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Short communication

Hair to document exposure to glibenclamide☆

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Abstract

Among the drugs that are used to incapacitate victims such as kids or elderly for sedation or for criminal gain such as sexual offences or robberies, glibenclamide, an antidiabetic was never mentioned. To document the interest of hair testing in such forensic situations, we have developed an original method to test for glibenclamide. A 30-year-old man was admitted to the Emergency Unit for coma and seizures after a party with some members of his family. Blood glucose was 0.40 g/l. A hair specimen was collected several weeks after the event and divided into two segments of 2 cm. Twenty milligrams of each segment cut into small pieces were incubated overnight in a phosphate buffer (pH 5.5), in presence of gliclazide used as internal standard (IS). A liquid/liquid extraction was realized with a mixture of diethyl ether/methylene chloride, and hair extract was separated on a XTerra MS C18 column using a gradient of acetonitrile and formate buffer. Detection of glibenclamide was achieved using two transitions: *m/z* 493.9 to 168.9 and 493.9 to 368.8. Linearity was observed from 5 to 1000 pg/mg ($r^2 = 0.956$) with a limit of quantification at 5 pg/mg and a clean-up recovery of about 61%. Within-batch precision and bias were 9.0 and 9.5%, respectively. Ion suppression tested on drug-free hair was about 50%. Glibenclamide tested positive in the two consecutive segments (root to 2 cm: 23 pg/mg and 2–4 cm: 31 pg/mg). These findings were in accordance with a repetitive exposure to the drug. The concentrations were compared with those obtained after a single and a daily dose administration. In the hair of a subject receiving a single 5 mg dose and collected 4 weeks later, glibenclamide was detected in the proximal segment at 5 pg/mg. After a 20 mg/day dose, the hair concentration of a subject under glibenclamide therapy was 650 pg/mg.

Keywords: Drug facilitated crime; Glibenclamide; Antidiabetic; Hair; LC–MS/MS

1. Introduction

Glibenclamide is a potent, second generation oral sulfonylurea antidiabetic agent widely used to lower glucose levels in patients with type II non-insulin-dependent diabetes mellitus. It acts mainly by stimulating endogenous insulin release from beta cells of pancreas [1].

Hypoglycemia presents important diagnostic and therapeutic problems. Severe and repetitive hypoglycemic episodes in patients without treatment may be difficult to explain. Failure to identify factitious hypoglycemia may lead to pancreatectomy. Hypoglycemia factitia is assessed as a manifestation of Munchausen's syndrome, which is characterized by factitious illness associated with hospital peregrination, mythomanic discourse that includes medical elements, and passivity and dependence at examinations [2]. The administration of glibenclamide by a parent (generally the mother) to his child is known as Munchausen's syndrome by proxy [3]. Beside classic overdosage due to suicide attempt, homicides with glibenclamide have been reported [4,5]. Sedation induced by glibenclamide can be observed as overdosage presents lethargy. This can be a cause of incapacitation of a potential victim. Diagnosis can be difficult to achieve with the focus of a discrimination between insulinoma and sulfonylurea-induced hypoglycemia. It seems that a case solution with disappearance of hypoglycemia is made possible by the removal of the perpetrator's presence.

For differential diagnosis of unclear hypoglycemia, antidiabetics must be screened. Various procedures applicable to

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plasma or urine, involving liquid chromatography have been published in the literature. Glibenclamide can be detected by UV [6], mass spectrometry [7] or tandem mass spectrometry [8,9]. The latter procedure [9] was applicable to test in equine biological specimens as the drug can also be used as a stopper in race-horses by reducing the blood glucose level. Alternatively, micellar electrokinetic capillary chromatography with diode-array detection has been used [10]. Unfortunately, no data are available to test for glibenclamide in hair which can be considered as the specimen of choice to complement blood and urine analysis in cases of drug-facilitated crimes [11,12]. Given the low concentration that is expected, a very sensitive method has to be used with respect to selectivity. Glibenclamide is one of the most potent antidiabetic drugs for which much lower dosages are required.

We present here an original method to test for glibenclamide in hair by LC–MS/MS and its application to a drug-facilitated case. Tandem mass spectrometry can provide a further dimension of sample clean up which makes it well-suited in handling complex sample matrices.

2. Materials and methods

2.1. Specimen

Hair from a male volunteer (1.77 m tall, 70 kg, 44-year-old) was collected 1 month after he was administered a single oral dose of Daonil (5 mg tablet). Strands of about 100 hairs were oriented (tied up at the root), cut with scissors in vertex posterior as close as possible to the scalp, and stored at room temperature.

The subject participated in the experimental part of the study through oral informed consent. His participation was incompliance with all applicable laws and practices in France.

Hair of a diabetic man, chronically treated at a daily dosage of 20 mg, was collected to document the incorporation in a case of therapeutic treatment.

A hair strand from a poisoned 30-year-old man was collected under close supervision of the medical staff, at the Emergency Unit of the Central Hospital of Strasbourg.

Glibenclamide-free hair samples were obtained from laboratory personnel.

2.2. Chemicals and reagents

Acetonitrile, diethyl ether and methylene chloride were of HPLC grade (Merck, Darmstadt, Germany). Chemicals for the 50% saturated phosphate buffer – KH_2PO_4, adjusted at pH 5.5 with ammonia solution – were purchased from Fluka (Saint-Quentin Fallavier, France). Gliclazide was purchased from Sigma (Saint-Quentin Fallavier, France). Glibenclamide was obtained from Aventis (Paris, France).

2.3. Extraction

Hair strands were twice decontaminated using methylene chloride (5 ml, 2 min) and then eventually segmented. Each segment was cut into small pieces (<1 mm). About 20 mg were incubated overnight in 0.75 ml of phosphate buffer at pH 5.5, in the presence of 5 ng of gliclazide used as internal standard (IS). After a liquid/liquid extraction with 3 ml of a mixture of methylene chloride/diethyl ether (50/50, v/v) and evaporation to dryness, the residue was reconstituted in 50 μl of acetonitrile/water (50/50, v/v).

Table 1
HPLC gradient profiles for the analysis of glibenclamide

Time (min)	Acetonitrile (%)	Formate buffer (%)
0	5	95
3	60	40
7	80	20
10	80	20
10.5	5	95
20	5	95

2.4. LC–MS/MS procedure

LC was performed using a Waters Alliance 2695 system. Chromatography was achieved using a XTerra MS C18 column (100 mm × 2.1 mm, 3.5 μm) eluted with a gradient delivered at a flow rate of 0.2 ml/min (Table 1). An injection volume of 10 μl was used in all cases. A Quattro Micro triple-quadrupole mass spectrometer (Micromass-Waters) fitted with a Z-Spray ion interface was used for analyses. Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of glibenclamide and the IS: capillary voltage, 1.0 kV; source block temperature, 120 °C; and desolvatation gas (nitrogen) heated to 350 °C and delivered at a flow rate of 550 l/h. In order to establish appropriate multiple reaction monitoring (MRM) conditions, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion of each compound and the collision energy (eV) was adjusted to optimize the signal for the two most abundant product ions (Table 2). Collision gas (argon) pressure was maintained at 4.0×10^{-3} bar. MassLynx 4.0 software was used for quantitation.

2.5. Method validation

A standard calibration curve was prepared in hair fortified with the drug and obtained by preparing spiked standards containing 5, 10, 20, 50, 200 and 1000 pg/mg of glibenclamide. Within-batch precision ($n = 6$) and bias were determined using blank hair spiked with glibenclamide at 20 and 100 pg/mg. The limit of detection (LOD) was evaluated by decreasing concentrations of glibenclamide until a response equivalent to three times the background noise was observed. Clean-up recovery ($n = 3$) was determined by comparing the representative peak area of glibenclamide in blank hair spiked before and after extraction at the final concentration of 20 pg/mg. Ion suppression was established by infusing glibenclamide at 1 mg/l and evaluating the deviation in signal response at its retention time when blank hair ($n = 20$) are injected at the same time.

Table 2
MRM transitions and conditions for the measurement of glibenclamide and the internal standard

Compound	Retention time (min)	Parent ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Glibenclamide	11.1	493.9	368.9	20	15
			168.9	20	40
Gliclazide	9.4	324.0	127.0	25	18
			110.0	25	21

3. Results and discussion

3.1. Validation

Under the chromatographic conditions used, there was no interference with the analytes by any extractable endogenous material present in hair. There was no blank effect.

Within the 20 min LC run, glibenclamide and the IS could be easily detected from the hair matrix. Confirmation of the drug was achieved by comparing two product ions as well as the retention time from the sample with those of a drug standard.

Linearity was observed for glibenclamide concentrations ranging from 5 to 1000 pg/mg with a correlation coefficient of 0.956. The limit of detection was 2 pg/mg and the limit of quantification at 5 pg/mg. Within-batch precision and bias at 20 pg/mg were 9.0 and 9.5%, respectively. Within-batch precision and bias at 100 pg/mg were 8.2 and 9.0%, respectively The clean-up recovery was of 61%. This was lower than after SPE [6] but chromatograms after hair extraction were cleaner. Ion suppression was about 50%, irrespective of the hair colour (=20). Stability analysis showed that glibenclamide and the IS are stable for at least 3 months when stored at 4 °C.

The hair was not digested, but suitable extraction was observed after a relatively gentle aqueous incubation. This is because this procedure belongs to a general screening in case of drug-facilitated crimes, first developed for benzodiazepines, compounds that require the same aqueous incubation [13].

3.2. Detection in hair

It is accepted by the scientific community that hair growth of about 1 cm/month (range 0.7–1.4 cm/month). In order to document any DFC case, it was the opinion of the authors to collect the hair after 3–5 weeks of the alleged offence and to divide the strand into three segments of 2 cm. That way, in case of a single dose administration, the drug will be present in the proximal segment (root to 2 cm) while not detected in the other segments.

The hair (light brown color) of the volunteer that had ingested a single 5 mg glibenclamide dose was segmented, analyzed and quantified. Glibenclamide tested positive in the proximal segment at a concentration of 5 pg/mg and was negative in the following segments. That demonstrates that a single 5 mg dose of glibenclamide is detectable in hair. Fig. 1 is the chromatogram obtained after extraction of the proximal segment of the hair.

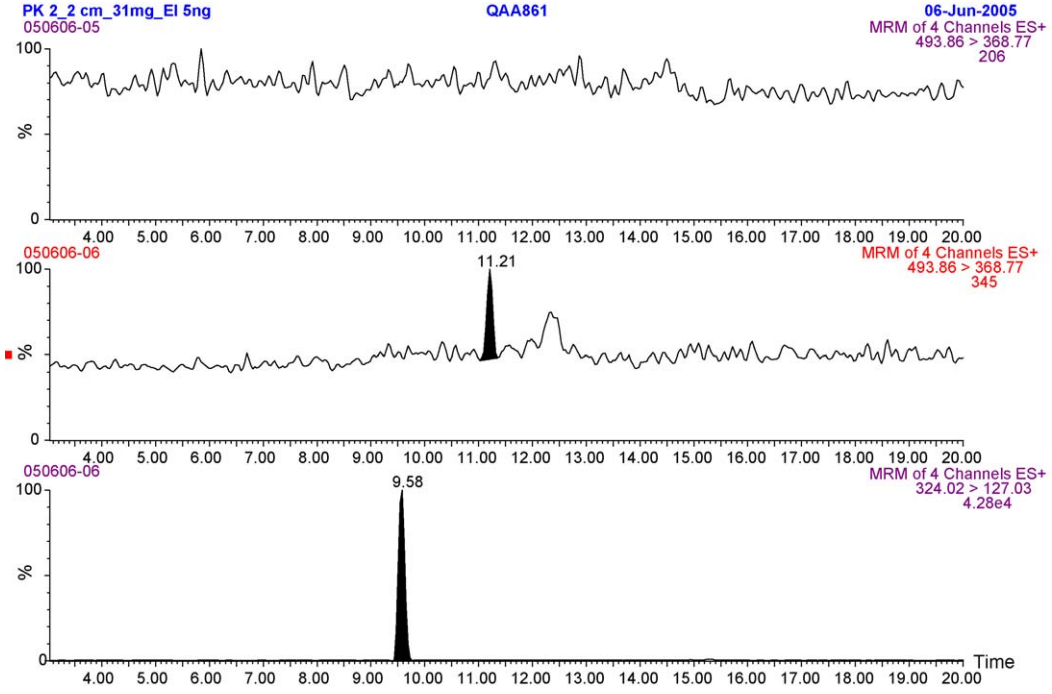

Fig. 1. Mixed chromatograms of a blank hair (on the top) and of the hair collected from a subject exposed to a single 5 mg tablet of glibenclamide; transition of quantification (*m/z* 493.9 → 368.9) of glibenclamide at RT 11.21 min (on the middle) and transition of quantification (*m/z* 324.0 → 127.0) of the IS at RT 9.58 min (on the bottom). Glibenclamide concentration was 5 pg/mg.

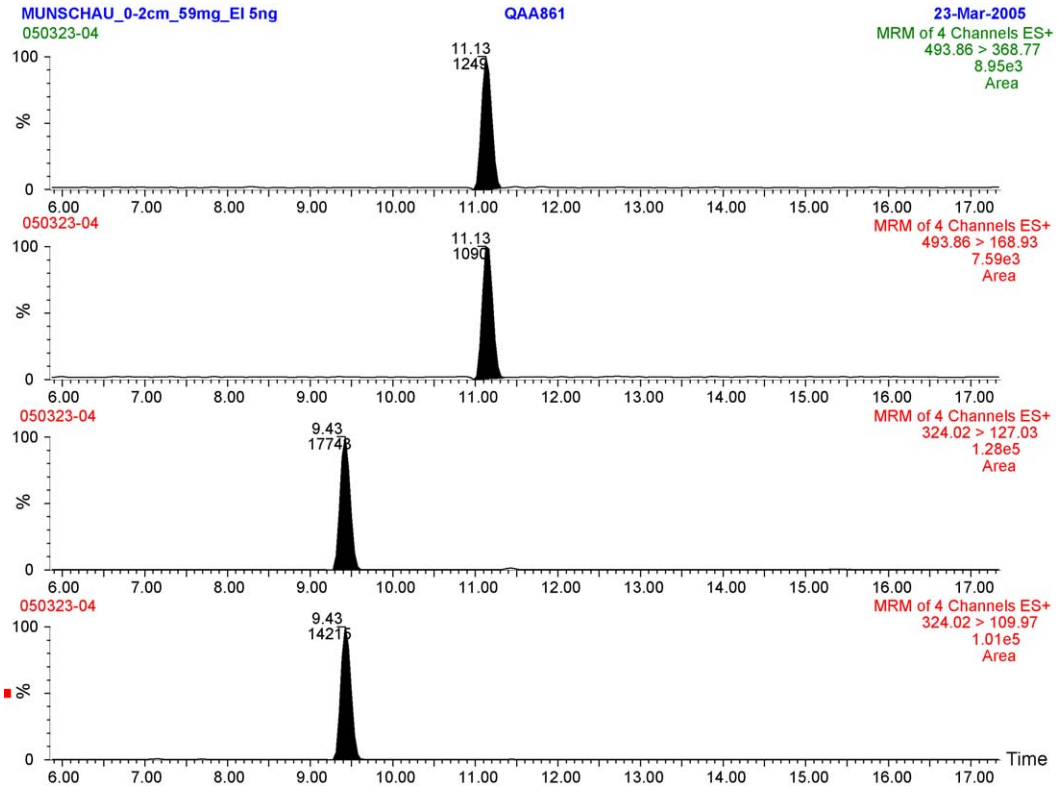

Fig. 2. Chromatogram of the proximal hair segment from a victim of poisoning. Glibenclamide concentration was 23 pg/mg. From the top to the bottom: the two transitions of glibenclamide (*m/z* 493.9 → 368.9 and 168.9) at RT 11.13 min and the two transitions (*m/z* 324.0 → 127.0 and 110.0) of gliclazide at RT 9.43 min (IS).

We also tested the hair of a patient under therapeutic treatment of glibenclamide. His dosage was 20 mg/day for at least 1 year and the concentration found in his 6-cm long hair was 650 pg/mg.

Hair analysis was applied to an authentic criminal case.

A 30-year-old man, was admitted at the Hospital after having consumed several beers, at home, the previous night. First glycemia at home was 0.33 g/l. The Emergency Unit treated him with a perfusion of diazepam and 30% glucose, but hypoglycemia persisted at 0.40 g/l. Despite intensive resuscitation attempts, vegetative coma occurred rapidly. Five months later, the patient was pronounced dead. Blood sample, collected at the admission, revealed the presence of 41 ng/ml of glibenclamide. At the time of the autopsy, blood concentration for glibenclamide was negative. Glibenclamide has been reported to be cleared quickly within 5 h in human plasma [14]. This is not confirmed, however, by Niopas and Daftsios [15] that detected the drug for at least 24 h following a single 5-mg dose in 18 volunteers, with a C_{max} at 167.7 ± 63.4 ng/ml and a T_{max} at 3.6 ± 2.3 h.

To discriminate between a single administration or repetitive administration, this laboratory was requested to analyze a hair strand. A 4-cm hair was divided into two 2-cm segments, that both tested positive at 23 and 31 pg/mg. The chromatogram of the proximal segment of hair is given in Fig. 2. The measured concentrations are in accordance with repetitive exposures during the last 4 months. Beside the medical investigations, hair analysis was the unique retrospective proof of glibenclamide exposure. Findings emphasize the importance of toxicological analysis when the clinical diagnosis or the cause of death are in doubt.

Among the causes for recurrent hypoglycemic episodes in seemingly healthy patients, discriminating between sulfonylurea-induced hypoglycemia and insulinoma is of utmost importance because of medico legal implications. Several unnecessary laparotomies and partial pancreatectomies because of erroneous diagnosis of insulinoma involving patients with surreptitious sulfonylurea exposure (inadvertent or factitious) have been reported, even leading to the death of a subject due to postoperative bleeding [16]. Therefore, toxicological analyses can be helpful. In case of late sampling, blood or urine have little interest and hair must be considered as the best opportunity to document exposure.

4. Conclusion

Hair testing should be used to complement conventional blood and urine analysis as it increases the window of detection and permits differentiation, by segmentation, of long-term therapeutic use from a single exposure. Selectivity and sensitivity of MS/MS are a pre-requisite, given the low concentrations that have to be measured. From this case, it appears that sulfonylurea screening should be established routinely before invasive evaluation for insulinoma. In such a context, hair analysis should be considered to a complement to blood or urine investigations, particularly to extend the window of detection.

References

[1] Physicians' Desk Reference, 52nd ed., Medical Economics Company, Montvale, NJ, 1998, p. 1217.
[2] T. Trenque, G. Hoizey, D. Lamiable, Diabetes Care 24 (2001) 792.
[3] M. Caruso, P. Bregani, B. Di Natale, A. D'Arcais, Minerva Pediatr. 41 (1989) 525.
[4] R. Fernando, Med. Sci. Law 39 (1999) 354.
[5] M.P. Heenan, R.M. Berezowski, in: Abstracts of the 41st International TIAFT Meeting, Melbourne, 2003, abstract 10.4.
[6] S. AbuRuz, J. Millership, J. McElnay, J. Chromatogr. B 817 (2005) 277.
[7] H.H. Maurer, C. Kratzsch, T. Kraemer, F.T. Peters, A.A. Weber, J. Chromatogr. B 773 (2002) 63.
[8] M.R. Moura, G. De Nucci, S. Rath, F.G. Reyes, Anal. Bioanal. Chem. 378 (2004) 499.
[9] E.N.M. Ho, K.C.H. Yiu, T.S.M. Wan, B.D. Stewart, K.L. Watkins, J. Chromatogr. B 811 (2004) 65.
[10] M.E. Roche, R.P. Oda, G.M. Lawson, J.P. Landers, Electrophoresis 18 (1997) 1865.
[11] M. Villain, V. Cirimele, P. Kintz, Clin. Chem. Lab. Med. 42 (2004) 1265.
[12] P. Kintz, M. Villain, V. Cirimele, B. Ludes, Ther. Drug Monit. 26 (2004) 211.
[13] M. Villain, M. Concheiro, V. Cirimele, B. Ludes, P. Kintz, J. Chromatogr. B 825 (2005) 72.
[14] S. Hsieh, K. Selinger, J. Chromatogr. B 772 (2002) 347.
[15] I. Niopas, A.C. Daftsios, J. Pharm. Biomed. Anal. 28 (2002) 653.
[16] E.G. Siegel, G. Mayer, M. Nauck, W. Creutzfeldt, Dtsch. Med. Wochenschr. 112 (1987) 1575.