Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, No. 2/3, pp. 187–191, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00

A safer methylation procedure with boron trifluoride–methanol reagent for gas chromatographic analysis of ritalinic acid in urine

TRINH VU-DUC* and ANDRÉ VERNAY

Unit of Drug Analysis, Institute of Occupational Health Sciences, 19 Rue du Bugnon, CH-1005 Lausanne, Switzerland

Abstract: Ritalinic acid (RITA), the major metabolite of methylphenidate (Ritaline®) is extracted with a solid-phase C_8 column. Following elution, methylation of the carboxylic group is performed with boron trifluoride–methanol as a reagent. The methyl ester of RITA which structurally corresponds to the parent compound methylphenidate is re-extracted at pH 9–11. Each step of the analytical method has been systematically studied, particularly the parameters governing the esterification reaction with BF_3–methanol. The recovery from the overall method is 96%. The limit of detection is 0.05 μg ml^{-1} with GC–NPD for a 10-ml urine sample. The method has been successfully applied to the detection and quantitation of RITA in urine specimens. The specificity of the methylated RITA peak has been verified by GC–MS in scan mode. Use of diazomethane or sulphuric acid is thus replaced by the use of a less hazardous reagent.

Keywords: *Methylphenidate; ritalinic acid; urine; elimination; derivatization; methylation with BF_3–methanol; drug screening; stimulant; analysis.*

Introduction

Methylphenidate [Ritaline®, methyl-α-phenyl-(2-piperidyl) acetate] is a sympathomimetic amine used for the treatment of hyperkinetic children [1–3] and against mild depression [4, 5]. The psychostimulant effect of methylphenidate (MEP) is similar to that of the amphetamines and its abuse has been observed in adult groups because of widespread clinical use [6]. MEP is rapidly metabolized in the body with 80–90% of the dose excreted in urine as non-conjugated ritalinic acid (RITA), 2.5% as oxo-methylphenidate and 5% as *p*-hydroxymethylphenidate [7, 8]. Less than 1% of MEP is excreted unchanged in the urine of hyperkinetic children [9], although this may be enhanced by acidosis.

Because of the hydrosolubility of the molecule, RITA cannot be extracted from urine by the commonly used organic solvents. This may explain why MEP abuse is not detected more frequently in drug screening programmes. RITA in urine can be determined by gas chromatographic (GC) methods following salting out liquid extraction [9] or solid-phase extraction [10] and methylation of the carboxylic functional group of RITA converting it back to the parent compound MEP. The methylation reaction is carried out with diazomethane [9] or with methanol and concentrated sulphuric acid [10, 11].

Both diazomethane and concentrated sulphuric acid are hazardous. Diazomethane is a powerful irritant for the skin and the eyes, and can cause cutaneous and pulmonary hypersensitivity upon acute and chronic exposure [12]. Its IDLH level (immediately dangerous to life and health) is low (10 ppm or 20 mg m^{-3}) and it is a potential carcinogen. The exposure limit to diazomethane is 0.4 mg m^{-3} for an 8-h work shift. Furthermore, diazomethane can cause severe explosions. The hazardous nature of concentrated sulphuric acid is well known with its risk of burns to the skin and the eyes.

This paper describes the development of an improved analytical method for the determination of RITA in human urine. The method employs a new clean-up scheme and a safer methylation technique using the ready-made boron trifluoride–methanol reagent. The methylated RITA is suitable for both qualitative screening by thin-layer chromatography and quantitative determination by GC.

* Author to whom correspondence should be addressed.

Materials and Methods

Reagents and materials

All solvents used were analytical grade from Merck. Methanol and chloroform were re-distilled. HPLC quality water was used.

Pure MEP and RITA were gifts from Ciba-Geigy (Basel, Switzerland) for use as standards. Stock solution of MEP was prepared in methanol at 1 μg μl^{-1}. RITA was in aqueous solution at 1 μg μl^{-1}. Diphenylamine of >99% purity was used as internal standard (Fluka, Buchs, Switzerland) at 0.5 μg μl^{-1} in chloroform.

BF_3–methanol reagent (12% by wt) was purchased from Supelco and kept refrigerated in Mini-Vials (Alltech No. 95050) in 5-ml fractions. Mini-Vials (3 ml) used for collection of eluate, esterification reaction and liquid–liquid re-extraction were purchased from Alltech (No. 95030). The Mini-vials were screw-capped with 18 mm TFE-lined silicone rubber septa (Alltech No. 95302) for maximum tightness. The Reacti-Vap evaporating accessory and Reacti-Therm heating module were obtained from Pierce Chemicals.

Disposable C_8 solid-phase columns (3 ml per 500 mg sorbent weight) were purchased from Baker. A stock drug-free urine was provided by a smoker (one of us). It was chosen to assess the potential interference by nicotine since many of the specimens for drug analysis in urines are from smokers.

Gas chromatography with NPD and/or FID

A Hewlett-Packard 5890 GC equipped with dual NPD/FID was used. The detectors are respectively connected to CP-Sil 8 CB and CP-Sil 5 CB, 25 m × 0.32 mm capillary columns in the same oven. By means of a two-hole graphite ferrule, the two columns are introduced in the same injector. A temperature programme of 120 to 155°C at a rate of 1.5°C min^{-1} was used. After 1 min at 155°C, the temperature was raised to 280°C at 30°C min^{-1} and maintained for 10 min to purge the columns. The nitrogen flow rates on both columns were 1.3 ml min^{-1}. Conditions for NPD were: 3 ml hydrogen, 77 ml air, 17 ml nitrogen make-up gas. Conditions for FID were: 34 ml hydrogen, 440 ml air, 28 ml nitrogen make-up gas. The temperatures for both detectors and injector were 300 and 280°C, respectively. Split ratio, 1:10.

Sample preparation procedure

Conditioning of the C_8 column. The column was eluted with 2 × 3 ml methanol followed by 2 × 3 ml water. The columns were not allowed to dry.

Solid-phase extraction of RITA. Ten millilitres of urine were introduced into the C_8 column and the flow rate adjusted so that it took about 4 min to pass the specimen through the column. The column was rinsed with 5 ml water, dried for 5 min under full vacuum and centrifuged for 10 min at 3000 rev min^{-1}.

Elution of RITA. The RITA was eluted into a 3-ml Mini-Vial by pushing 3 ml of MeOH–glacial acetic acid (99.9:0.1) slowly through the column with a plunger and evaporated to dryness at 60°C under nitrogen.

Methylation with BF_3–methanol. One millilitre of BF_3–methanol reagent was added, the vial stoppered carefully and incubated in an air-circulated oven at 90°C for 4 h. After the vial had reached the oven temperature (15 min), it was rapidly vortexed for 5 s at hourly intervals. At the end of incubation, the vial was cooled to room temperature and the reaction stopped by adding 0.5 ml of water.

Liquid–liquid re-extraction of methylated RITA. The reaction solution was made alkaline (pH 9–11) by adding 0.3 ml NaOH 37% then 1 ml chloroform and vortexed for 1 min. The mixture was centrifuged for 3 min at 1000 rev min^{-1} and 60 μl of diphenylamine was added. The organic phase was quantitatively transferred to a Mini-Vial and evaporated to dryness under nitrogen and brought to 100 μl final volume with chloroform.

Results

Methylation yield with BF_3–methanol. Influence of temperature and reaction time

Orientation experiments showed that the esterification of RITA by BF_3–methanol to the parent compound MEP requires more drastic conditions of temperature and time than the 15 min at 50°C reported for other compounds [13]. Temperatures from 60 to 110°C were studied for a 2-h reaction time in the vial. The esterification yield was still very low at 70°C, and even at 90°C, only 85% of RITA was

converted. At 110°C there is risk of over pressure from the gas phase reagent inside the vial upon heating and subsequent leakage by the Teflon lined-septum. For this practical reason, the temperature of 90°C was chosen to study the required time to complete the reaction. The amount of BF_3–methanol reagent used in the procedure was sufficient for the methylation of 100 μg of RITA in the vial. Five experiments were repeated at each time with a relative standard deviation (RSD) <8%. A plateau is observed between 4–5 h (Fig. 1). The determined yield of esterification is 97% ± 7 (N = 8) at 90°C and 4 h incubation time.

Recovery studies

The recovery studies were carried out by spiking 10-ml volumes of drug-free urine with 5 μg of RITA per ml. The steps of interest were then conducted in each case. The recovery of RITA from urine after the solid-phase extraction with the C_8 column is 100% ± 8 for N = 3 experiments. The recovery of methylated RITA from the liquid–liquid re-extraction step with chloroform was assessed by spiking an aqueous BF_3–methanol solution at various pH values from 9 to 14 with standard methylated RITA. The extraction yields of methylated RITA by chloroform at pH values 9–12 were nearly quantitative (N = 2). pH does not have much influence on the extraction efficiency but a slight drop is observed with pH over 12 (Fig. 2). Overall recovery of RITA throughout the whole procedure has been determined in N = 9 repeated experiments. The overall recovery of the procedure is 96% ± 9.

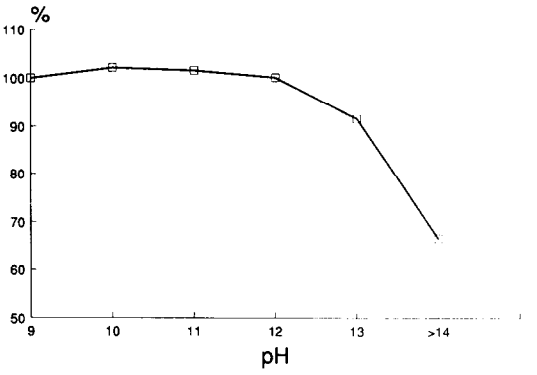

Figure 2
Influence of pH range on the re-extraction recovery of methylated RITA

Chromatography

Quantitative determination is made with capillary GC–NPD relative to diphenylamine added to the final solution to eliminate injection error. This procedure is acceptable since the overall recovery of the procedure approaches 100%. The linearity of the calibration curve was studied by spiking 10-ml volumes of drug-free urine with standard RITA at various concentration levels from 0.5 to 7.5 μg ml^{-1}. The linearity of the area ratio of methylated RITA relative to diphenylamine as a function of concentration is shown in Fig. 3. The equation of the regression line is $Y = 0.4674X - 0.0071$. The sensitivity of the NPD to methylated RITA (i.e. MEP) was 1 ng at a signal to noise ratio of 10. The practical limit of detection of the method was 50 ng of RITA per ml urine in the conditions adopted. Using the FID, the limit of detection was 100 ng.

The method was applied to a few urine samples suspected to contain MEP due to drug

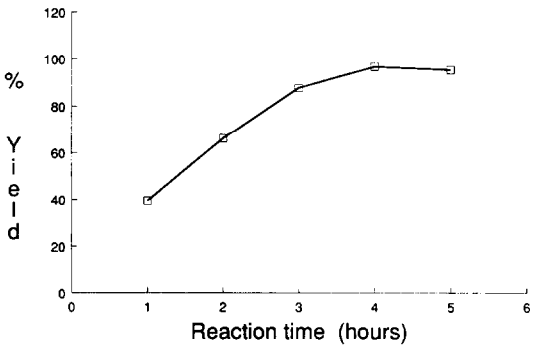

Figure 1
Optimization of esterification yield of RITA versus reaction time and temperature.

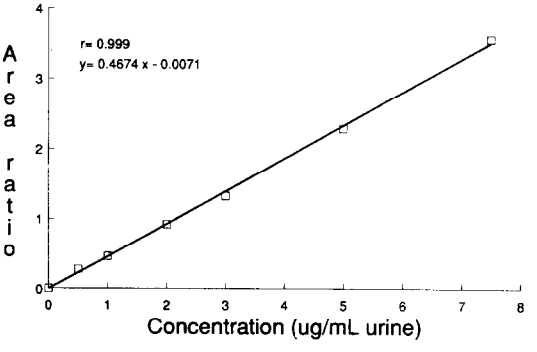

Figure 3
Nitrogen phosphorus detector response to increasing concentrations of methylated RITA extracted from urine. Area ratio of methylated RITA to internal standard diphenylamine.

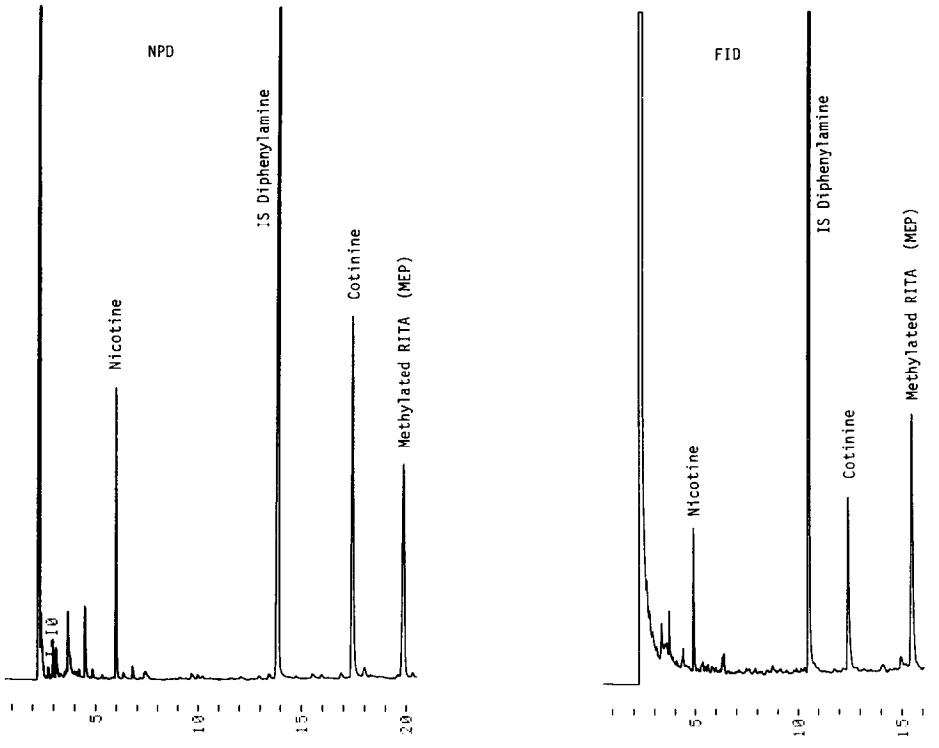

Figure 4
NPD and FID chromatographic profiles of a MEP positive urine extract. (NPD Att.X6 and FID Att.X4.)

abuse. The NPD and FID chromatograms of a positive MEP sample are presented in Fig. 4. The peak is equivalent to 120 ng of methylated RITA corresponding to 1.2 μg ml^{-1} in the urine specimen. The presence of nicotine and its metabolite cotinine indicates that the subject was a smoker.

Discussion

The mechanism of ester formation from alcohol and carboxylic acid in the presence of an electron-pair acceptor like BF_3 has been described [14]. Esterification by BF_3–methanol can be rapid and has found applications in pesticide analysis, fatty acids and nitrosoamino acids [13]. The BF_3–methanol esterification method has not been studied by others for the analysis of RITA in urine. The commercial availability of BF_3–methanol as well as the less hazardous character of the reagent led us to investigate the conditions for methylation of the carboxylic functional group in the RITA molecule. The reagent presents the advantage of a long shelf life (6 months), in contrast to diazomethane which should be freshly prepared.

The sample preparation within the method is convenient since transfer of solutions is limited, although potential problems with the methylation process are the long incubation time and possible leakage. Several types of septa have been tested for the tightness of the 3 ml reaction Mini-Vials to ensure no gas leakage when BF_3 is brought to 90°C during 4 h. The TFE-lined silicone rubber septa provided the highest efficiency and safety. In the final stage of the procedure outlined, methylated RITA is recovered in chloroform and appears to be stable. Hydrolysis of the methyl-ester is not apparent since the results with extracts injected the day after the preparation showed the same profile.

Because of the hydrophylic properties of RITA, an extraction with classical organic solvent would not yield good and consistent recovery. The clean-up procedure described in this paper is different from the previous column extraction techniques which uses C_{18} and has a reported detection sensitivity of ≥ 1 μg ml^{-1} [10]. Quantitative recovery is obtained with C_8 column and, moreover, better purification and enrichment results in high signal to noise ratio as shown by the

chromatogram baseline. This laboratory is equipped with a GC where two capillary columns of different polarities are permanently mounted onto two detectors, NPD and FID, respectively [15]. This facility provides more confidence and reliability in routine results but the use of two detectors is not a necessity of the method. The efficiency of the clean-up scheme allows the use of either detector but NPD is preferred for its better specificity. The absence of interfering substances at the retention time of the methylated RITA peak and its definite identity have been verified by injection of the urine extract into a GC–MS system (Finnigan's ITS40). The mass spectrum is similar to that of reference MEP, and mass chromatograms exhibit no mass fragment at the retention time of methylated RITA.

Acknowledgements — We gratefully acknowledge the gift of pure methylphenidate and ritalinic acid by the Ciba-Geigy Company (Switzerland) and Mr Blaser from Spectronex AG for the use of their GC-ITS40 system.

References

[1] R.J. Schain and C.L. Reynard, *Pediatrics* **55**, 709–716 (1975).
[2] B.G. Winsberg, S.S. Kupietz, J. Sverd, B.L. Hungund and N.L. Young, *Psychopharmacology* **76**, 329–332 (1982).
[3] R. Klorman, J.T. Brumaghim, L.F. Salzman, J. Strauss, A.D. Borgdtedt, M.C. McBride and S. Loeb, *Psychophysiology* **27**, 328–337 (1990).
[4] R.N. Wharton, J.M. Perel, P.G. Dayton and S. Malitz, *Am. J. Psychiat.* **127**, 55–61 (1971).
[5] G.M.J. van Kempen, J.G. Goekoop, H.B. Jong, P.M. Edelbroek and F.A. de Wolff, *Biol. Psychiat.* **28**, 638–640 (1990).
[6] R.M.J. Haglund and L.L. Howerton, *Int. J. Addict.* **17**, 349–356 (1982).
[7] J.M. Perel and P.G. Dayton, in *Methylphenidate in Psychotherapeutic Drugs*, Part II (E. Usdin and J.S. Forest, Eds), pp. 1287–1316. Marcel Dekker, New York (1977).
[8] H. Sheppard, W.H. Tsien, W. Rodegkar and A.J. Plummer, *Toxicol. Appl. Pharmacol.* **2**, 353–362 (1960).
[9] R. Wells, K.B. Hammond and D.O. Rodgerson, *Clin. Chem.* **20**, 440–443 (1974).
[10] H.W. Allen and B. Sedgwick, *J. Anal. Toxicol.* **8**, 61–62 (1984).
[11] B.R. Manno, J.E. Manno and C.A. Dempsey, *J. Anal. Toxicol.* **10**, 116–119 (1986).
[12] U.S. Department of Health and Human services, in *NIOSH Pocket Guide to Chemical Hazards*, p. 94. U.S. Government Printing Office, Washington D.C. (1985).
[13] A.J. Sheppard and J.L. Iverson, *J. Chromatogr. Sci.* **13**, 448–452 (1975).
[14] J.D. Roberts and M.C. Caserio, in *Alcohols and Ethers: C. Ester formation in Basic Principles of Organic Chemistry* (W.A. Benjamin, Ed.), p. 388. Benjamin, W.A. Inc., Menlo Park, CA (1964).
[15] T. Vu-Duc and A. Vernay, *Biomed. Chromatogr.* **4**, 65–69 (1990).

[Received for review 29 April 1991; revised manuscript received 27 August 1991]