Sandra Valtier,[1] *B.S. and John T. Cody,*[1] *Ph.D.*

Metabolic Production of Amphetamine Following Administration of Clobenzorex*

REFERENCE: Valtier S, Cody JT. Metabolic production of amphetamine following administration of clobenzorex. J Forensic Sci 1999;44(1):17–22.

ABSTRACT: Many of the anorectic drugs that are metabolized to amphetamine and/or methamphetamine pose significant concerns in the interpretation of amphetamine-positive drug testing results. One of these drugs—clobenzorex—has been shown to produce amphetamine. Thirty milligrams of clobenzorex hydrochloride, in the form of a single Asenlix capsule (Roussel, Mexico), were administered orally to five human volunteers with no history of amphetamine, methamphetamine or clobenzorex use. Following administration, urine samples (total void volume) were collected *ad lib* for seven days and pH, specific gravity and creatinine values were determined. To determine the excretion profile of amphetamine and parent drug, samples were extracted, derivatized, and analyzed by gas chromatography/mass spectrometry (GC/MS) using a standard amphetamine procedure with additional monitoring of ions at *m/z* 91, 118, 125 and 364 for the detection of clobenzorex. Peak concentrations of amphetamine were detected at 4 to 19 h postdose and ranged from approximately 715 to 2474 ng/mL amphetamine. Amphetamine could be detected (>5 ng/mL) in the urine in one subject for up to 116 h postdose. GC/MS was also used to determine the enantiomeric composition of the metabolite, amphetamine. This analysis revealed the metabolically derived amphetamine was only the *d*-enantiomer. This differs from previous literature which indicates clobenzorex is the racemic N-orthochlorobenzyl derivative of amphetamine.

KEYWORDS: forensic science, clobenzorex, GC/MS, enantiomer analysis, amphetamine, metabolism, anorectic drug, precursor drug, Asenlix

Clobenzorex, N[(2-Chlorophenyl)methyl]-α-methyl-benzeneethanamine, is an anorectic drug that is not available in the United States but is available in many countries as a prescription (i.e., Mexico) or over-the-counter (i.e., Panama) drug. The recommended starting dose is 30 mg (one capsule) per day. It is one of several so called ''precursor drugs'' which are metabolized to amphetamine by the body and excreted in the urine. Because it is metabolized to amphetamine, therapeutic use of clobenzorex may lead to suspicion of amphetamine abuse. Drugs that are metabolized to amphetamine and/or methamphetamine are potentially significant concerns in the interpretation of amphetamine positive drug testing results. The presence of the parent compound or a unique metabolite can be conclusive evidence for the involvement of this drug.

Published data on clobenzorex consists of a report (1) which documents the results of a single subject administered one dose of the drug with samples taken at four time points. The last time point was the maximum level observed, therefore peak levels could not be determined from these data. Another report (2) indicates amphetamine was found in urine in pooled samples collected 0 to 8 h and 8 to 24 h following administration of clobenzorex. While this is important information, it does not allow for the evaluation of the presence (or absence) of the parent in individual samples. In a study by Maurer et al. (3), three volunteers received a single oral dose of 60 mg of clobenzorex. Following administration, the parent drug was detected for only 4 h. However, subject urine samples were collected every 4 h. As a result, detectable levels of clobenzorex may have been missed that otherwise may have been detected had samples been collected *ad lib.* In the study by Maurer et al., the limit of detection in urine was 50 ng/mL clobenzorex, which may also explain the short duration of detection. In the present study, the limit of detection was 1 ng/mL. No data regarding the enantiomeric composition, peak levels or excretion profile were presented in the previous studies.

Methods and Materials

Materials—Amphetamine, methamphetamine, and methamphetamine-D_{11} (1-phenyl-D_5-2-methyl-D_3-aminopropane-3,3,3-D_3), amphetamine-D_5 (1-phenyl-2-aminopropane-1,2,3,3,3-D_5), methamphetamine-D_5 (1-phenyl-2-methyl-D_3-aminopropane-1,2-D_2) were obtained from Radian Corporation. Amphetamine-D_6 (1-phenyl-2-aminopropane-1,1,2,3,3,3-D_6) was obtained from Alltech. Clobenzorex hydrochloride was obtained through colleagues in Spain. Heptafluorobutyric anhydride (HFBA), fenfluramine hydrochloride and benzphetamine hydrochloride were obtained from Sigma Chemical Company. Trifluoroacetyl-*l*-prolyl chloride (*l*-TPC) was obtained from Aldrich Chemical Company. Clobenzorex administered to experimental subjects, in the form of Asenlix, was purchased from a pharmacy in Mexico.

Drug Administration and Sample Collection—Thirty milligrams of clobenzorex, in the form of a single Asenlix capsule (Roussel, Mexico), was administered orally to five healthy volunteers (two female and three male) with no history of amphetamine, methamphetamine, or clobenzorex use. Following administration, urine was collected for the next seven days. Samples were provided *ad lib* and refrigerated until analysis. No attempt was made to physiologically control urine pH.

[1] Clincial Research Squadron, Wilford Hall Medical Center, Lackland AFB, TX 78236-5319.

* Presented at the 50th Annual Meeting of the American Academy of Forensic Sciences, San Francisco, CA, Feb. 1998. This work was supported in part by a Lucas Research Grant. The voluntary fully informed consent of the subjects used in this research was obtained as required by AFI 40-403. The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Defense or other Departments of the U.S. Government.

Received 21 April 1998; and in revised form 23 June 1998; accepted 25 June 1998.

Copyright © 1999 by ASTM International

Sample Preparation and Analysis

Sample pH was measured using a Fisher Accumet 50 pH meter and specific gravity determined using an AO Scientific Instruments refractometer. Creatinine levels were determined at the Wilford Hall Medical Center clinical laboratory using standard clinical laboratory procedures. GC/MS analyses were performed using a Hewlett-Packard 5890 II gas chromatograph (GC) coupled with an HP 5971 mass spectrometer (MS) using a 7673 autoinjector.

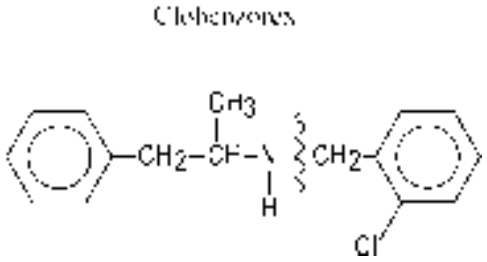

FIG. 1—*Chemical structure of clobenzorex. Curved line indicates point of cleavage to release amphetamine.*

Quantitative Analysis—Quantitation and detection was based on single-point calibration using a calibration standard containing 500 ng/mL of amphetamine, methamphetamine, clobenzorex and 250 ng/mL of internal standards. Low-concentration samples were quantitated based on single point calibration using a standard at 25 ng/mL of each of the analytes of interest and 50 ng/mL of internal standards. Two-milliliter urine samples were aliquoted into glass tubes (optional: add 1.0 mL of 0.35 M sodium periodate and allow to stand at room temperature for 15 min). Aliquots were extracted and derivatized with heptafluorobutyric anhydride (HFBA) as previously described (4). GC conditions were as follows: splitless injection using a 7673 autoinjector with injection port and interface temperatures set at 270°C. Conditions for the HP-1 column (12-m 0.2 mm i.d. and 0.33 μm film thickness [Hewlett-Packard]) consisted of a temperature program with an initial time of 1 min at 80°C, programmed to 180°C at 20°C per min, then programmed to 250°C at 28°C per min with a 1 min final time. Conditions for the DB-17 column (30-m 0.18 mm i.d. and 0.30 μm film thickness [J & W Scientific]) were the same as for the HP-1 with the exception of a final time of 2.0 min. Ions monitored were: amphetamine: *m*/*z* 240, 118, 91; methamphetamine: *m*/*z* 254, 210, 118; amphetamine-D_6: *m*/*z* 244, 123; methamphetamine-D_{11}:

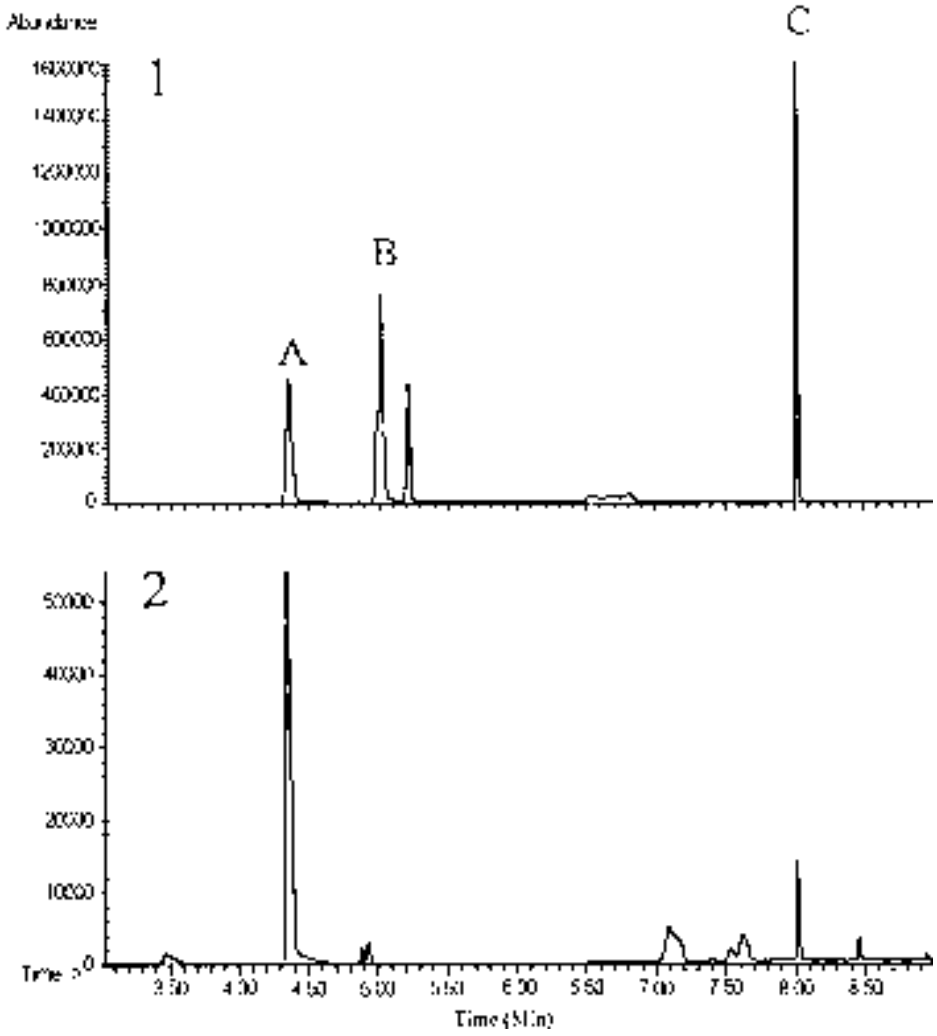

FIG. 2—*(1) Chromatography of amphetamine and amphetamine-d6 (peak A), methamphetamine and methamphetamine-d11 (peak B) and clobenzorex (peak C) from calibration standard containing 500 ng/mL of each constituent. (2) Chromatography of amphetamine and clobenzorex from a urine sample collected following use of clobenzorex.*

m/*z* 260, 213; fenfluramine: *m*/*z* 159, 240, 268; benzphetamine: *m*/*z* 65, 91, 148 and clobenzorex: *m*/*z* 91, 118, 125 and 364.

Enantiomer Analysis—The calibrator contained 50% of both enantiomers of amphetamine and methamphetamine. Urine samples (2 mL) containing 500 ng/mL each of amphetamine-d5 and methamphetamine-d5 were analyzed. Extraction was accomplished by addition of 0.3 mL 1M NaOH and 5 mL 1-chlorobutane. Tubes were shaken for 10 min at approximately 120 cycles per minute (cpm) and then centrifuged for 5 min at approximately 1500 rpm to separate the layers. The organic layer was transferred to a clean dry tube; 50 μL of N-trifluoroacetyl-*l*-prolyl chloride (*l*-TPC) was added and the mixture allowed to stand at room temperature for 15 min. NaOH (3 mL, 0.01M) was added and the samples shaken for 15 min and centrifuged as described above. The organic layer was transferred, evaporated under nitrogen at 50 to 60°C, reconstituted in ethyl acetate, and injected into the GC-MS. Instrumental conditions for the enantiomer analysis were as follows: HP-1 column; splitless injection; injector and interface temperatures were set at 270°C; oven temperature, 120°C for 2 min then 4°C per min to 200°C. Ions monitored were *m*/*z* 237, 241, 251, 255 for *d*- and *l*-amphetamine; *d,l*-amphetamine-D5; and *d*- and *l*-methamphetamine and *d,l*-methamphetamine-D5, respectively.

Results and Discussion

As expected, ingestion of clobenzorex resulted in the metabolic production of amphetamine. Amphetamine was detected in the urine of all five subjects. Methamphetamine was not detected in any of the subject samples (LOD for methamphetamine, 5 ng/mL). Following administration of the drug, the subjects were asked if they noticed any subjective effect from the drug. Three of the five subjects reported having some kind of effect. One subject reported moderately increased alertness and sense of well-being that started one and one half hours after taking the drug and lasted for approximately 5 h. Two subjects reported mild excited/jittery sensation and one of those subjects also reported moderately decreased appetite, mild headache and significant irritability.

Figure 1 shows the structure of clobenzorex, revealing its relationship to amphetamine. Figure 2 illustrates a typical chromatographic result from the quantitative analysis of subject samples. Clobenzorex was easily identified using the same extraction, derivatization, and instrumental analysis parameters used for the determination of amphetamine and methamphetamine with only minor modification of the GC over temperatures and monitoring of ions *m*/*z* 91, 118, 125 and 364 for the detection of clobenzorex (see Fig. 3). Although all four ions are suitable for the monitoring of clobenzorex, the 91 ion exhibited interference with some urine samples at low clobenzorex concentrations. Therefore, the remaining three ions were routinely used for the identification of clobenzorex.

Quantitating clobenzorex proved to be a challenging task. A number of different procedures were evaluated for the quantitative analysis of clobenzorex. HFBA and other derivatizing reagents (TFAA, HFB.Cl) were tested using higher temperatures (70 to 80°C) in the derivatization step. Solid phase extraction using a C18 column and fenfluramine as the internal standard were used to

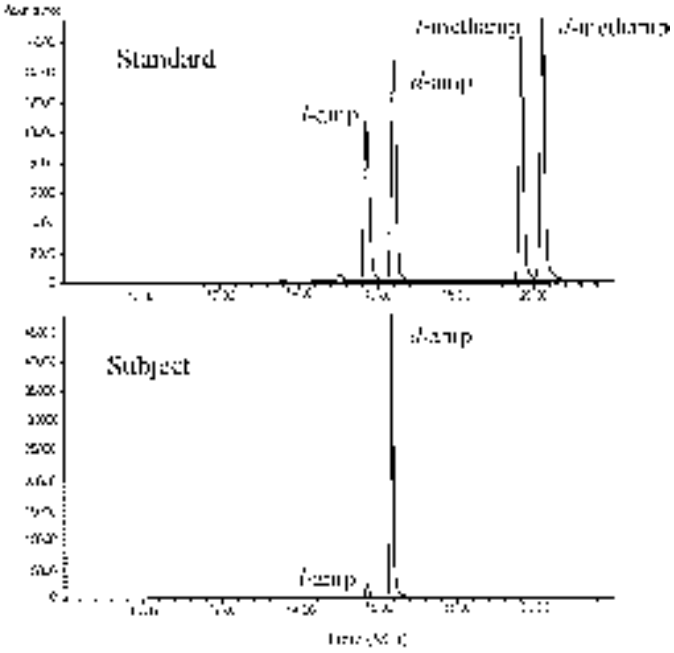

FIG. 4—*(1) Chromatography of l-amphetamine and l-amphetamine-d5, d-amphetamine and d-amphetamine-d5, l-methamphetamine and l-methamphetamine-d5, d-methamphetamine and d-methamphetamine-d5 from calibration standard containing 500 ng/mL each of racemic drug and deuterated internal standard. (2) Chromatography of d-amphetamine enantiomer from a urine collected following use of clobenzorex.*

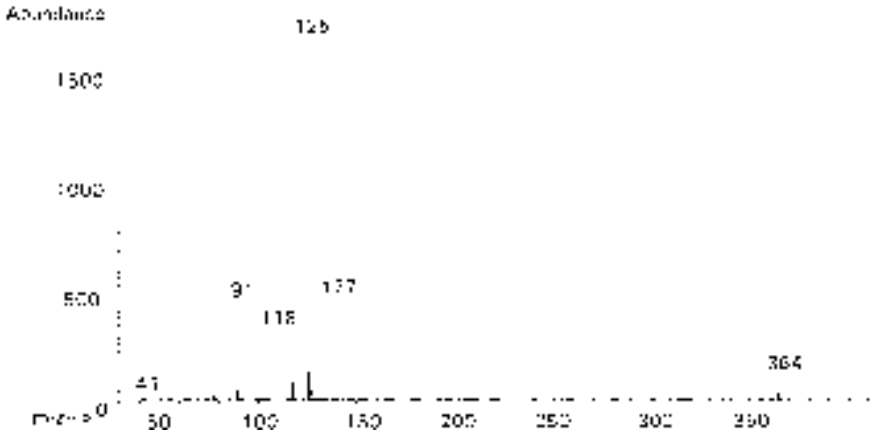

FIG. 3—*Mass spectrum of HFBA derivatized clobenzorex.*

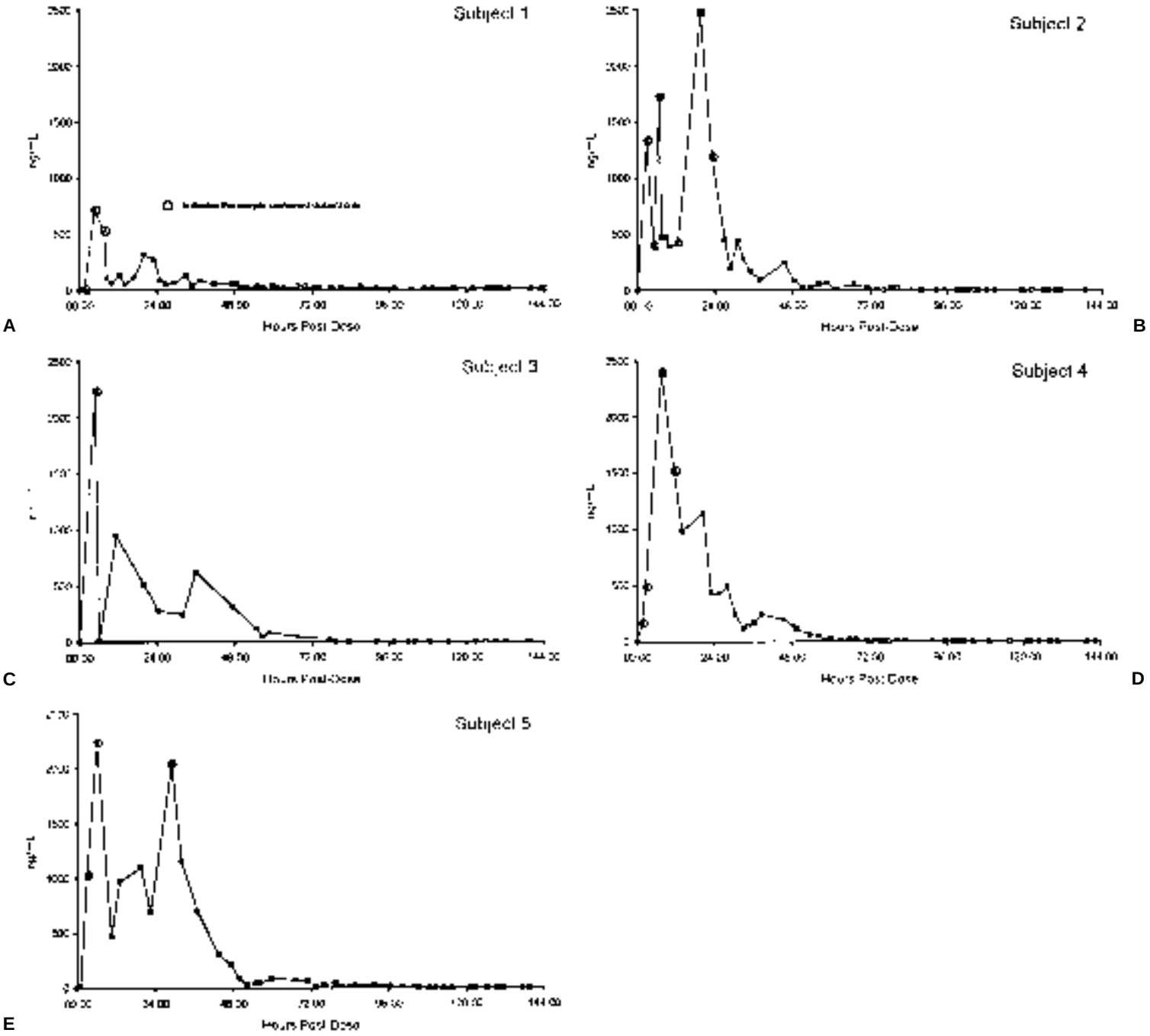

FIG. 5—*Amphetamine excretion profiles.*

TABLE 1—*Summary of amphetamine and clobenzorex detection.*

Subject	No. of Amp Positives	No. of Clobenzorex Detected	Amp First Detected (≥5 ng/mL) (hours postdose)	Amp First Positive (≥500 ng/mL) (hours postdose)	Amp Last Positive (≥500 ng/mL) (hours postdose)	Amp Last Detected (≥5 ng/mL) (hours postdose)	Maximum Concentration Amp (ng/mL)
1	2	3	4:30	4:30	7:30	116:00	715
2	4	6	2:40	2:40	23:00	95:00	2474
3	4	1	5:00	5:00	35:59	77:30	2233
4	4	4	1:30	7:30	20:30	91:30	2399
5	8	3	1:00	3:00	37:00	104:00	2232

detect clobenzorex in a study by Franceschini et al. (5). Our laboratory evaluated a solid phase extraction procedure using C18 cartridges and several attempts were made using either methamphetamine-D_{11} or fenfluramine as internal standards for the of quantitation of clobenzorex. These results proved to be unacceptable due to poor reproducibility of the quantitative values (>20% of target levels). In addition, benzphetamine was evaluated as an internal standard but it too proved unsatisfactory for accurate quantitation. Therefore, clobenzorex is reported as either detected or not detected. Clobenzorex was shown to elute after methamphetamine (relative retention time, 1.58 on HP-1, 1.66 on DB-17). See Fig. 2. Enantiomeric composition of the amphetamine produced showed the presence of *d*-enantiomer only (see Fig. 4). Individual subject excretion profiles for amphetamine and detection of clobenzorex are shown in Fig. 5.

The first detectable level of amphetamine (LOD = 5 ng/mL) ranged from 1:00 to 5:00 h post dose. A summary of amphetamine and clobenzorex containing samples is given in Table 1. The number of amphetamine positives (≥500 ng/mL; the Health and Human Services and Department of Defense administrative cutoff level for amphetamine in urine) ranged from two to eight for the five subjects and time of first positive ranged from 2:40 to 7:30 h postdose. The last positive amphetamine (≥500 ng/mL) sample was seen as soon as 7:30 and as long as 37:00 h post dose. Amphetamine was last detected from 77:30 up to 116:00 h postdose. In four of the subjects, amphetamine concentrations greater than 500 ng/mL could be seen for more than 20 h. One subject had only two samples, collected within the first 8 h after administration, that contained more than 500 ng/mL of amphetamine (Subject 1). Peak concentrations of amphetamine were seen in samples collected 4:30 to 19:30 h postdose where the concentrations reached 715 to 2474 ng/mL. As seen from this range, the peak concentration of amphetamine varied considerably between subjects. Subject 1 demonstrated the lowest peak concentration of 715 ng/mL. The other four subjects' were much more consistent where peak values ranged from 2232 to 2474 ng/mL. Evaluation of the creatinine, specific gravity and pH show subject one to have more dilute urine than the other subjects. This leads to the conclusion that the difference in concentrations is the result of more dilute urine rather than significant differences in metabolism. This is further supported by the fact that the percentage of clobenzorex metabolized to amphetamine was 6.6 in Subject 1 and the range for all subjects was 5.9 to 15.4%.

Although quantitative values for clobenzorex were inconsistent, the parent compound could easily be detected to 1 ng/mL with high signal-to-noise ratios for all ions except *m/z* 91 (see Fig. 6). Clobenzorex was detected (RT ± 2%, ion ratios, ±20%, s/n > 3) in urine samples for up to the first 7 to 29 h postdose. It was not detected in all samples positive for amphetamine, nor was it detected in all samples within the first 24 h. In one subject, clobenzorex was detected in 4 of 4 (Subject 2) amphetamine positive samples and in another subject 3 of 8 amphetamine positive samples (Subject 5) showed the presence of clobenzorex. Although not detected in some samples with high levels of amphetamine, clobenzorex was detected in a sample that had no detectable amphetamine at 1:30 h post dose.

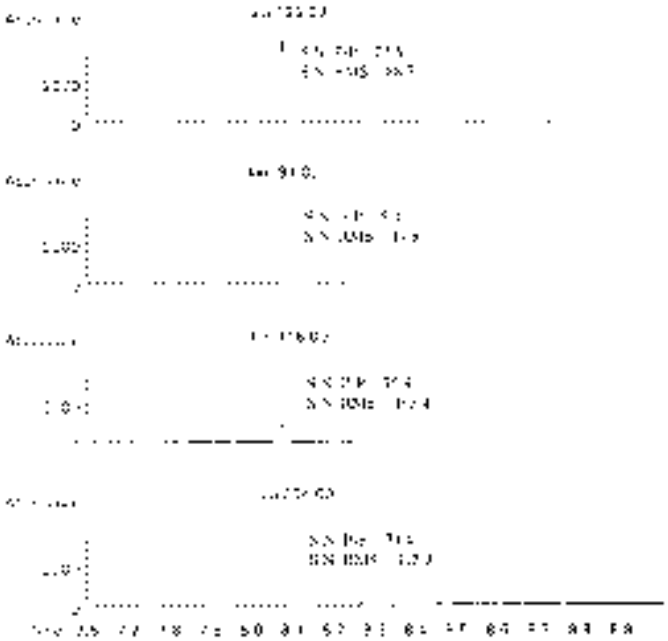

FIG. 6—*Ion chromatograms for 1 ng/mL clobenzorex showing typical signal-to-noise ratios. P-P = peak to peak, RMS = root mean square.*

Conclusion

Drugs, such as clobenzorex, that are metabolized to amphetamine are potentially significant concerns in the interpretation of amphetamine positive drug testing results. Interpretation of analytical results can be helpful in determining the possibility of involvement of this drug. Based on this study, a single dose of the drug can result in detectable levels of amphetamine in urine for up to 116 h post dose. Peak concentrations reached as high as 2474 ng/mL for amphetamine. Because clobenzorex is metabolized to amphetamine only, samples which contain methamphetamine would be inconsistent with clobenzorex use. Enantiomeric composition of the amphetamine derived from clobenzorex in this study showed the presence of only the *d*-enantiomer. Samples which contain the *l*-enantiomer would, therefore, not be consistent with clobenzorex use. This differs from previous literature which indicates clobenzorex to be the racemic N-orthochlorobenzyl derivative of amphetamine (6). This single fact has a significant impact on the interpretation of drug testing results. Given the previous report, an individual accused of illicit use of amphetamine whose analytical results indicated the presence of *d*-amphetamine without substantial amounts of *l*-amphetamine would have reasonably been judged as not being consistent with the use of clobenzorex.

Evaluation of assay characteristics using a minor modification of an existing amphetamine procedure proved to be viable for detection of this compound. Although quantitation of the parent drug was not accomplished as part of this study, the enantiomeric composition, peak levels and presence of the parent drug in urine samples were clearly established. Further work is necessary to develop a reliable method to accurately quantitate clobenzorex and to study any possible contribution of its metabolites to the interpretation of analytical results.

Acknowledgments

Many thanks to Donna Hensley, Sue Worthy and Sally Banfield for assistance in the processing and analysis of samples. Thanks to Maria Antonia Martinez Gonzalez for arranging for the clobenzorex used as standard material in this study. Thanks also to WHMC clinical laboratory personnel for creatinine measurements.

References

1. Tarver JA. Amphetamine-positive drug screens from use of clobenzorex hydrochlorate. J Anal Toxicol 1994;18:183.
2. de la Torre R, Badia R, Gonzalez G, Garcia M, Pretel MJ, Farre M, et al. Cross-reactivity of stimulants found in sports drug testing by two fluorescence polarization immunoassays. J Anal Toxicol 1996;20:165–70.
3. Maurer HH, Kraemer T, Ledvinka O, Schmitt CJ, Weber AA. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) in toxicological analysis. Studies on the detection of clobenzorex and its metabolites within a systematic toxicological analysis procedure by GC-MS and by immunoassay and studies on the detection of α and β-amantin in urine by atmospheric pressure ionization electrospray LC-MS. J Chromatog B, 1997;689:81–9.
4. Valtier S, Cody JT. Evaluation of internal standards for the analysis of amphetamine and methamphetamine. J Anal Toxicol 1995;19: 375–80.
5. Franceschini A, Duthel JM, Vallon JJ. Use of gas chromatography-mass spectrometry for urinary sympathomimetic amine detection in anti-doping control. J Chromatog 1991;541:109–20.
6. Harris LS. The stimulants and hallucinogens under consideration: a brief overview of their chemistry and pharmacology. Drug and Alcohol Dependence 1986;17:107–18.

Additional information and reprint requests:
Sandra Valtier, B.S.
Clinical Research Squadron
1255 Wilford Hall Loop
Lackland AFB Texas 78236-5319