

Detection of Tamoxifen Metabolites by GC-MSD

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Abstract

Tamoxifen is an antiestrogen used in the adjuvant endocrine therapy of early breast cancer and malignant breast disorders. It is also used in women with anovulatory infertility caused by its stimulating effect on the secretion of the pituitary gonadotrophic hormones. In males it could increase the endogenous production of androgens. Because of these properties tamoxifen may be misused in some sports to treat the androgens suppression caused by the extensive abuse of anabolic androgenic steroids. A method for identification and confirmation of tamoxifen metabolites is described. Hydroxymethoxytamoxifen is detected in urine by gas chromatography and mass spectrometry in a selective ion monitoring method followed by the routine postrun in the screening of anabolic steroids. Once the hydroxymethoxytamoxifen is detected, confirmation of reported metabolites could be performed with a 5973 mass selective detector in the scan mode after solid-phase extraction by cationic exchange. This study also reports an excretion profile for a single dose of tamoxifen equivalent to 40 mg administrated orally to two males volunteers.

Introduction

Tamoxifen is an antiestrogen that is used in the adjuvant endocrine therapy of early breast cancer and malignant breast

disorders. It is also used in women with anovulatory infertility caused by its stimulating effect on the secretion of the pituitary gonadotrophic hormones. In males it could increase the endogenous production of androgens. Because of these properties, tamoxifen may be misused in some sports to treat the androgens suppression caused by the extensive abuse of anabolic androgenic steroids. For this reason it was first included in the forbidden list by the International Olympic Committee (IOC) and later by the World Antidoping Agency (WADA) as an antioestrogenic agent prohibited only in males. Tamoxifen is extensively metabolized and excreted mainly in feces. Biphasic excretion has been reported, and the terminal half-time may be longer than 7 days (1). J. Grobe et al. (2) reported two metabolites in urine: a carboxylic acid (M1) and a hydroxymethoxytamoxifen (M2) (Figure 1). R. Mihailescu et al. (3) reported three metabolites in urine: a hydroxy compound, *N*-desmethyltamoxifen, and another fenolic compound from the cleavage of the ethyldimethylamine moiety with a detection time within 6 days after administration. W. Schänzer et al. (4) reported that the main metabolite of tamoxifen is the hydroxy-methoxy compound (M2) in agreement with Grobe and Mihailescu (2,3).

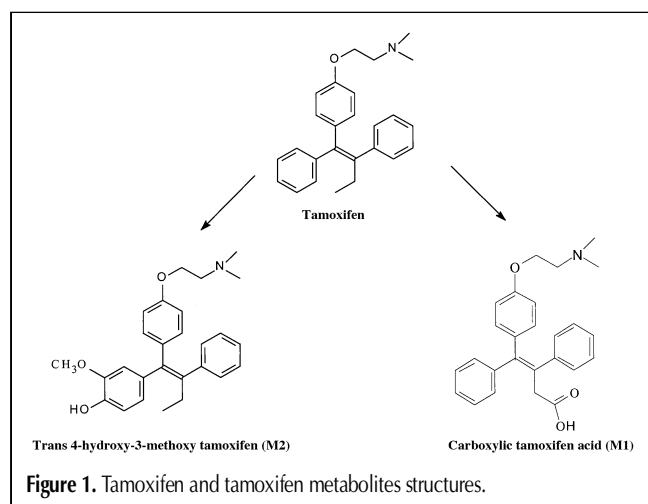
We carried out an excretion profile for a single dosis of 40 mg of tamoxifen administered orally to two male volunteers in order to select those metabolites that are suitable using the normal screening procedure for anabolics. Urine samples were collected from 0 to 168 h post-administration.

Hydroxymethoxytamoxifen was detected in urine by gas chromatography (GC)-mass selective detection (MSD) using a selective ion monitoring (SIM) method and a postrun macro, as is routine for the screening of anabolic steroids. Once the hydroxy methoxytamoxifen was detected, confirmation of reported metabolites could be performed with a Hewlett Packard 6980/5973 (GC-MSD) in the scan mode, with previous extraction by solid phase with a copolymeric bonded phase containing hydrophobic and sulfonic functional groups (ZSDAU020).

Experimental

Drug administration

A 40-mg dose of tamoxifen (Taxus) was administered orally to two healthy male volunteers. Both were 28-years old and didn't



receive any kind of medication before or during the excretion study. The study was conducted according to the rules of our Faculty Ethical Committee (Universidad de Chile, Santiago, Chile). Urine samples were collected prior to and 2, 4, 8, 12, 24, 48, 60, 72, 96, 120, 144, and 168 h after administration.

Materials

Tamoxifen (Taxus) was obtained from Tecnofarma (Santiago, Chile), tert-butyl methyl ether was from Merck (Darmstadt, Germany), β -glucuronidase from *E. Coli* (the strength of solution at 37°C was at least 140 U/mL) was from Roche (Mannheim, Germany), *N*-methyl-*N*-trimethyl-silyl-trifluoroacetamide (MSTFA) was from Aldrich (St. Louis, MO), and chlortrimethylsilan (TMSCI) was from Merck. Dithioerythritol (2,3-dihydroxybutano-1,4-dithiol) was from Sigma (Ontario, Canada). Chromatographic-grade water, dichloromethane, and isopropanol were from EMD Chemicals (Gibbstown, NJ). $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, K_2CO_3 , and NH_4I as a solid reagent were from Merck. The copolymeric bonded solid-phase extraction

(SPE) column was from United Chemical Technologies (UCT, Bristol, PA) worldwide monitoring (ZSDAU020).

Instrumental

The analyses were performed in a Hewlett Packard 6890/5973 GC-MSD system. The GC was carried out using a 100% polydimethylsiloxane fused silica capillary column from Agilent Technologies (Palo Alto, CA) (HP1, 25-m \times 0.2-mm i.d, 0.11- μm), operated in SIM and scan modes. The methods and GC-MSD parameters were different for the screening and confirmation procedures and are given below.

Methods

Screening

Two milliliters of urine were hydrolyzed with 50 μL of β -glucuronidase from *E. coli* at pH 6 with 750 μL of phosphate buffer (1.04 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 6.07 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ resulting in a concentration of 100mM) during 1 h at 50°C. Extraction was carried out with 5 mL of tert-butyl methyl ether at pH 9.5 with 80 μL of 25% potassium carbonate solution. The extract was dried under nitrogen at 40°C. The dried extract was derivatized with 100 μL MSTFA- NH_4I -dithioerythritol (1000:4:3) at 75°C for 30 min. Three microliters was injected into the GC-MSD in the split mode (1:10).

The GC-MSD parameters included a column flow of 0.2 mL/min of Helium. The injector temperature was 280°C in the split mode. The oven temperature was programmed from 190°C to 235°C at a rate of 2°C/min and then from 235°C to 300°C at a rate of 15°C/min. The final time was 7 min. It was operated in the SIM mode using a postrun macro for anabolic steroids including ions m/z 58, 72, and 489 at the retention time of metabolite 2 of tamoxifen. (Figure 2).

Confirmation

Five milliliters of urine was hydrolyzed with 50 μL of β -glucuronidase from *E. coli* at pH 6 with 750 μL of phosphate buffer (100mM) during 1 h at 50°C. Tamoxifen was used as the internal standard at 100 ng/mL. The samples were applied over the solid-phase column and eluted with dichloromethane-isopropanol-ammonia (78:20:2) (5). The extract was dried under nitrogen at 40°C. Dried extracts were derivatized with 100 μL of MSTFA-TMSCI (1%, v/v) to reduce chromatographic background produced by the derivatization mixture used in the screening step. Two microliters was injected into the GC-MSD in the splitless mode.

The GC-MSD parameters included a constant column flow of 1 mL/min. The injector temperature was 250°C. The oven temperature was programmed from 130°C to 310°C at a rate of 17°C/min, with a final time of 5 min. Operation was in the scan mode (scan range, 50–550 amu).

Results and Discussion

Analysis of urine samples showed that unchanged tamoxifen was not excreted. The

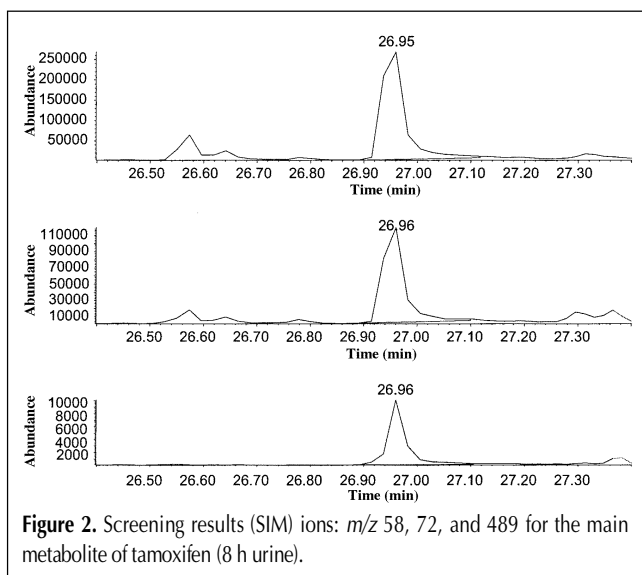


Figure 2. Screening results (SIM) ions: m/z 58, 72, and 489 for the main metabolite of tamoxifen (8 h urine).

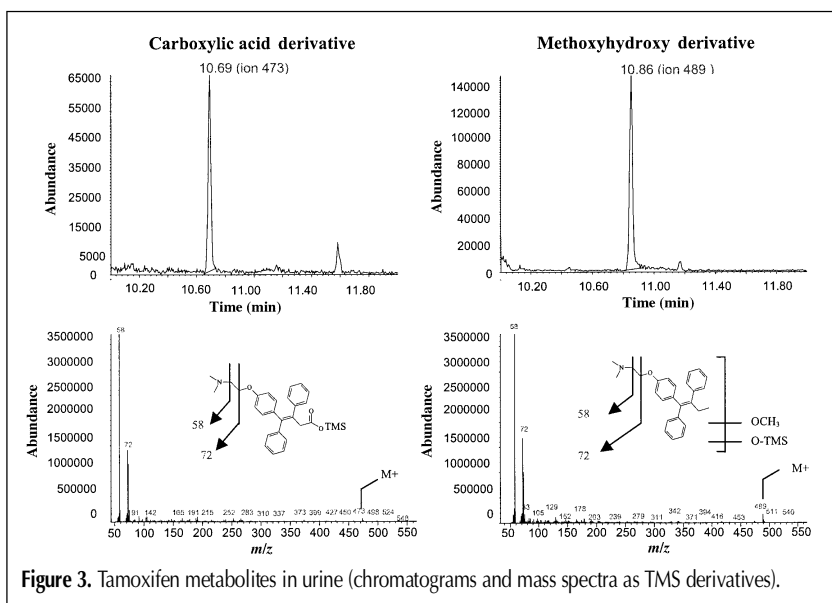
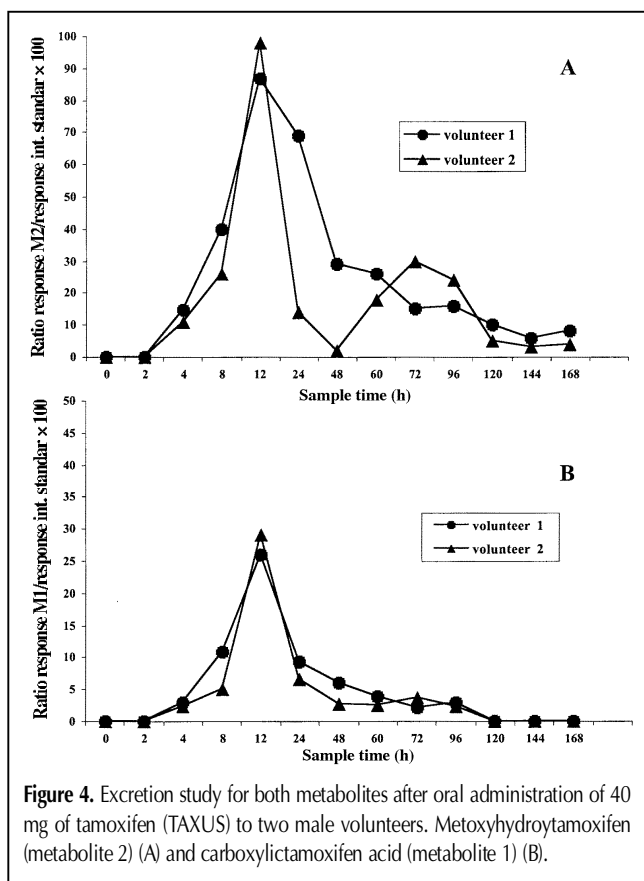


Figure 3. Tamoxifen metabolites in urine (chromatograms and mass spectra as TMS derivatives).



identification of both reported metabolites of tamoxifen, carboxylic acid (M1), and methoxyhydroxy (M2) were carried out by monitoring the molecular ions m/z 473 and 489 (Figure 3), respectively.

Samples without enzymatic hydrolysis indicated a lower extraction recovery for metabolite M2, but metabolite M1 resulted in similar recoveries for hydrolyzed and nonhydrolyzed samples. This could suggest a nonconjugated excretion of metabolite M1.

The fragmentation pattern of both metabolites was similar to the parent drug. The fragmentation generates, after α -cleavage, the ion at m/z 58 as the base peak, followed in abundance by the ion at m/z 72 (Figure 3).

A metabolic profile for M1 and M2 was obtained using tamoxifen as the internal standard because previous analysis of specimens showed that the parent drug was not detected. The results of both excretion studies are presented in Figure 4, showing a

peak of excretion at 12 h for both metabolites. Using this method, the main metabolite, hydroxymethoxytamoxifen (M2), could be detected up to 7 days after administration.

Hydroxymethoxytamoxifen was detected in urine by the routine GC-MSD screening procedure for anabolic steroids by a SIM method followed by a post-run macro (Figure 2). Once the hydroxymethoxytamoxifen was detected, confirmation of both reported metabolites could be performed by GC-MSD in the scan mode, changing liquid extraction by SPE because of the amphoteric nature of M1 metabolite.

Conclusion

It is possible to include the detection of tamoxifen in the usual routine screening procedure for anabolic steroids by liquid-liquid extraction, detecting the major metabolite hydroxymethoxytamoxifen by SIM method with the characteristic ions at m/z 58, 72, and 489. Confirmation of both metabolites could be accomplished previously by extraction with a copolymeric cationic-exchange solid phase using 5973 (MSD) in full-scan mode. The discussed metabolites are not commercially available, and their identification was based on their chemical structure and fragmentation pattern.

References

1. Martindale. *The Complete Drug Reference*. 32nd ed. Ed Pharmaceutical Press, Tanton, MA, 1999. pp. 563-65.
2. J. Grobe, R. Lang, R.K. Muller, and D. Thieme. Detection of tamoxifen metabolites in urine by GC/MS. *Recent Advances In Doping Analysis, Proceedings of the 12th Cologne Workshop on Dope Analysis* April 1994. Ed. Sport-Koln. Germany, 1995, pp. 317-27.
3. R. Mihailescu, H.Y. Aboul-Enein, and M.D. Efstatide. identification of tamoxifen and metabolites in human male urine by GC/MS. *Biomed. Chromatogr.* **14**: 180-83 (2000).
4. U. Mareck-Engelke, G. Sigmund, G. Opfermann, H. Geyer, and W. Schänzer, Eds. "Screening for tamoxifen, clomiphene and cyclofenil in doping analysis". *Recent Advances In Doping Analysis, Proceedings of the 19th Cologne Workshop on Dope Analysis*, March 2001. Sport-Koln, Cologne, Germany, 2002, pp. 53-61.
5. *Clean Screen Extraction Column. Applications Manual*. World Wide-Monitoring Corp., Horsham, PA, 1998.