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Short communication

Simultaneous detection of methylphenidate and its main metabolite, ritalinic acid, in doping control☆

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Abstract

Two analytical methods for the simultaneous detection in urine of methylphenidate and its main metabolite, ritalinic acid, are described. Both procedures are based on solid-phase extraction of urine samples on Bond Elut Certify columns, and capillary gas chromatographic–mass spectrometric detection of O-trimethylsilyl, N-trifluoroacetyl derivatives. The former method is used as a general screening procedure for the detection of basic polar nitrogen-containing compounds in urine such as stimulants, narcotic and adrenergic drugs. The latter procedure is proposed as a specific method to confirm methylphenidate ingestion. The two methods are sensitive enough to detect methylphenidate and ritalinic acid in urine at least for 24 h after administration of a therapeutic dose (20 mg oral dose) of methylphenidate.

1. Introduction

Methylphenidate (MPH, α-phenyl-2-piperidineacetic acid methyl ester, also known as ritalinic acid methyl ester), a sympathomimetic agent with stimulant effects on the central nervous system, is the drug of choice in the treatment of attention-deficit disorder and narcolepsy [1]. The abuse of this drug has been described and MPH is submitted to international restriction (Schedule II, United Nations Convention). In recent years, MPH has been abused in sport events because of its stimulant properties. Consequently, MPH was banned by the International Olympic Committee (IOC) Medical Commission [2]

MPH is rapidly metabolised in man, predominantly giving a de-esterified product, commonly known as ritalinic acid (RA). This metabolite, which has little or no pharmacological activity, is the main substance detected in urine after MPH administration [3–6].

In doping control, MPH detection is usually included in an analytical procedure aimed to detect the presence of basic volatile nitrogen-containing stimulant drugs. This method consists of a liquid–liquid extraction of urine with organic solvents at alkaline pH. The final extract is then analyzed by capillary gas chromatography coupled to a nitrogen–phosphorous specific detector. Although this procedure enables good extraction and detection of MPH, its main me-

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tabolite in urine, RA, can not be measured [7–9]. Moreover, MPH suffers from spontaneous hydrolysis to RA in non-stabilized urine [6]. Detection of MPH abuse by urinalysis must therefore be focused on analytical methods able to handle both substances.

In this paper two analytical methods for the isolation of MPH and RA based on solid-phase extraction and gas chromatographic–mass spectrometric (GC–MS) detection are described. The former is a general screening procedure for the simultaneous analysis and detection of basic polar nitrogen-containing compounds in urine, such as stimulants, adrenergic drugs and narcotics [10], and is a modification of a previously published method [11]; the latter is a confirmatory procedure for MPH and RA.

2. Experimental

2.1. Chemicals and reagents

MPH hydrochloride and RA were provided by Sigma Chemicals (St. Louis, USA). The internal standard levallorphan tartrate was kindly donated by Roche (Basel, Switzerland).

Methanol HPLC grade, chloroform, glacial acetic acid, phosphoric acid, hydrochloric acid and acetone analytical grade were purchased from Merck (Darmstadt, Germany). Isopropyl alcohol and ammonium hydroxide 25% reagent grade were supplied by Scharlau (Barcelona, Spain). Deionized water was obtained by Milli-Q system (Millipore, Mulheim, France). N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and N-methyl-bis-trifluoroacetamide (MBTFA) were obtained from Macherey-Nagel (Düren, Germany).

2.2. Standard solutions

Stock solutions (1 mg/ml, free bases) of MPH hydrochloride, RA and levallorphan tartrate were prepared in methanol. Working solutions of 100 μg/ml were prepared by dilution of stock solutions. These solutions were checked by UV spectrophotometry and stored at −20°C.

2.3. Excretion studies

Three excretion studies involving drug administration and urine collection were performed by three healthy male volunteers under the authorization of Hospital del Mar Ethical Committee (Barcelona, Spain) and Spanish Ministry of Health (assay No. 88/135). MPH was administered at a single therapeutic dose (Ritalin, 20 mg p.o.) and urines were collected for a period up to 24 h. The volunteers were under medical supervision throughout the study.

2.4. Equipment

Bond-Elut Certify columns were provided by Analytichem International (Harbor City, MI, USA) and the Visiprep vacuum manifold was obtained from Supelco (Bellefonte, PA, USA). A Turbo-Vap LV Evaporator from Zymark Corporation (Hopkinton, MA, USA) was used to evaporate organic phases to dryness under a nitrogen stream.

Gas chromatography was performed with a Hewlett-Packard (HP; Palo Alto, CA, USA) Model 5890 coupled to a mass selective detector HP Model 5970. Data acquisition and reduction were done by HP Chemstation 59940 (HP UNIX series). Separation of analytes was carried out using an Ultra2 HP crosslinked capillary 12 m × 0.2 mm I.D., 5% diphenyl and 95% dimethylpolysiloxane gum (0.33 μm film thickness). Injector (split mode, ratio 1:10) and detector temperatures were 280°C. Oven temperature was programmed from 100°C to 290°C at 20°C/min (final time, 4 min). Helium flow was 0.8 ml/min and the sample injection volume was 2 μl. The mass spectrometry detector was operated in the electron-impact ionization (EI, 70 eV) mode and in the full scan acquisition mode (70–400 a.m.u.).

2.5. Extraction procedure

Two different extraction procedures were used: the first corresponds to a general screening procedure for basic polar nitrogen-containing drugs applied in doping control analysis; the

second is a specific confirmatory procedure for MPH and RA.

For testing the procedures, urine samples (2.5 ml) were spiked with MPH, RA and levallorphan (5 μg/ml).

Screening procedure of basic nitrogen-containing compounds including MPH

Bond Elut Certify columns were inserted into a vacuum manifold and conditioned by washing once with 2 ml of methanol and 2 ml of deionized water. The columns were prevented from drying out before applying specimens. Samples were adjusted to pH 8–9 with 1 *M* KOH. The mixtures were centrifuged at 770 *g* for 5 min to remove any precipitate. Supernatant urine samples were poured into each column reservoir and drawn slowly through the column. The columns were washed with 2 ml of deionized water, 1 ml of 0.1 *M* acetate buffer pH 4 and 2 ml of methanol. Elution of analytes was performed with 2 ml of chloroform–isopropyl alcohol (80:20, v/v) containing 2% of ammonium hydroxide. The eluates were collected and evaporated to dryness under a stream of nitrogen in a 50°C water bath.

Confirmatory procedure for MPH and RA

Bond Elut Certify columns were inserted into a vacuum manifold and conditioned by washing once with 2 ml of methanol and 2 ml of 0.1 *M* phosphate buffer pH 7. The columns were prevented from drying out before applying specimens. Then, 1 ml of 0.1 *M* phosphate buffer pH 7 was added to the urine samples. The mixture was vortex-mixed and centrifuged at 770 *g* for 5 min. Supernatant urine samples were poured into each column reservoir and drawn slowly through the column. The columns were washed with 3 ml of deionized water, 3 ml of 0.1 *M* hydrochloric acid and 9 ml of methanol. Elution of analytes and organic phase evaporation was performed as described above.

2.6. Derivatization procedure

Trimethylsilyl/trifluoroacetyl derivatives

MSTFA (100 μl) was added to the dried residue, and the mixture was vortex-mixed and kept at 60°C for 5 min. After cooling to room temperature, 20 μl of MBTFA were added and the mixture was vortex-mixed and incubated for 10 min at 60°C [12]. The following derivatives of MPH, RA and the internal standard were obtained by this procedure: MPH-N-trifluoroacetamide (MPH-N-TFA), RA-O-trimethylsilyl-N-trifluoroacetamide (RA-O-TMS-N-TFA) and levallorphan-O-trimethylsilyl derivative (see Fig. 1).

3. Results and discussion

Difficulties in the determination of MPH abuse by means of urinalysis are mainly caused by its extensive metabolism to RA. In addition to the in vivo metabolism of MPH, there is some evidence that at least part of the conversion of MPH to RA occurs in aqueous alkaline solutions during storage, although it may be retarded by acidification or addition of EDTA [6].

The two analytical methods proposed are based on solid-phase extraction on Bond-Elut Certify columns [13], the main difference between the procedures being the pH at which the columns and samples are conditioned and washed.

Recoveries and within-day coefficients of variation ($n = 5$) were calculated for each extraction procedure. Recoveries obtained with the former method were 86.0% (C.V. = 9.5%) for MPH-N-TFA, 5.0% (C.V. = 89.0%) for RA-O-TMS-N-TFA and 82.1% (C.V. = 2.3%) for the internal standard derivative. For the latter method, recoveries were 98.9% (C.V. = 2.0%) for MPH-N-TFA, 23.1% (C.V. = 2.7%) for RA-O-TMS-N-TFA and 96.7% (C.V. = 3.7%) for the internal standard derivative. Although the recovery of RA in the screening procedure method is poor, the fact that less than 1% of the MPH dose is excreted unchanged in 24 h whereas ca. 80% is eliminated as RA [1], makes it suitable for screening purposes. This screening procedure enables the detection and identification of more than 100 drugs and their metabolites, including many stimulants (amphetamine, ephedrine, methoxyphenamine, etc.), adrenergic agents, either blockers (metoprolol, nadolol, proprano-

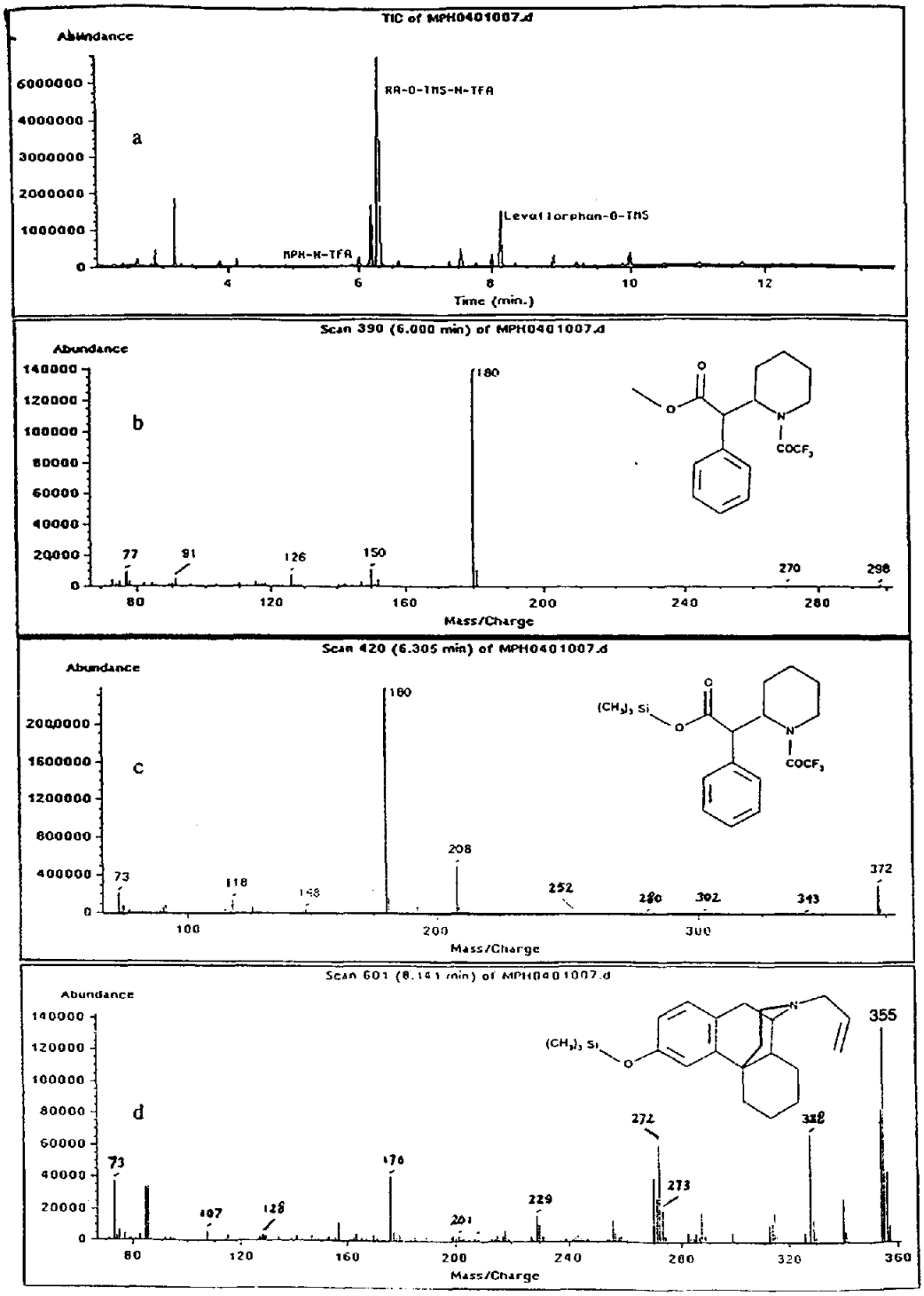

Fig. 1. GC–MS detection of a urine sample containing MPH, RA and the internal standard levallorphan. The EI mass spectra of the TMS-TFA derivatives are also included. (a) Total ion chromatogram (TIC). (b) Mass spectrum of MPH-N-TFA derivative. (c) Mass spectrum of RA-O-TMS-N-TFA derivative. (d) Mass spectrum of levallorphan-O-TMS derivative

lol, etc.) or agonists (clenbuterol, salbutamol, etc.) and narcotic drugs (nalbuphine, morphine, pentazocine, etc.) [10,11]. As a general rule in doping control, when a substance is detected in a sample a second independent analysis must be done to confirm unequivocally its presence. This second analysis consists of a reextraction of the presumptive positive sample in a batch which also includes a urine obtained after a real excretion study of the banned substance and other quality control samples. The batch can be processed with another suitable analytical method. In the case of MPH screening detection, the second procedure has been designed to achieve more favourable extraction conditions for MPH and RA and allows confirmation of the results obtained with the first method.

Thus, the latter analytical method was applied to the analysis of urine samples from three excretion studies of MPH in three healthy volunteers. MPH and RA were identified and quantified in two different time collection periods: 0–8 and 8–24 h (see Table 1). The cumulative mean recovered dose in 24 h was 80.94% for RA and 0.95% for MPH. These results are in agreement with those obtained by others [1,14].

In conclusion, two analytical methods are proposed which have the advantages of solid-phase extraction procedures: speed, cleanliness and automation. A few modifications made to a general screening extraction procedure allowed the development of a more appropriate confirmatory method for RA and MPH. Both methods were applied in doping control of urine samples from athletes participating in the 25th Olympic Games held in July 1992 in Barcelona.

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Table 1
Urinary excretion of MPH and RA after a 20 mg oral dose of MPH in three healthy volunteers

Volunteer	Time (h)	Volume (ml)	MPH		RA	
			μg/ml	% dose	μg/ml	%dose
A	0–8	600	0.35	0.70	21.84	43.68
	8–24	850	0.10	0.27	9.55	27.07
B	0–8	800	0.17	0.68	15.61	62.45
	8–24	850	0.07	0.28	7.25	30.79
C	0–8	750	0.24	0.61	18.19	45.48
	8–24	1000	0.09	0.31	10.01	33.36
Mean ± S.D.	0–8			0.66 ± 0.04		50.54 ± 8.45
	8–24			0.29 ± 0.02		30.41 ± 8.49