W. Van Thuyne, P. Van Eenoo and F.T. Delbeke

Comprehensive screening method for the detection of narcotics and stimulants using single-step derivatisation.

Doping Control Laboratory – Ghent University - UGent, Technologiepark 30, B-9052 Zwijnaarde, Belgium

Introduction

Nowadays, narcotic agents such as morphine, heroin and pethidine and stimulating agents including amphetamine and ephedrine are most frequently analysed using gas chromatography. Volatile and unconjugated stimulants are frequently analysed using nitrogen-phosphorus detection [1], while conjugated narcotics and stimulants are very often analysed using GC-MS after hydrolysis of phase-II metabolites and selective derivatisation [2].

Screening methods using GC-NPD are only based on retention time criteria. However, the unequivocal determination of suspicious substances should be achieved by the combination of both retention time and mass spectrometric criteria [3]. Therefore, different confirmation procedures need to be developed and validated.

Screening methods using GC-MS frequently relied on derivatisation using MSTFA and MBTFA creating O-TMS and N-TFA functionalities. However, the combination of both agents does not always result in the formation of one derivative. Analysis of beta-blocking agents for instance can result in multiple derivatives [2].

Because GC-MS is most often used for confirmation purposes, the use of a derivatising agent for volatile substances is mandatory. A reagent very often used is trifluoroacetic acid anhydride or TFAA generating sufficient diagnostic ions meeting the WADA confirmation criteria [4].

However, the use of TFA derivatising agents such as MBTFA and TFAA is pernicious for column lifetime. So the goal of this work was to develop a comprehensive screening method which combines the former screening methods I and II and that can also be used for confirmation purposes. In addition, it was our aim to increase productivity by avoiding frequent column replacements.

Experimental

Chemicals and reagents

Morphine, oxymorphone, buprenorphine and EDDP (metabolite of methadone) were purchased from Cerrilliant (Round Rock, Texas, USA). Codeine was purchased from Boehringer-Ingelheim (Brussels, Belgium). Hydromorphone, ethylmorphine, heroin (diacetylmorphine), dextromoramide, oxycodone, fentanyl, pethidine, dimethylamphetamine HCl, mephentermine sulphate, amiphenazole, phendimetrazine HCl and methadone were obtained from Sigma (Bornem, Belgium). Normethadone was obtained from Bios-Coutelier (Brussels), nalorphine (internal standard) from Janssen-Phamraceutica (Beerse, Belgium) and pentazocine from Whintrop Laboratories (Newcastle, United Kingdom).
Bambuterol, MDA, MDEA and MDMA were a kind gift from the Lisbon doping control laboratory in Lissabon. Fencamfamine HCl, norephedrine HCl, norpseudoephedrine HCl, pseudoephedrine HCl, amiloride and methamphetamine HCl were purchased from Merck (Darmstadt, Germany), pipradrol HCl from Merrell-DOW (Cincinnati, Ohio, USA) and amphetamine sulphate and triamterene from GlaxoSmithKline (Philadelphia, USA). Phenmetrazine, etilefrine, fenoterol and prolintane HCl were a gift from Boehringer & Sohn (Ingelheim am Rhein, Germany). Heptaminol HCl was purchased from Ets. A De Bournonville (Braine L'Alleud, Belgium), norfenfluramine HCl from Eutherapie Benelux (Brussels), ephedrine HCl from Hoechst AG (Frankfurt, Germany) and fenfluramine HCl, amineptine, amineptine C5-metabolite and fenspiride HCl from Laboratoires Servier (Orleans, France). Methylephedrine HCl was purchased from Laboratoire G.A. (Cochard, France), phentermine HCl from NV Certa Noville (Mehaigne, Belgium), nikethamide and methylphenidate from Ciba-Geigy (Groot-Bijgaarden, Belgium) and mefenorex from Produits Roche (Brussels). Chlorphentermine HCl was purchased from Tropon GmbH (Cologne, Germany). Isopropylhexedrine was purchased from Veride (Diegem, Belgium) and ethylamphetamine HCl from Will-Pharma Benelux (Brussels). Crotethamide, cropropamide, bromantane, carphedone and benzylpiperazine were purchased from NMI (Pymble, Australia), fencamine from Laboratoires Miquel S.A. (Barcelona, Spain), pholedrine from Knoll AG (Ludwigshaven, Germany), fenethylline from Chemiwerk Hamburg (Germany), etamivan from Sinclair Pharmaceuticals Ltd (Godalmings, UK) and benzoylecgonine from Lipomed (Arlesheim, Switserland). Furfenorex and clobenzorex were obtained from Roussel Uclaf (Romainville, France), methoxyphenamine and benzphetamine from Upjohn (UK),

amfepramone from Lab. Pharm. R.H. Trenker (Brussel, Belgium), dimefline from Recordate Industria Chemica & Farmaceutica (Milan, Italy), lidocaine from Astra Chemicals (Brussels, Beligium), propoxyphen from Park Davis (Bornem, Belgium) and formoterol from Novartis (Arnhem, The Netherlands). Aminogluthetimide was purchased from European Pharmacopeia (Strasbourg, France).
Excretion urines of aromatase-inhibitors clomiphene, cyclophenyl, tamoxiphen, anastrazole and letrozole as well as from the stimulants prolintane and amfepramone were obtained after the controlled administration of a therapeutic dose.
N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Chem. Fabrik Karl Bucher (Waldstedt, Germany). All other chemicals were of analytical grade

GC/MS conditions

The GC/MS analysis is carried out on an Agilent 5973 mass spectrometer directly coupled to an Agilent 6870 gas chromatograph equipped with an J&W-Ultra 1 column with a length of 17 m, internal diameter of 0.2 mm and a film thickness of 0.11 µm. The GC is operated in constant flow mode at a flow rate of 0.6 ml/min. The oven temperature is as follows:
60°C (0 min) → 90°C/min → 100°C (5 min) → 20°C/min → 300°C (3 min).
Half a microliter is injected in the splitless mode.
The mass spectrometer is operated in the full scan mode between m/z 50 and 550.

Extraction

Extraction is performed on 8 ml of urine divided in aliquots of 5 ml and 3 ml.
1 ml of phosphate buffer (pH 7), 50 µl of β-glucuronidase and 50 µl of the internal standard nalorphine (20 µg/ml, MeOH) are added to 3 ml of urine after which the samples are hydrolysed overnight at 42 °C. Extraction is performed using 5 ml of CH_2Cl_2/MeOH (9/1) after the aliquot was made alkaline using 0.5 ml of an ammonium buffer (pH 9.5). After rolling for 20 min and centrifugation the organic layer was separated and evaporated under oxygen free nitrogen at 52°C.
To a second aliquot of 5 ml of urine, 50 µl of the internal standard cyclopentamine (100 µg/ml, MeOH) and 0.5 ml KOH (5 M) were added together with ± 1 g NaCl and 1 ml MTBE. After rolling for 1 h and centrifugation, the organic layer was added to the dried residue of the extraction performed at pH 9.5 and evaporated under oxygen free nitrogen at room temperature. To avoid the loss of very volatile substances, methanolic HCl can be added

before evaporation. The final residue of the combined extracts is derivatised using MSTFA for 10 min at 80°C.

Results and discussion

Preliminary tests for robustness have shown that the addition of KOH is not crucial for the extraction of diphenylamine, a substance frequently used as internal standard for the determination of volatile stimulating agents [2] while this is essential for the extraction of other volatile stimulating agents. Therefore, cyclopentamine was used as internal standard in this screening method.

This method allows for the detection of more than 80 different components including all narcotic agents mentioned on the WADA doping list. For methadone, the parent compound as well as the metabolites normethadone and EDDP are monitored. Because EDDP is sold as a racemic mixture, two not completely separated peaks can be observed.

Numerous stimulating agents can also be screened with this comprehensive method. Because most of these stimulants elute rather early and their spectra often show an identical base ion, differentiation should be done using minor ions. As an example, ethylamphetamine and fenfluramine show an identical base ion and are not completely resolved at the MRPL level of 500 ng/ml. Using retention time locking and the minor ions m/z 159 and m/z 220, identification is rather easy (Figure 1).

Both narcotic agents and stimulants were validated at the MRPL level of 200 ng/ml and 500 ng/ml respectively. Besides these categories, two beta-agonists, formoterol and bambuterol, were also included in this screening method. These two substances were validated at a level of 250 ng/ml. No extraction recovery could be obtained for fenoterol using the mixture CH_2Cl_2/MeOH as an extraction solvent. Changing this extraction solvent to ethyl acetate for the extraction at pH 9.5 shows comparable recoveries to the other beta-agonists. Unfortunately, extraction recoveries for benzoylecgonine are very poor using ethyl acetate. Therefore, ethyl acetate was not used as the extraction solvent.

Finally, this method is also capable of detecting several metabolites or parent compounds of agents with anti-estrogenic activity such as aminogluthetimide, clomiphene, cyclofenyl, anastrazole, letrozole and tamoxiphen. Using this full scan screening procedure, positive screening results could be obtained for hydroxy-methoxy-tamoxiphen, the main metabolite of tamoxiphen, in a WADA-PT testing sample in 2005 while results obtained in the SIM mode of screening IV results were not conclusive.

The validation procedure of this method was performed using prolintane excretion urine. The detection of the four main metabolites of prolintane, all excreted as glucuronides, are used as a direct indication of the effectiveness of the enzymatic hydrolysis. As an alternative, the mono-TMS derivatives of both androsterone and etiocholanolone can also be used.
Based on the high MRPL of stimulants and the good sensitivity, the analytical method was revalidated using only 1 ml of urine for the extraction at pH 14 instead of 5 ml. Besides the need for less urine, this also results in less interference of high amounts of pseudoephedrine in the retention time window of ephedrine. Selectivity and specificity were also tested and fulfilled all criteria.
This method comprises several substances with a urinary threshold level according to WADA criteria [5]. These substances are morphine, ephedrine, cathine and methylephedrine. In order to avoid unnecessary confirmation procedures of suspicious peaks with abundances far below these threshold levels, confirmation thresholds were introduced.
The confirmation procedure for morphine is identical to this screening method excepting the omission of the extraction at pH 14. As the extraction recovery of morphine at this pH and the prevalence of unconjugated morphine are negligible, screening results can be quantified using the same calibration curve of the confirmation procedure. A validation procedure pointed out that a confirmation threshold of 0.5 µg/ml is reasonable.
As the confirmation procedure for ephedrines is performed at pH 14 and these substances also show a substantial recovery at pH 9.5, a different approach is needed compared to morphine. Analysis of samples spiked at half of the threshold level, which is a safety margin of 50 %, show that suspicious cathine screening results only should be confirmed if the ratio of m/z 116 of cathine to m/z 130 of the internal standard is higher than 0.5. Similar, methylephedrine should only be confirmed if the ratio of m/z 72 to m/z 130 is higher than 2. The ratio in samples spiked at the threshold levels of 5 µg/ml and 10 µg/ml respectively were below the proposed confirmation thresholds.
In contrast to cathine which possesses a primary amine function that is always derivatised resulting in the bis-TMS derivative and methylephedrine showing only the mono-TMS derivative, derivatisation of ephedrine using MSTFA results in the detection of both the mono-TMS and bis-TMS derivative, the latter being the most abundant. Using the same procedure as for cathine and methylephedrine, ephedrine suspicious results should be confirmed when the ratio of the ion m/z 130 of ephedrine-bis-TMS to the ion m/z 130 of the internal standard is higher than 2. Samples spiked with ephedrine at the threshold level

showed a ratio of 4 proving the ratio of mono-TMS to the bis-TMS derivative of ephedrine is concentration independent.

Conclusion

A comprehensive screening method was developed which combines the former screening methods I and II. This method only requires derivatisation using MSTFA resulting in an increased column lifetime of approximately 1000 injections. In contrast to former NPD methods, this method provides mass spectral data reducing the number of confirmations and because of the good sensitivity of this method, positive results can be obtained at very low concentrations. In order to reduce the number of confirmations of substances with a threshold level, confirmation cut-off levels were introduced.

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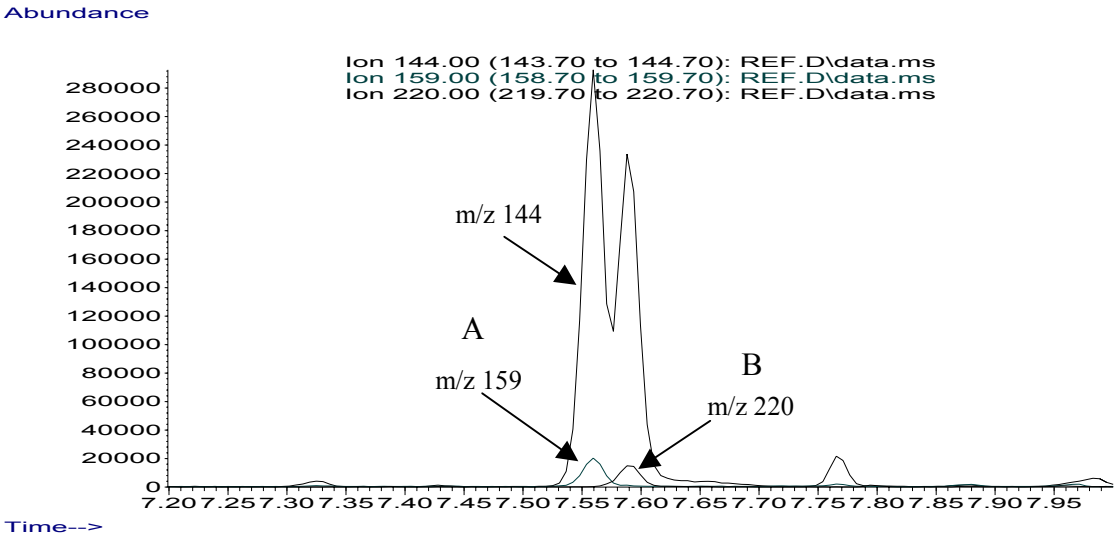

Figure 1: Identification of ethylamphetamine (A) and fenfluramine (B) using the minor ions m/z 159 and m/z 220.

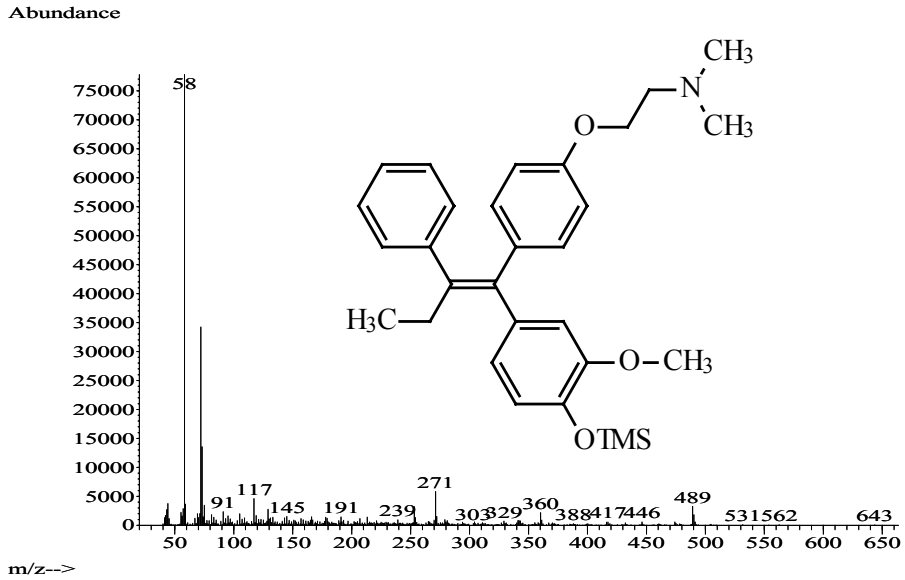

Figure 2: Positive screening results for trimethylsilyl-hydroxy-methoxy-tamoxiphen.

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