urine for *N*-acetylcysteine and thioglycolic acid, and 50–500 and 2.5–25 μM urine for cysteine and cysteinylglycine, respectively, with correlation coefficient better than 0.999. The lower limit of detection was 0.25 μM for *N*-acetylcysteine and thioglycolic acid, and 0.12 μM urine for cysteine and cysteinylglycine.

4.3. Red-ox status of main urinary thiols

Kuśmierek and Bald took advantage of HPLC-UV method for evaluation of thiol redox status, defined as reduced – to – oxidized ratio, of main urinary aminothiols cysteine and cysteinylglycine [27]. In their work contents of reduced and total thiol were measured directly, but oxidized forms for each urine specimen were calculated as a difference between total and reduced amounts. First morning urine of 45 apparently healthy volunteers, distributed for age, was analyzed and redox status of cysteine and cysteinylglycine was calculated. The results have shown that cysteine redox status in human urine is not age-dependent, but on the contrary the reduced to oxidized cysteinylglycine ratio in children is more oxidized than that in adults [27].

5. Determination of thiol drugs

Thiol-containing drugs are incorporated as therapeutic agents in a variety of pharmaceutical preparations, and are commonly used as drugs in the treatment of many diseases. Among these compounds cysteamine, tiopronin, D-penicillamine, captopril, mesna, *N*-acetylcysteine, thyreostats and thiopurines are the most important. Severe adverse reactions to oral thiol-drugs use have been described in subjects in which abrupt incremental dosing of the drugs were started. This suggests that monitoring the concentrations of these compounds in biological fluids, over the course of therapy, is warranted and consequently a number of different methods of quantitating these thiols have been described. Table 2 summarizes the conditions of HPLC analyses of urine for thiol drugs that are addressed in this article.

5.1. Cysteamine

Cysteamine (mercaptamine 2-aminoethyl mercaptan, 2-aminoethanethiol) is used therapeutically as a radioprotective agent [55] and to prevent a severe liver damage after paracetamol poisoning [56]. First of all, it is a drug widely used for the treatment of nephropathic cystinosis, a rare autosomal recessive disease which manifests itself in raised intracellular levels of the cystine to 50–100 times normal levels [57]. Cysteamine lowers intracellular levels of cystine by forming a cysteamine–cysteine mixed disulfide, which is structurally similar to lysine and can egress the lysosome using the pathway for lysine excretion [58].

In accordance with our knowledge, only five works offer procedures for the determination of cysteamine in human urine [32,38,59,60,61], however, only one takes advantage of an HPLC-UV technique [61]. This method is based on derivatization of cysteamine with CMQT followed by ion-pairing reversed-phase liquid chromatography separation and ultraviolet-absorbance detection at 355 nm. Total cysteamine was determined by reductive conversion of its oxidized fraction by sodium borohydride to the thiol form before the derivatization step. The imprecision ranges for reduced and total cysteamine were within 1.4–11.7% and 0.7–10.6% (RSD), respectively. The analytical accuracy for determined compound was from 98.8 to 109.8%. This method requires neither extraction nor preconcentration and uses only simple sample preparation in the form of derivatization and offers procedure for determination of two redox forms of cysteamine. Cysteine and cysteinylglycine, known to form CMQT derivatives, can be measured concurrently if needed.

5.2. Tiopronin

Tiopronin (*N*-(2-mercaptopropionyl)-glycine, Thiola) is a synthetic compound that acts as a potent free radical scavenger [8]. It has been shown to protect against ischemic/reperfusion-mediated injury [62] and could also be effective against radiation-induced damages, even at low doses [63]. Tiopronin is used in the treatment of rheumatoid arthritis [64], hepatic diseases and as a mucolytic in respiratory disorders [5,8] and is efficient antidote to heavy metal poisoning [64,65]. Moreover, it has been frequently used for the treatment of cystinuria, an autosomal recessive genetic defect of the transepithelial transport of cystine and other dibasic amino acids in the kidneys [22,66]. Tiopronin prevents the formation of cystine stones in kidneys when there is too much cystine in the urine. Tiopronin transforms cystine into a mixed disulfide, 50 times more soluble than cystine itself [22].

A number of methods have been proposed for the determination of tiopronin in human urine, such as CE [38] and HPLC with fluorescence [32,67] and ultraviolet [68] detection. The HPLC-UV method, developed by Kuśmierek and Bald [68], relies on transformation of the determined compound to its *S*-quinolinium derivative in the reaction with CMQT, separation by ion-pairing reversed-phase chromatography, and ultraviolet detection and quantitation. The lower limits of detection and quantitation were 0.5 μM and 1.0 μM urine, respectively. The tiopronin standards added to the urine show that the response of the detector is linear within the range studied, from 1 to 200 μM urine. The imprecision ranges for tiopronin were within 1.6–8.2%. The method is able to measure simultaneously, in one analytical run, tiopronin, cysteine and D-penicillamine.

One more HPLC method with ultraviolet detection is described in the literature. It was based on derivatization with *p*-bromophenacyl bromide [69], but was applied only to plasma samples.

5.3. D-Penicillamine

D-Penicillamine (2-amino-3-mercapto-3-methylbutanoic acid, β,β-dimethylcysteine, 3-mercaptovaline) is a thiol drug used in the treatment of Wilson's disease, an autosomal recessive disorder of copper transport [70]. It is also used as antifibrotic agent to treat scleroderma [71] and as antirheumatic drug to treat patients with active rheumatoid arthritis [72]. Moreover, similarly as tiopronin, it is used for treatment of heavy metal poisoning [64,65] and cystinuria [22,66].

Several methods have been reported for the determination of D-penicillamine in human urine, including capillary electrophoresis [38] and liquid chromatography with electrochemical [73], chemiluminescence [74] or fluorescence [47] detection. Only one method takes advantage of liquid chromatography followed by ultraviolet detection [68]. The developed analytical procedure [68] makes possible to measure concurrently cysteine, D-penicillamine and tiopronin in human urine after derivatization with CMQT. The method reported by Amarnath et al. [37], based on the conversion of D-penicillamine to 5,5-dimethyl-2-thioxothiazolidine-4-carboxylic acid in the cyclization reaction with TCDI, also use HPLC with UV detection system, but its applicability to the urine sample was not shown.

5.4. Captopril

Captopril (1-[3-mercapto-2-(*S*)-methyl-1-oxopropyl]-(*S*)-proline), the angiotensin-converting enzyme inhibitor, is used in the treatment of essential hypertension [75] and to reduce mortality in patients with acute myocardial infraction [76].

Among several methods for measurement of captopril in human urine, HPLC has been widely used [77–81]. Hayashi et al. [77] reported method which enables determination of captopril and its mixed disulfides in plasma and urine. Captopril was derivatized with *N*-(4-benzoylphenyl)maleimide and the derivative was extracted with chloroform and assayed using a liquid chromatograph equipped with an ultraviolet detector at 254 nm. Mixed disulfides of captopril with thiol compounds such as cysteine, glutathione and plasma proteins are reduced with tri-*n*-butylphosphine to form captopril, followed by derivatization with *N*-(4-benzoylphenyl)maleimide. Accurate determinations were possible over a concentration range of 0.5–11.5 μM for captopril in urine. A column-switching HPLC procedure for the determination of captopril in plasma and urine has been developed by Shen et al. [78]. This method was based on derivatization of captopril with *p*-bromophenacyl bromide (Fig. 4A) to form a product that shows ultraviolet-absorbing properties. Captopril derivative was detected at 260 nm. The calibration curve was linear in the range 0.05–1.0 μM in urine. The recovery averaged 99.5% and the coefficient of variation was less than 10%. *p*-Bromophenacyl bromide may also react with carboxylic groups but in anhydrous environment and at elevated temperature. Sypniewski and Bald [79] performed an HPLC method with UV-detection for determination of captopril and its disulfides in whole blood and urine. The procedure involves a pre-column derivatization with 1-benzyl-2-chloropyridinium bromide followed by solid-phase extraction and chromatographic separation with ultraviolet detection at 314 nm. Chemical derivatization reaction equation is presented in Fig. 4B. Oxidized captopril was converted to reduced form by the use of triphenylphosphine and derivatized and quantified in the same manner. Accurate measurements were possible over a concentration range of 0.2–5.5 μM for captopril and 0.2–13.8 μM for captopril disulfide in urine. The detection and quantitation limits were 0.01 and 0.05 μM, respectively. Khedr and El-Sherief [80] reported method for determination of captopril in urine after liquid–liquid extraction and derivatization with 3-bromomethyl-4-

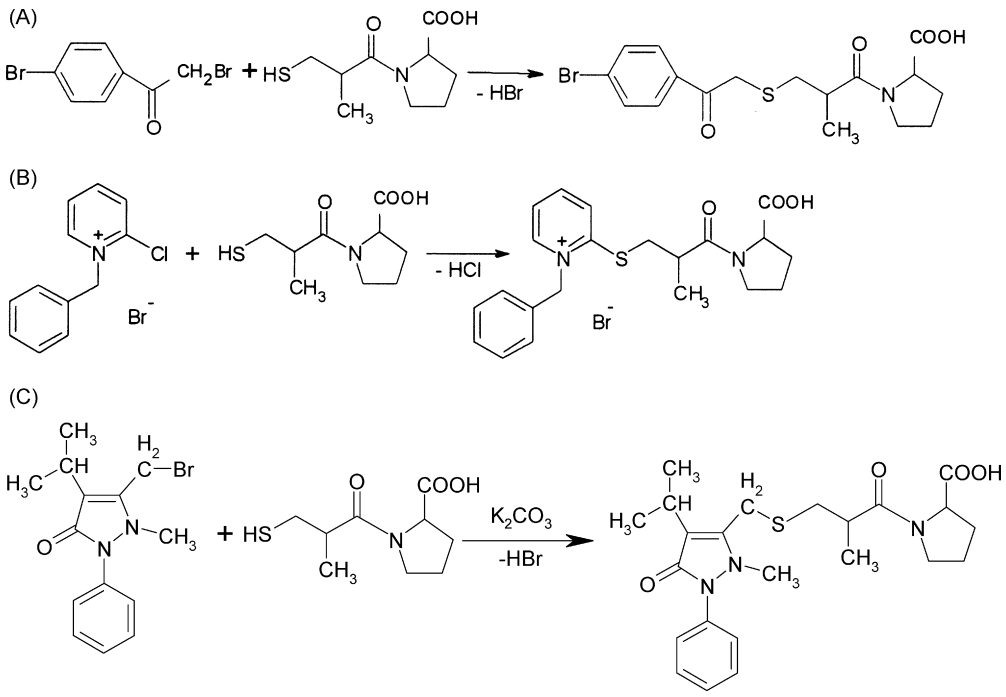

Fig. 4. Derivatization reaction equation of captopril with: (A) *p*-bromophenacyl bromide; (B) 1-benzyl-2-chloropyridinium bromide; (C) 3-bromomethyl-4-izopropylphenazone.

isopropylphenazone (BMP). The reagent reacts with the sulfhydryl group of captopril in acetone using anhydrous potassium carbonate as hydrobromide acceptor (Fig. 4C). The derivatized compound has shown maximum UV-absorption at 243 nm. The calibration curve was linear over the range from 3.6 to 73.6 nmol per injection. The detection limit was 1.4 nmol per injection at a signal-to-noise ratio of 5. Recently, Kuśmierek and Bald [81] performed liquid chromatography method for determination of captopril after reduction with tris-(2-carboxyethyl)phosphine and derivatization with CMQT. The calibration curve for the derivatized captopril showed linearity in the range 0.1–200 μM urine with a regression coefficient corresponding to 0.9999. The detection and quantitation limits were 0.05 and 0.1 μM, respectively. The procedure requires neither extraction, nor preconcentration and uses only sample preparation in the form of simple 2 min room temperature derivatization with CMQT. The overall analysis time, encompassing –S–S– bond reduction, derivatization, centrifugation and final HPLC separation and quantitation, amounts to 25 min. Earlier described methods [77–80] for determining captopril are much more labour and time demanding.

5.5. Mesna

Mesna (sodium 2-mercaptoethanesulfonate, sodium salt of 2-mercaptoethanesulfonate) can antagonize the dose-limiting effects of alkylating anticancer agents on the genitourinary tract. The superiority of mesna as a chemoprotector to other available thiols has been confirmed clinically in an ifosfamide trial [82]. The urotoxic oxazaphosphorine metabolites are detoxified by their reactions with the sulfhydryl group of mesna [83]. Mesna does not block the antitumor action of oxazaphosphorines most likely due to its rapid formation of the inactive dimer dimesna in the blood-stream [84].

Several HPLC methods have been used to measurement of mesna in biological samples. The most frequently used and sensitive method to determine mesna and total mesna is HPLC with electrochemical detection [85–88]. A HPLC method using ultraviolet detection at 220 nm [89] without derivatization shows very low sensitivity, and post-column derivatization approach with Ellman's reagent [85] requires special equipment which may not be readily available. Mesna and total mesna in urine can be easily and reliably determined by a HPLC-UV method after derivatization with CMQT [90]. For determination of mesna (reduced form) the reduction step is omitted. The content of oxidized forms of mesna, that is sum of dimesna and mesna mixed disulfides, were assessed by subtraction of the result for mesna from that of total mesna. The assay for mesna and total mesna in urine was proved to be linear over the studied ranges of 0.2–30 and 0.2–800 μM urine, respectively. The mean recovery over the calibration ranges was 95.4% for mesna and 99.7% for total mesna. The lower limits of detection and quantitation were 0.1 and 0.2 μM for both the procedures, respectively.

5.6. Thyreostats

Thyreostats, also referred to as anti-hormones, are a group of compounds widely used in medicine for treatment of hyperthyroidism [91,92]. Antithyroid drugs act primarily to inhibit thyroid hormone synthesis within the thyroid gland by interfering with thyroid peroxidase-mediated utilization of iodine. The most important and powerful thyreostatic drugs, hitherto used, are methimazole (1-methyl-2-mercaptoimidazole, tapazole) and propylthiouracil (6-propyl-2-thiouracil) [91,92].

A variety of methods for determination of thyreostats (especially methimazole and propylthiouracil) in urine have been reported. In most cases, these methods take advantage of less commonly used techniques such as flow-injection with spectrophotometry detection [93], GC–MS [94,95], CE with electrochemical detection at a carbon fiber microdisk electrode [96] and LC-APCI-MS [97]. Methimazole and propyltiouracil, as distinct from aliphatic thiols, show structural properties necessary for the production of signal compatible with ultraviolet detector. Therefore, they can be determined directly without derivatization step as shown by Hollos et al. [98]. They reported method for the simultaneous determination of methimazole and selected metabolites in fish homogenates

by mixed mode solid phase extraction, and reversed-phase liquid chromatography with ultraviolet detection at 255 nm. However, applicability of this method to a urine sample was not shown. To the best of our knowledge, only three methods offer procedures for measurement of methimazole [99,100] and propylthiouracil [101] in urine by HPLC-UV. The recently published method [99] for methimazole requires neither extraction, nor preconcentration and uses simple sample preparation in the form of a single derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate. The response of the UV detector, set at 345 nm, is linear within the concentration range studied, that is, from 2 to 440 μM urine. Lower limits of detection and quantitation were 1 and 2 μM urine, respectively. Zakrzewski [100,101] reported selective post-column reaction detection method for the determination of methimazole [100] and propylthiouracil [101] based on their sensitizing induction on iodine-azide reaction following HPLC separation. Sulfur (II) compounds (e.g. methimazole and propylthiouracil) induce a reaction between azide and iodine and allow sensitive detection of thiols. The procedure relies on the separation of the analyte on a chromatographic column and subsequent measurement of the iodine comsumption in the iodine-azide reaction [102]. In the absence of these compounds a constant absorbance from iodine is recorded when a HPLC system is supplied with azide ions from the mobile phase and iodine solution from post-column reagents. The presence of methimazole or propylthiouracil in the chromatographic band is accompanied by the increase in the iodine consumption in the iodine-azide reaction and subsequent appearance of a negative peak at 350 nm. The methimazole standards added to normal urine have shown that the response of the detector, corresponding to the iodine consumption in the post-column iodine-azide reaction, was linear within the concentration range 2–10 μM in urine. Limits of detection and quantitation were 1 and 2 μM in urine, respectively. The same procedure was applied to analysis of urine for propylthiouracil concentration [101]. The linear range, the lower limit of detection and quantification for propylthiouracil were 0.4–1.0 μM, 0.3 μM and 0.4 μM in urine, respectively. Very narrow calibration range, resulted from difficulties in post-column reaction control and the nature of a negative peak, may seriously hamper application of the method.

5.7. Thiopurines

The thiopurine antimetabolites 6-thioguanine and 6-mercaptopurine are important chemotherapeutic drugs in the treatment of childhood acute lymphoblastic leukaemia [103–105]. Measurement of metabolites of these thiopurines is important because correlations exist between their concentration and the prognosis in childhood acute lymphoblastic leukemia.

Because 6-thioguanine and 6-mercaptopurine are chromophores, they can be determined directly without derivatization. A few methods for determination of both or one or the other compounds in human urine by HPLC with UV detection were described [106–109]. Almost all methods [106–108] make use of natural structural properties of thiopurines, which possess a well-defined absorption maximum in the ultraviolet spectrum. Recently, Zakrzewski [110] reported a post-column reaction detection method for HPLC determination of thioguanine, based on the sensitizing induction of thioguanine on iodine-azide reaction. The analysis was accomplished in the optimum conditions for iodine-azide detection system and HPLC separation. The values for the limits of detection and quantitation amounted to 0.4 and 0.5 μM in urine, respectively. The method was linear in the range from 0.8 to 1.7 μM in urine. Very narrow calibration range, resulted from difficulties in post-column reaction control and the nature of a negative peak, may seriously hamper application of the method.

6. Conclusions

The measurement of thiols in urine has received less attention than in plasma so far, although urine can be obtained less invasively than blood. This lack of analysis of urine may be partly attributed to the difficulty in resolving the number of peaks in chromatograms generated from urine. Moreover, the concentration of the analytes in urine depends on the constitution of the different individuals. Therefore, to facilitate the comparison between the data of particular cases, and to compensate for different urine volume excreted, the urinary thiol concentrations should be normalized against creatinine. The non-invasive nature of urine collection and the fact that many drugs and metabolites are concentrated in urine make this physiological fluid very attractive as an object of analysis in clinical practice.

Urinary thiols can be determined using different analytical methods but HPLC has proven to be widely applicable. HPLC measurements are sensitive, selective, reproducible, accurate and convenient. Ultraviolet detection is less specific and less sensitive than fluorescence one, nevertheless, its sensitivity is sufficient for detection and quantitation of endogenous and exogenous thiols in urine in physiological and pathological conditions. Moreover, equipment for HPLC-UV analysis is often a part of the existing, standard instrumentation in hospital laboratories and staff is usually well experienced in its use. The UV detector is known for its stability and low demand in terms of maintenance. Hopefully, the present review provides satisfactory information on the HPLC-UV methods currently available for thiols in urine, and helps in choosing the most appropriate analytical approach for use in the future studies on the role of endogenous and exogenous thiols in human metabolism.

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