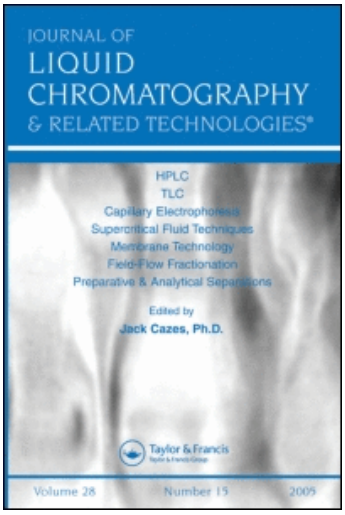

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Use of High Pressure Liquid Chromatography and Thin Layer Chromatography for the Separation and Detection of Testosterone and its Metabolites from in vitro Incubation Mixtures

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USE OF HIGH PRESSURE LIQUID CHROMATOGRAPHY AND THIN LAYER CHROMATOGRAPHY FOR THE SEPARATION AND DETECTION OF TESTOSTERONE AND ITS METABOLITES FROM IN VITRO INCUBATION MIXTURES[1]

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ABSTRACT

Testosterone and its 6β-, 7α-, and 16α-hydroxylated metabolites were resolved by high pressure liquid chromatography and thin layer chromatography. Separation by HPLC was achieved in less than 45 min on a microparticulate silica gel column using isocratic elution with isopropanol:tetrahydrofuran:hexane (5:15:80) as the mobile phase. TLC systems utilizing silica gel on glass and plastic plates, and polysilicic acid on glass fiber sheets are presented. The monohydroxylated metabolites of testosterone formed during incubation of (^{14}C)-testosterone with liver postmitochondrial preparations from adult male rats pre-treated with phenobarbital or Aroclor 1254 were separated and quantitated by both HPLC and TLC. The results using both techniques are compared with those obtained by paper chromatography.

INTRODUCTION

The metabolism of testosterone (T) by liver microsomal mixed-function oxidases has been followed by measuring the formation of 6β-, 7α-, and 16α-hydroxytestosterone (1,2). Studies by Conney and co-workers

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(3-6) have demonstrated that hydroxylation at each of these positions is catalyzed by a different cytochrome P-450 and that the levels of each activity can be selectively altered by compounds which affect liver microsomal drug metabolism. By using T an investigator can study the action of three different hemeproteins on one substrate (5,6).

The standard method for measuring the hydroxylated metabolites of T employs descending paper chromatography (1,2). Basically, the method involves pre-equilibration of filter paper with lower phase of the solvent mixture after spotting the sample. The chromatogram is then developed with the upper phase by descending chromatography for 24-48 hours during which time, T runs off the end of the sheet leaving only the polar metabolites. The chromatograms are then dried and cut into 1 cm pieces and the distribution of radioactivity measured by scintillation counting.

Although high pressure liquid chromatography has been applied in the separation of steroids (7) and bile acids (8,9), only one such method (9) has been reported for the specific separation and detection of the hydroxylated metabolites of bile acids. One HPLC technique using reversed-phase methodology has been reported for the separation of some polar metabolites of T (10). Previously-reported thin-layer chromatographic (TLC) methods require multiple developments to adequately separate the 7α- and 16α-hydroxylated metabolites (11,12).

This paper reports HPLC and TLC methods for the separation of androstene-dione, testosterone, and the 6β-, 7α-, and 16α-hydroxytestosterones. Another TLC system is also described which can be used to separate the non-polar metabolites from testosterone and its hydroxylated metabolites. In contrast to previously reported methods, our TLC systems require only one development. These two chromatographic techniques have been successfully applied to the separation and detection of major polar metabolites of testosterone produced <u>in vitro</u> by incubation of testosterone

with liver postmitochondrial fractions from adult male Sprague-Dawley rats pretreated with either phenobarbital or Aroclor 1254. HPLC and TLC results are compared with those obtained by conventional paper chromatography.

MATERIALS AND METHODS

Reference Standards and Reagents - (^{14}C)-Testosterone was obtained from Amersham Searle Co., (Chicago, IL), and used without further purification; testosterone, 16α-, and 6β-hydroxytestosterones were from Steraloids Inc., (Wilton, NH); 7α-hydroxytestosterone was obtained from the Steroid Reference Bank of the Medical Research Council in Great Britain. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 5α-Androstan-3,17-dione, 5α-Androstan-17β-ol-3-one, 5α-Androstan-3α-ol-17-one, Δ^4-Androsten-3,17-dione, and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). Phenobarbital was obtained from Sterling-Winthrop (Phila., PA) and Aroclor 1254 (a commercial mixture of polychlorinated biphenyls) from Analabs (New Haven, CN). All other reagents were of the highest purity commercially available.

Preparation of Microsomes and Testosterone Metabolism - The 9000 x g post-mitochondrial supernatant (S9) fractions were prepared from 25% homogenates of livers from adult male rats (Sprague-Dawley strain, 250 g or greater) obtained from the NIH animal colony. The microsomal enzymes were induced by treating the animals with intraperitoneal injections of either PB (100 mg/kg/day for 3 days) or AC (500 mg/kg once 5 days before being euthanized). Protein concentrations were determined by the method of Lowry et al. (13).

Testosterone metabolism was determined essentially by the method of Conney and Klutch (14). The following reaction components were contained in a total volume of 1 ml: 100 μmole potassium phosphate, pH 7.5, 3 μmoles $MgCl_2$, 0.5 μmole NADPH, 1 unit glucose-6-phosphate dehydrogenase,

5 μmoles glucose-6-phosphate, 125 nmoles (^{14}C)-testosterone (1 μCi) and 1.25-5 mg liver S9. The reactions were started by the addition of substrate, incubated for 5 min at 37°C, and stopped by the addition of 10 ml methylene chloride. After vortexing for 30 sec and centrifuging to separate the phases, five μl aliquots of the CH_2Cl_2 were counted to calculate recovery (average recovery was 96%) and then 9 ml were taken to dryness under nitrogen. The residue was dissolved in 2 ml methanol, divided in half and each 1 ml portion taken to dryness again. One half of the sample was dissolved in 200 μl methanol and 100 μl aliquot analyzed using the paper chromatographic method (1), and the remainder separated by TLC as described below. The other half of the sample was dissolved in 100 μl CH_2Cl_2 and 25 μl subjected to HPLC analysis.

High Pressure Liquid Chromatography. A modular HPLC system was used for this study. The unit consisted of a Laboratory Data Control Constametric II pump (Riviera Beach, FL), a Chromatronix dual-channel UV absorbance detector (Spectra-Physics, Santa Clara, CA), a high-pressure sixport sampling valve fitted with a Glenco (Houston, TX) Model-VIS valve injection syringe, and a Fisher Recordall R Series 5000 recorder (Fisher Scientific, Silver Spring, MD) operated at 0.2-in/min chart speed and a 10 mV setting.

A 25 cm x 4.6 mm, 10 μm Partisil-10 column (Whatman, Inc., Clifton, NJ) operated at ambient temperature was used to separate testosterone metabolite standards, or actual metabolites which were eluted with HEX/THF/IPA (80:15:5) at a flow rate of 1 ml/min. A precolumn (4 cm x 2.1 mm) packed with Waters Associates pellicular Corasil (37-50 μm), was used to ensure the stability of the analytical column. Peak areas and retention times were determined with a Hewlett-Packard (Avondale, PA) 3352A laboratory data system linked through a Hewlett-Packard 1865 A/D

Converter to the UV detector output of the liquid chromatograph. When the metabolism extracts were analyzed by HPLC, 1 ml fractions were collected in scintillation counting vials and 10 ml PCS cocktail (Amersham-Searle, Arlington Heights, ILL) added.

Thin-layer chromatography - Standard rectangular glass tanks were used for plate development. A viewing cabinet with long-wavelength (366 nm) and short-wavelength (254 nm) UV lamps (Brinkman, Westbury, N.Y.) was used to locate the spots on the plate. Three different analytical plates were used, silica gel 60 (E. Merck, Elmsford, N.Y.) Baker flex silica gel 1B-F (J.T. Baker Chem. Co., Phillipsburg, N.J.) and polysilicic acid gel-impregnated glass fiber sheets ITLC-SA (Gelman Instrument Co., Ann Arbor, MI). After development and drying, plates were sprayed with 50% methanol-sulphuric acid and heated in the oven at 110°C for 5 min.

As mentioned earlier, after the aliquot of the extract was used for PC analysis, the remainder of the extract was separated on TLC using the Gelman ITLC-SA sheets. A standard mixture of AD, T, 6β-, 7α-, and 16α-H-T were chromatographed in lanes on the outer perimeter of the chromatograms. Individual sheets were developed with solvent system 1 (ethyl ether: ethyl acetate, 9:2, v/v). After the solvent front had migrated about 18 cm, the sheets were removed from the tank, dried in air and marked at 1 cm intervals starting at the bottom of the sheet. The individual sample and standard lanes were separated and the latter sprayed with 50% methanol-sulfuric acid and heated at 110°C for 5 min in order to locate the metabolite standards. The sample lanes were cut into 1 cm sections and placed into scintillation vials containing 10 ml PCS scintillation cocktail.

Radioactivity measurements - All samples, whether they were 1 cm sections of paper chromatograms, glass-fiber sheets or 1 ml HPLC fractions, were quantitated for their levels of (^{14}C)-metabolites using

an Isocap/300 Liquid Scintillation System (Nuclear-Chicago, Des Plains, ILL). Samples were counted against an external standard to correct for quenching. Counting efficiency with all these samples was usually 80%.

RESULTS

Fig. 1 shows a typical HPLC chromatogram of the separation of a mixture of the compounds being studied. Retention times, peak areas and peak shapes were highly reproducible under the HPLC conditions used

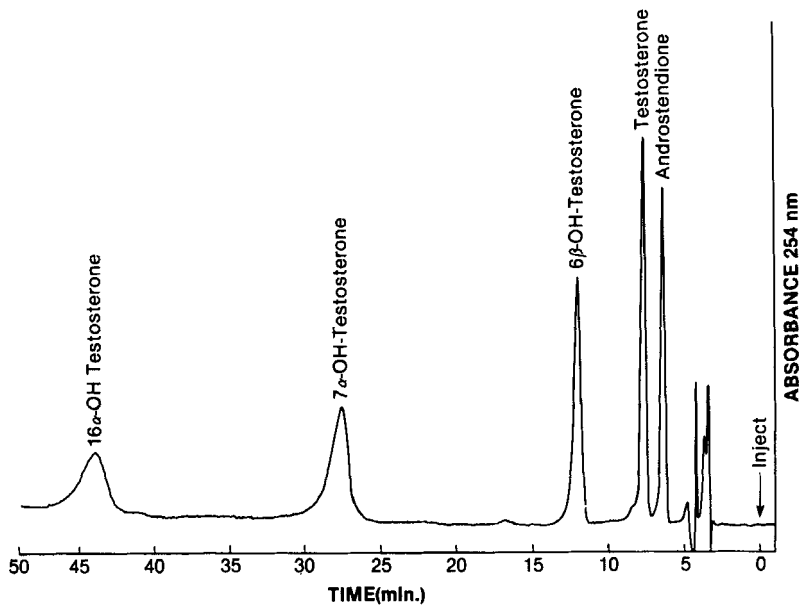

Figure 1 HPLC profile of mixed standards. Approximately 1 µg each of AD, T, 6β-HT, 7α-HT and 16α-HT were separated on a microparticulate silica gel column using isocratic elution with HEX/THF/IPA (80:15:5). UV-absorbance was monitored at 254 nm with full scale sensitivity at 0.04.

(see Materials and Methods) and the average retention times (min) ± S.D. of individual compounds over 3 days for 12 replicate injections were as follows: 6.4 ± .05 (AD), 7.7 ± .05 (T), 12 ± .12 (6β-HT), 27.2 ± .41 (7α-HT), and 42.2 ± .61 (16α-HT). Standard calibration curves (Fig. 2) covering the range of concentrations from 0.125 to 5 μg was linear.

Table 1 shows the separation of T and seven of its metabolites by TLC. Four of these compounds (1 thru 4) were less polar while the other four (6 thru 8) were more polar than T. The table is divided into three parts depending on the type of plate used: Silica gel on glass sheets,

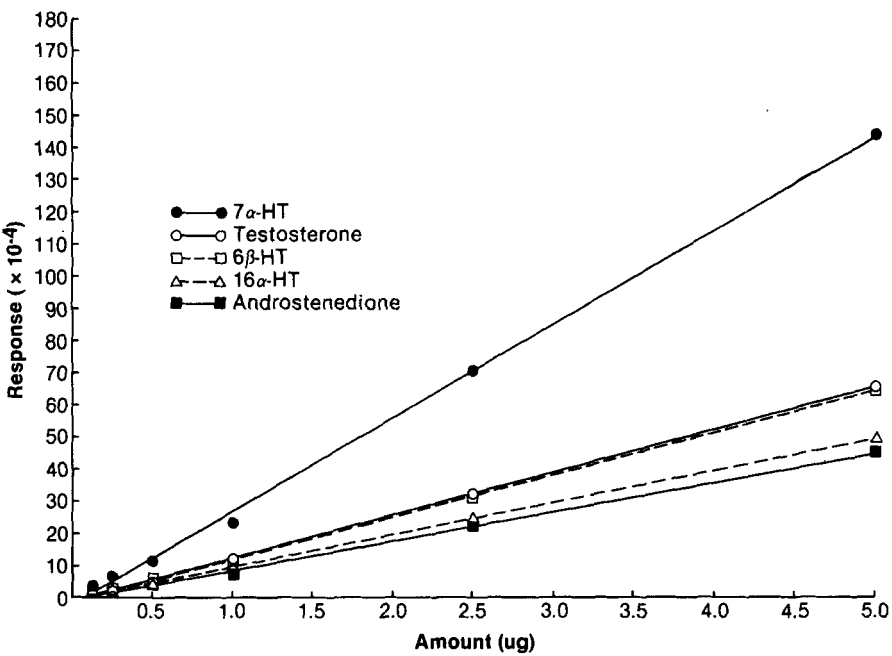

Figure 2 Calibration curve for testosterone and metabolite standards. Peak area counts in mV (response) as a function of varying amounts of T and metabolites. HPLC conditions same as in Figure 1.

TABLE 1. Thin Layer Chromatographic Separation of Testosterone and its Metabolites

#	Compound	Color	Rf x 100 SG 1	SG 2	BF 1	BF 2	ITLC-SA 1	ITLC-SA 2	ITLC-SA 3*
1	5α-Androstan-3,17-dione	blue	82	74	84	71	86	74	60
2	5α-Androstan-17β-ol-3-one	blue	70	62	78	63	80	74	53
3	5α-Androstan-3α-ol-17-one	brown	72	57	78	63	83	80	57
4	Δ^4-Androsten-3,17-dione	light blue	67	53	71	54	76	70	43
5	Δ^4-Androsten-17β-ol-3-one (Testosterone)	yellow green	58	45	64	45	69	58	38
6	Δ^4-Androsten-6β,17β-diol-3-one (6β-HT)	blue	39	23	45	24	48	35	10
7	Δ^4-Androsten-7α,17β-diol-3-one (7α-HT)	brown	17	7	21	8	22	11	0
8	Δ^4-Androsten-16β,17β-diol-3-one (16α-HT)	blue green	11	3	13	6	13	9	0

1 Ethyl Ether:Ethyl Acetate (9:2)

2 Ethyl Ether:Cyclohexane (9:1)

3 Ethyl Ether:Cyclohexane (9:5)

* Solvent system # 3 is primarily used to separate the polar metabolites.

silica gel on plastic sheets and polysilicic acid on glass fiber sheets. The three backings were selected to meet the needs of various analysts. Glass sheets were easier to scan; plastic and fiber sheets were easier to cut when they are to be placed in a vial for counting the labeled components. The plastic sheets gave better reproducibility than the glass fiber sheets, however the glass plates gave the best reproducibility.

Each type of plate was developed in either of two solvent systems (1 and 2) in order to separate the two classes of metabolites, polar and non-polar, respectively. Using silica gel on glass plates, all of these components were separated with these solvent systems, but a third solvent system ethyl acetate:cyclohexane (9:5) was required for the separation of the non-polar mixture on glass fiber sheets.

These methods were subsequently applied to the separation of hydroxylated testosterone metabolites produced by incubation of (^{14}C)-testosterone with liver S9-fractions from rats pretreated with PB or AC. These results are listed in Table 2. All three methods show reasonably good agreement, especially at the low protein concentration as shown in Table 2. Besides these three major metabolites, several other unidentified polar metabolites were observed with the HPLC assay both by UV-absorbance and by the appearance of new radioactive peaks (see Fig. 3). These unknown metabolites were observed between 6β-H-T and the 7α-H-T and others migrated throughout the region between 7α- and the 16α-H-T's. The appearance of these metabolites was not totally unexpected since several other potential metabolites (2,11,15) would be expected to migrate in chromatography systems that separate the polar metabolites of T.

DISCUSSION

This paper describes HPLC and TLC methods for the separation and detection of metabolites of T. The HPLC and TLC results were comparable

TABLE 2. Comparison of Paper Chromatographic, TLC and HPLC Analysis of Testosterone Metabolism

MG S9 added	NMOLES HYDROXYLATED METABOLITE FORMED/5 MIN								
	16α-hydroxy-T			7α-hydroxy-T			6β-hydroxy-T		
	PC	TLC	HPLC	PC	TLC	HPLC	PC	TLC	HPLC
1.25 AC-S9	0.43 ±0.07	0.50 ±0.10	0.40 ±0.08	0.43 ±0.13	0.43 ±0.02	0.48 ±0.07	3.51 ±0.87	5.25 ±0.28	4.83 ±0.43
2.50 AC-S9	0.96 ±0.12	1.05 ±0.29	1.25	1.18 ±0.11	1.07 ±0.13	1.54	7.89 ±0.05	9.12 ±0.74	10.32
3.75 AC-S9	1.20	2.12 ±0.23	1.74	1.47	1.49 ±0.24	1.63	11.76	14.75 ±1.30	13.69
5.00 AC-S9	1.60 ±0.19	2.44 ±0.16	— —	1.69 ±0.29	1.89 ±0.13	— —	13.64 ±1.14	18.69 ±0.32	— —
1.25 PB-S9	0.68 ±0.07	1.10 ±0.01	0.76 ±0.09	0.26 ±0.01	0.43 ±0.09	0.31 ±0.04	3.57 ±0.04	5.04 ±0.21	4.22 ±0.28
2.50 PB-S9	1.43 ±0.13	1.64 ±0.24	1.76	0.65 ±0.04	0.75 ±0.17	0.96	7.10 ±0.04	8.80 ±1.08	7.38
3.75 PB-S9	2.24	2.19 ±0.54	2.19	0.70	1.64 ±0.45	1.49	10.99	14.00 ±1.16	12.30
5.00 PB-S9	2.41 ±0.33	2.75 ±0.10	2.95	1.03 ±0.01	1.93 ±0.07	2.00	12.64 ±0.55	18.42 ±0.57	13.51

Extracts from duplicate incubations mixtures were analyzed individually or pooled using each of the methods as described in the text. The duplicates of the 1.25 mg S9 samples were run with each method, while only one of those assays mixtures containing higher amounts of protein were run on HPLC. Duplicates assay mixtures were analyzed for all the TLC and PC points except that only one 3.75 mg S9 sample was run on PC. Results are presented as the means ± S.D. where appropriate.

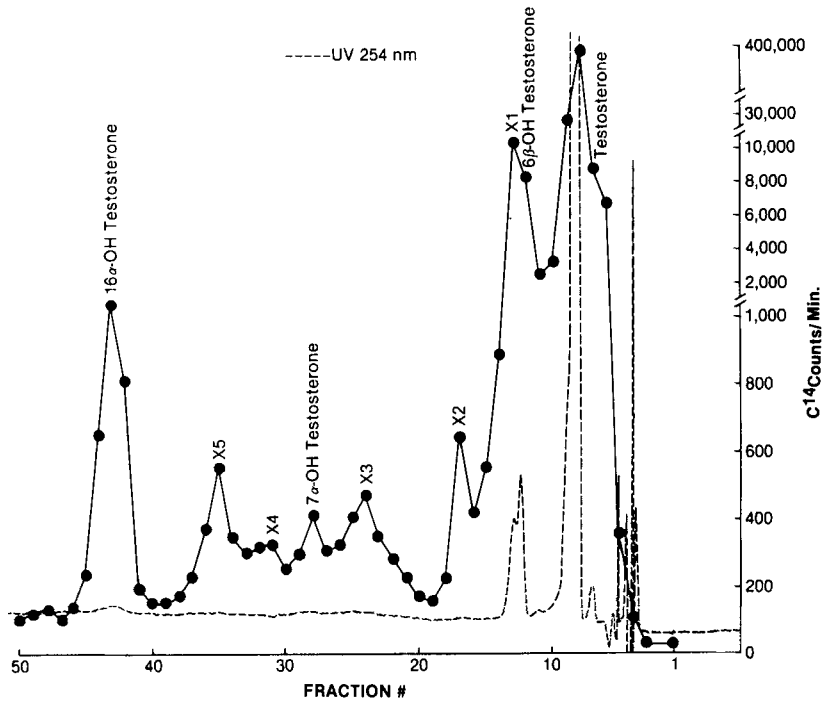

Figure 3 HPLC profile of a typical metabolism extract. One-half of the total lipid extracts from incubation mixtures, containing 1.25 mg PB-S9, were dissolved in 100 µl CH_2Cl_2 and 25 µl applied to the HPLC column. Fifty fractions containing 1 ml column effluent were collected and the levels of (^{14}C)-labelled metabolites quantitated as described in the text. The position of known compounds are indicated as such; unknown metabolites are identified as X_1, etc.

with those obtained using conventional paper chromatography. Both the HPLC and TLC methods resolved T and its polar metabolites. The TLC methods also separated the non-polar metabolites of T. Use of the Gelman

ITLC-SA sheets allowed a quantitative analysis of the radioactive metabolites since the entire sheet can be cut up and the complete distribution of radioactive metabolites determined.

To demonstrate the feasibility of these techniques, both the HPLC and TLC systems were used to separate and quantitate the levels of polar hydroxylated metabolites of T generated in in vitro incubations containing liver S9-fractions from PB- or AC-treated rats. Even though total lipid extracts were used, the contaminating lipids did not interfere with the detection or elution of the compounds of interest in either system.

The application of the HPLC method to the separation of the major hydroxylated metabolites of testosterone is clearly demonstrated in chromatogram shown in Fig. 3. The radioactivity located in HPLC fractions corresponding to those of known metabolites, supported the validity and sensitivity of the method. The selection of (80:15:5) HEX:THF:IPA as the HPLC mobile phase afforded a useful degree of selectivity in metabolite separation, since additional (unidentified) metabolites were resolved along with the expected metabolites of T, namely, 6β-, 7α-, and 16α-hydroxytestosterone (see Fig. 3). The unidentified metabolites noted in the present study represent the possible formation of other hydroxylated metabolites of T (e.g., 2β-hydroxytestosterone (2,11,15), but further experiments would be necessary to establish their identities.

Some of the advantages of the TLC procedure are its ability to separate both the polar and non-polar metabolites of T, to handle large numbers of samples in a relatively short time, and to detect some of the non-UV absorbing metabolites which are not seen by HPLC.

The results obtained from a comparison of the PC, TLC, and HPLC methods show relatively good consistency. Although HPLC and TLC give slightly higher values than that obtained by PC, the results are well

within a reasonable margin of error, even at high concentrations of protein. The fact that the TLC procedure can be performed with such ease and speed will greatly facilitate use of this substrate for studying drug metabolism. That other, as yet unidentified, metabolites were separated using the HPLC procedure indicated that this system can be used to isolate essentially all of the polar metabolites of testosterone. Hence, the availability of both TLC and HPLC procedures provides the researcher with powerful tools to analyze for polar and non-polar metabolites of testosterone.

LIST OF ABBREVIATIONS

T-	Testosterone (Δ^4-Androsten-17β-ol-3-one)
6β-HT-	6β-hydroxytestosterone (Δ^4-Androsten-6β,17β-diol-3-one)
7α-HT-	7α-hydroxytestosterone (Δ^4-Androsten-7α,17β-diol-3-one)
16α-HT-	16α-hydroxytestosterone (Δ^4-Androsten-16α,17β-diol-3-one)
AD-	Androstenedione (Δ^4-Androsten-3,17-dione)
PB-	Phenobarbital
AC-	Aroclor 1254 (a commercial mixture of polychlorinated biphenyls)
S9-	9000 x g postmitochondrial supernatant
HPLC-	High pressure liquid chromatography
PC-	Paper chromatography
HEX-	Hexane
THF-	Tetrahydrofuran
IPA-	Isopropanol
TLC-	Thin layer chromatography
SG-	Silica Gel 60
BF-	Bakerflex silica gel
ITLC-SA-	Polysilicic acid gel impregnated glass fiber sheets

REFERENCES

1. A.H. Conney and K. Schneideman, J. Pharmac. Exp. Ther. 146, 225 (1964).
2. M. Jacobson, W. Levin and R. Kuntzman, Biochem. Pharmacol. 18, 2253 (1969).
3. A.H. Conney, W. Levin, M. Ideda, R. Kuntzman, D.Y. Cooper and O. Rosenthal, J. Biol. Chem. 243, 3912 (1968).
4. A.H. Conney, W. Levin, M. Jacobson and R. Kuntzman, In: Microsomes and Drug Oxidations. J.R. Gillette, A.H. Conney, G.J. Casmides, R.W. Easterbrook, J.R. Fouts and G.J. Mannering, Eds., 279-295, Acad. Press (1969), New York, NY.
5. P.E. Thomas, A.Y.H. Lu, D. Ryan, S.B. West, J. Kawalek and W. Levin, Mol. Pharmacol. 12, 764 (1976).
6. P.E. Thomas, A.Y.H. Lu, S.B. West, D. Ryan, G.T. Miwa and W. Levin, Mol. Pharmacol. 13, 819 (1977).
7. P.G. Satyaswaroop, E. Lopez de la Osa and E. Gurpide, Steroids 30, 139 (1977).
8. R. Shaw and W.H. Elliott, Anal. Biochem. 74, 273 (1976).
9. B. Shaikh, N.J. Pontzer, J.E. Molina and M.I. Kelsey, Anal. Biochem. 85, 47 (1978).
10. M.J. O'Hare, E.C. Nice, R. Magee-Brown and H. Bullman, J. Chromat. 125, 357 (1976).
11. B.P. Lisboa, J.A. Gustofsson and J. Sjoval, Eur. J. Biochem. 4, 496 (1968).
12. K.T. Shiverick and A.H. Neims, Fed. Proc. 37, 304 (1978).
13. A.H. Conney and A. Klutch, J. Biol. Chem. 238, 1611 (1963).
14. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193, 265 (1951).
15. J.A. Gustofsson and N. Ingelman-Sundberg, J. Biol. Chem. 250, 3451 (1975).