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High-performance liquid chromatography determination of glyoxal, methylglyoxal, and diacetyl in urine using 4-methoxy-*o*-phenylenediamine as derivatizing reagent

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ABSTRACT

Bioanalytical relevance of glyoxal (Go) and methylglyoxal (MGo) arises from their role as biomarkers of glycation processes and oxidative stress. The third compound of interest in this work is diacetyl (DMGo), a component of different food products and alcoholic beverages and one of the small α-ketoaldehydes previously reported in urine. The original idea for the determination of the above compounds by reversed phase high-performance liquid chromatography (HPLC) with fluorimetric detection was to use 4-methoxy-*o*-phenylenediamine (4MPD) as a derivatizing reagent and diethylglyoxal (DEGo) as internal standard. Acetonitrile was added to urine for matrix precipitation, and derivatization reaction was carried out in the diluted supernatant at neutral pH (40 °C, 4 h); after acidification, salt-induced phase separation enabled recovery of the obtained quinoxalines in the acetonitrile layer. The separation was achieved within 12 min using a C18 Kinetex column and gradient elution. The calibration detection limits for Go, MGo, and DMGo were 0.46, 0.39, and 0.28 μg/L, respectively. Within-day precision for real-world samples did not exceed 6%. Several urine samples from healthy volunteers, diabetic subjects, and juvenile swimmers were analyzed. The sensitivity of the procedure proposed here enabled detection of differences between analyte concentrations in urine from patients at different clinical or exposure-related conditions.

Introduction

Oxidative stress and glycation reactions play a detrimental role in the development of chronic degenerative diseases and in aging processes. In routine clinical control of patients, the evaluation of appropriate biomarkers provides information on endogenous processes and also on adherence to dietary recommendations. For such purposes, urine is a convenient sample because of its easy noninvasive collection and relatively simple chemical matrix as compared with other biofluids or tissues. It has been demonstrated that the analysis of spot samples or samples of morning void urine offers reliable and useful results in the follow-up of patients in human exposure-related and epidemiological studies [1–3].

The two small α-ketoaldehydes, methylglyoxal (MGo)[1] and glyoxal (Go), are generated endogenously, mainly during metabolic conversion of glucose and oxidative degradation of lipids [4,5]. Their presence in fermented beverages and in food products should also be mentioned as a potential exogenous source [6,7]. Within the cell, MGo and Go form adducts or cross-links with biomolecules, thereby compromising their biological activity and inactivating antioxidant machinery [8]. Under normal physiological conditions, both molecules are efficiently scavenged by the glyoxalase system, aldose reductase, betaine aldehyde dehydrogenase, and 2-oxoaldehyde dehydrogenase [9,10]; however, the impairment of enzymatic defense as well as the increased concentrations of MGo and Go have been associated with chronic diseases and aging [8,11]. Because the two compounds act as the precursors of advanced glycation end products (AGEs) and are considered as biomarkers of lipid peroxidation [4,12], their determination in clinical samples is relevant. In particular, it has been proposed that monitoring of MGo and Go in diabetic patients would help to assess the risk of progression of diabetic complications [13–16].

Diacetyl (DMGo), the third compound of interest in the current work, has also been associated with oxidative and carbonyl stress as a potential mediator of electron transfer reactions, an intermediate of Maillard processes and precursor of AGEs [17]. This minor metabolite of acetaldehyde derived from ethanol is easily reduced

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[1] *Abbreviations used:* MGo, methylglyoxal; Go, glyoxal; AGE, advanced glycation end product; DMGo, diacetyl (or dimethylglyoxal); 4MPD, 4-methoxy-*o*-phenylenediamine; DEGo, diethylglyoxal (or 3,4-hexanedione); HPLC, high-performance liquid chromatography; TEA, triethylamine; SPE, solid phase extraction; FLD, fluorimetric detector; RSD, relative standard deviation; DL, detection limit; QL, quantification limit.

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to acetoin and 2,3-butanediol [18]; therefore, it has not always been detected in biological samples [19,20]. On the other hand, diacetyl is present in many beverages and various food products as a metabolite of microbial fermentation or as a buttery flavor additive [21,22]. Its determination in urine would be of interest in studies on alcohol toxicity and addiction [17] and in evaluation of recent exposure to exogenous sources [21]. In this regard, recent findings showed that exogenous dicarbonyl compounds react with digestive enzymes, which reduces their bioavailability and might favor their elimination in urine [23].

Quantification of α-ketoaldehydes in clinical samples has often been reported; however, only few studies focused on urine analysis (Table 1) [20,24–31]. It is worth noting that urine has been suggested as the most practical sample for such analysis because spontaneous de novo formation of MGo from triose phosphates occurring in more complex biological matrices can be avoided [27,32,33]. Brief reviews and comparisons of analytical procedures, the great majority of them based on suitable precolumn derivatization followed by chromatographic or electrophoretic separation, can be found in the introductory parts of several previous articles [24,25,29,31,34] and in comprehensive reviews [9,35]. In regard to liquid chromatography, the use of 1,2-diamino-substituted aromatic compounds that yield fluorescent quinoxalines should be highlighted [36]. In particular, 1,2-diaminobenzene [12,33], 1,2-diamino-4,5-dimethoxybenzene [27,32,37], 1,2-diamino-4,5-methylenedioxybenzene [38], 4,5-dichloro-1.2-diaminobenzene [20], and 2,3-diaminonaphthalene [30] has been reported so far. As already mentioned, diacetyl has rarely been detected in urine (Table 1), and some authors used this compound as internal standard [25,27,29]. It is worth noting, however, that 4-methoxy-o-phenylenediamine (4MPD) was proved to be useful for fluorimetric determination of diacetyl in wine [39].

The goal of this work was to establish a new procedure for the determination of Go, MGo, and DMGo in urine at physiological levels. To this end, diethylglyoxal (DEGo) was proposed as internal standard, 4MPD was examined as a derivatizing agent, and the fluorescent quinoxalines were separated by reversed phase high-performance liquid chromatography (HPLC). Using an original sample pretreatment, the results obtained in the analysis of real-world samples demonstrated that the proposed procedure would enable quantification of the three compounds in samples from subjects presenting diverse exposure-related or clinical conditions.

Materials and methods

Instrumentation

An Agilent series 1200 liquid chromatographic system equipped with a quaternary pump, a well plate autosampler, a column oven, a fluorimetric detector, and a ChemStation (Agilent Technologies, Palo Alto, CA, USA) was used; the chromatographic column (Kinetex C18, 150 × 3 mm, 2.6 μm) and the C18 guard column were obtained from Phenomenex (Torrance, CA, USA).

Chemicals and samples

All chemicals were of analytical reagent grade. Deionized water (18.2 MΩ cm, Labconco, Kansas City, MO, USA) and HPLC-grade acetonitrile (Fisher Scientific, Pittsburgh, PA, USA) were used throughout.

The standard solutions containing 1 mg/mL Go (ethanedial, Fluka), MGo (2-oxopropanal, Sigma), and DMGo (butane-2,3-dione, dimethylglyoxal, Fluka) were prepared in deionized water. The following Sigma reagents were also used: 4MPD (derivatizing reagent), hydrochloric acid, acetic acid, potassium phosphate dibasic, boric acid, sodium hydroxide, 2-mercaptoethanol, sodium chloride, and triethylamine (TEA).

The first morning urine samples were provided by volunteers characterized as follows: three healthy adults, these same adults after alcohol ingestion the night before, three members of a youth swimming team, and three diabetic patients. Additional samples from healthy individuals were used for the evaluation of the method detection and quantification limits.

Procedures

Small dicarbonyl compounds are unstable yet ubiquitous; hence, special care was needed during preparation of standards, reagents, and samples. In particular, all aqueous solutions were purified by derivatization of potentially present dicarbonyls with

Table 1
Some examples of analytical procedures proposed for the determination of Go, MGo, or DMGo in urine.

Urine	Reagent	Analytical technique	Detection limits and concentrations found (range or mean ± SD)						References
			Go		MGo		DMGO		
			DL	*c*	DL	*c*	DL	*c*	
Not specified	DCDB	GC–ECD	–	nd	–	nd	860 μg/L	1.73 ± 0.04 nmol/mg[a]	[20]
Not specified	TRI	HPLC–FLD	32 pmol	13.18 μM	11 pmol	1.50 μM	99 pmol	2.10 μM	[28]
Healthy	DDB2	HPLC–FLD	–	50–250 μM 2.9–14.9 mg/L 4.7 ± 1.4 μg/mg[a]	–	20–100 μM 1.4–7.2 mg/L 2.2 ± 0.7 μg/mg[a]	–	nd	[27]
Healthy	DDP	HPLC–DAD/FLD	5.30 μg/L	19.02 μg/L 0.43–1.50 μg/mg[a]	6.71 μg/L	24.07 μg/L 0.30–0.90 μg/mg[a]	–	nd	[26]
Not specified	DAN	SBSE–HPLC–DAD	15 ng/L	268.9 ± 6.3 μg/L	25 ng/L	94.1 ± 3.2 μg/L	–	nd	[30]
Diabetic	DAP	GC–FID	–	nd	40 μg/L	170–250 μg/L	50 μg/L	nf	[29]
Diabetic	DDB1	GC–FID	20 μg/L	170–400 μg/L	10 μg/L	190–360 μg/L	10 μg/L	nf	[25]
Control diabetic	TBA	CE–AD	1.0 μg/L	20.1–21.1 μg/L 64.1–71.4 μg/L	0.2 μg/L	11.7–12.2 μg/L 29.4–127.2 μg/L	–	nd	[24]
Control diabetic	TRI	HPLC–FLD	0.16 μg/L	0.30–1.1 μg/mg[a] 0.57–0.84 μg/mg[a]	0.44 μg/L	0.1–0.3 μg/mg[a] 2.0–3.8 μg/mg[a]	0.43 μg/L	nf	[31]
Healthy diabetic	4MPD	HPLC–FLD	0.46 μg/L	17.0–43.2 μg/L 71.2–175 μg/L	0.39 μg/L	17.3–27.0 μg/L 53.8–249 μg/L	0.28 μg/L	13.2 ± 1.6 μg/L 64.6 ± 3.4 μg/L	This work

Note: SD, standard deviation; DL, detection limit; *c*, concentration; nd, not determined in this work; nf, not found; AD, amperometric detection; CE, capillary electrophoresis; DAN, 2,3-diaminonaphthalene; DAP, 1,2-diaminopropane; DCDB, 4,5-dichloro,1.2-diaminobenzene; DDB, 2,3-diamino-2,3-dimethylbutane; DDB2, 1,2-diamino-4,5-dimethylenedioxybenzene; DDP, 5,6-diamino-2,4-hydroxypyrimidine sulfate; DMB, 1,2-diamino-4,5-dimethoxybenzene; SBSE, stir bar sorptive extraction; TBA, 2-thiobarbituric acid; TRI, 6-hydroxy-2,4,5-triaminopyrimidine.

[a] Normalized to urine creatinine.