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Screening of some Dope Agents by Immunological Methods

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INTRODUCTION

A positive result for a sample consists of the demonstration of the presence of the suspected drug or its metabolites. To achieve this result, well known criteria of analytical chemistry should be used in terms of sensitivity, repeatability, reproducibility, accuracy, recovery, and linearity or systematic error. In doping control, additionally, the metabolism and pharmacokinetics of banned drugs must be considered.

Current methodology to detect presumptively banned substances includes chromatographic methods (GC, HPLC). The confirmation of presumptive positive cases involves the use of chromatographic approaches coupled with mass spectrometry (GC/MS and LC/MS). The principal difference of this analytical setup as compared with other related fields in clinical toxicology (i.e. drugs of abuse testing, horse testing...) is the low incidence of immunological techniques for the presumptive screening of drugs. One of the main reasons for such situation is the large menu of substances to be covered (more than 100) as compared with the drugs of abuse field (i.e. NIDA Five). To cope such a large menu solely with immunological techniques is until today nearly impossible. The inability to cover the full range of substances and the low level of specificity have been some of the criticisms of these kind of techniques. In some cases such analytical techniques can be an extremely useful tool if used judiciously (i.e. methods with low substance specificity but high group specificity). Some compounds, specially peptide hormones, can only be detected by using immunological techniques (1,2). Some of the characteristics of these techniques that can be considered as advantageous in doping control are rapid turnaround time, low cost, high sensitivity (if required), and inexpensive equipment.

IMMUNOLOGICAL METHODS IN DOPING CONTROL IN MAJOR SPORT EVENTS

In some previous Olympic Games and other major sport events, the laboratory has already included the use of immunological techniques for the analysis of cocaine, cannabinoids, amphetamines, benzodiazepines and barbiturates (3,4,5) and some peptide hormones like ß-hCG (5). The main reasons why these techniques have been used are listed below:

1.- They allow the screening of groups of substances with a high chemical homology (i.e. **phenylalkylamine derivatives**, ß-blockers, ß-agonists, anabolic steroids, corticosteroids, **opiates, barbiturates, benzodiazepines** ...) with a very fast turnaround time. Results may be available in the time required for sample preparation for chromatographic analysis. Then presumptive positive cases can be analyzed with priority.

2.- Substances requiring a dedicated chromatographic screening (i.e. **cannabinoids, cocaine**) can be screened by highly specific immunological methods. Only those presumptive positive cases may then be analyzed chromatographically.

3.- There are some substances where immunological methods are the analytical technique of choice (i.e. peptide hormones as **ß-hCG**).

USEFULNESS OF IMMUNOLOGICAL METHODS WITH HIGH GROUP SPECIFICITY IN DOPING CONTROL

Some immunological methods designed for the detection of groups of substances (i.e. amphetamine like compounds), have a higher degree of cross-reactivity with structurally similar compounds, as compared with those immunological methods highly specific for a particular substance (i.e. cocaine). In doping this is not a drawback because a subsequent confirmation will determine the actual identity of the detected compound. The absence of a high substance specificity, but the maintenance of a high group specificity, may become an important advantage in presumptive detection in doping control.

Previous studies (6,7) demonstrated that an enzymeimmunoassay with polyclonal antibodies designed for the detection of amphetamine and methamphetamine can detect a number of related substances (see Table 1) of interest in doping control. Most of these substances are phenylalkyllamine derivatives. In this particular test, the specificity can be increased by using an additional chemical reaction: the pretreatment of samples with alkaline periodate eliminates the cross-reaction of α-hydroxy-phenyl ethylamines (i.e. ephedrine derivatives).
This demonstrates the fact that chemical reactions can be combined with immunological techniques for the modulation of their specificity. Another finding of interest was that some substances with no cross-reactivity with the test (as publicized by the manufacturer), can give positive results. The metabolism of these substances (i.e. amphepramone) in the body gives rise to urinary metabolites with a high cross-reactivity. These metabolites are responsible for these "unexpected" results.

THE CONCEPT OF BIOLOGICAL CROSS-REACTIVITY

As stated above, preliminary results with the enzymeimmunoassay test showed that there is a large difference between studies on cross-reactivity done by manufacturers in urines spiked with the parent compound and those results obtained when a urine from a healthy subject administered with the same substance is analyzed. This finding gives rise to a more practical concept for drug testing which is the **biological cross-reactivity for a given substance**. The biological cross-reactivity refers to the ability of a given immunological test of detecting a particular substance in a biological fluid after its administration in humans. Manufacturers do not do cross-reactivity studies with metabolized urines because this gives results that are less predictable than the studies of cross-reactivity with spiked urines. There are many factors that influence the final excretion for a particular substance, like the dose administered, interindividual variations in clearance, sample time collection.... Nevertheless if cross-reactivity studies for doping control are done with metabolites in the urine a better picture for a particular immunological test is obtained.

BIOLOGICAL CROSS-REACTIVITY OF AMPHETAMINES AND OPIATES BY FLUORESCENCE POLARIZATION IMMUNOASSAY TESTS (FPIA).

Amphetamines

Two FPIA techniques for the detection of amphetamines (Amphetamine Class and Amphetamine-Methamphetamine tests) were selected to conduct the present study. Table 2 shows the wide differences in cross-reactivity (spiked urines) between antibodies used in both techniques. Urine samples analyzed were from healthy volunteers administered with doses suggested by the IOC Medical Commission for the generation of reference urines. Urinary collection periods for each substance are indicated in tables and figures. Tables 3 and 4 show differences between cross-reactivities reported by the manufacturer (spiked urines) and the results obtained after analyzing the corresponding excretion study for each assay and for some selected compounds. Those compounds detected by chromatographic techniques and, when available, their cross-reactivity are also listed for each compound. Table 5 lists the excretion studies evaluated and Figures 1 and 2 the results obtained. Table 6 lists substances detected or non detected by combining both tests. Table 7 lists those substances that can be only detected by one of the evaluated tests.

When reviewing substances reported by IOC accreditated laboratories in the last 4 years as positives belonging to the Class A: Stimulants, as much as a 95% of cases, could had been detectable by combining both immunological methods.

Opiates

A single FPIA technique designed for the detection of opiates has been used in the present evaluation. Table 8 shows excretion studies evaluated and Figure 3 shows the results obtained.

When reviewing substances reported by IOC accreditated laboratories in the last 4 years as positives belonging to the Class B: Narcotics, as much as a 98% of cases, could had been detectable by this immunological method.

CONCLUSIONS

In addition to those substances whose analysis is only amenable by immunological methods (i.e. peptide hormones) the following conclusions can be drawn in regards to the usefulness of these techniques in doping control:

a) Immunological techniques with broad cross-reactivities can be a useful tool for doping control.

b) The specificity of the results can be increased by combining immunological techniques with antibodies directed to structurally similar compounds but with different cross-reactivities.

c)Immunological techniques deserve further attention in the screening of samples to be analyzed by chromatographic techniques.

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List of Stimulants Given to Healthy Volunteers to Generate 24-hours Urines to be Studied by EMIT assay

Bold face indicates positive response in Syva EMIT d.a.u. Amphetamine assay

DRUG	DOSE (mg)
Amphetamine	**10**
Clorphentermine	**25**
Diethylpropion*	**50**
Dimethylamphetamine	**30**
Dimethylpropion*	**50**
Ephedrine	**50**
Etaphedrine	**50**
Fencamfamine	**30**
Fenfluramine*	**20**
Heptaminol* **	**200**
Isoetharine*	**20**
Isoprenaline*	**30**
Methoxyphenamine	**50**
Methamphetamine	**10**
Micoren (cropropamide + crotethamide)**	**400+400**
Nikethamide*	**250**
Norpseudoephedrine	**50**
Phendimetrazine	**30**
Phenmetrazine	**25**
Phentermine	**30**
Phenylephrine*	**25**
Phenylpropanolamine	**50**
Tranylcypromine*	**10**

* Not explicitly included in I.O.C. lists (revision April 1986).
** Not penylalkylamine (PhAA) structure.
# Other details of these studies may be found in references 6 and 7.

TABLE 1

PUBLICIZED AMPHETAMINE TESTS SPECIFICITY
FPIA Abbott Laboratories

SUBSTANCE	CONCENTRATION ADDED (μg/mL)	% CROSS REACTIVITY	CONCENTRATION ADDED (μg/mL)	% CROSS REACTIVITY
	AMPHETAMINE CLASS		AMPHETAMINE/METHAMPHETAMINE	
Benzphetamine	1	**24.0**	10	**n.d.**
Diethylpropion	10	**n.d.**	1000	**n.d.**
Ephedrine	1	**66.0**	3000	**n.d.**
Fenfluramine	10	**6.4**	10	**19.0**
Isoxsuprine	1	**12**	10	**n.d.**
Methamphetamine	1	**198.0**	1	**78.0**
3,4-Methylenedioxy-N-ethylamphetamine (MDE)	1	**21.0**	1	**54.0**
3,4-Methylenedioxy-methamphetamine (MDMA)	1	**28.0**	1	**99.0**
Mephentermine	10	**17.4**	10	**4.7**
Phenmetrazine	10	**8.2**	10	**1.0**
Phentermine	10	**19.5**	10	**44.0**
Phenylpropanolamine	1	**17.0**	1000	**n.d.**
Propylhexedrine	1	**93.0**	1	**34.0**
l-Pseudoephedrine	1	**12.0**	3000	**n.d.**

Substances were tested by adding a known quantity of the test compound to a drug-free urine (Ref. Intramural studies, Abbott Laboratories).
n.d. = none detected. Concentration less than the sensitivity of the assay (0.10 μg/mL).

TABLE 2.- Observed differences in tests specificity after analyzing some spiked urines.

AMPHETAMINE CLASS
FPIA

SUBSTANCE	STANDARD URINE (a)		GENERATED URINE (b)					STANDARD URINE (a)	
	CONCENTRATION ADDED (ng/mL)	% CROSS REACTIVITY	ROUTE OF ADMINISTRATION	DOSE GIVEN	URINE COLLECTION PERIOD	RESULTS (FPIA) (ng/mL)	OBSERVED SUBSTANCES (GC/NPD)	CONCENTRATION ADDED (ng/mL)	% CROSS REACTIVITY
Amfepramone	10000	n.d.	oral	75 mg	0-12 h	1769	Amfepramone	10000	n.d.
	100000	0.4			12-24 h	950	Diethylnorephedrine		?/+
					0-24 h	1395	Dinordiethylpropion		?
							N-Ethylnorephedrine		?/+
							Nordiethylpropion		?
							Norephedrine	1000	17.0
Etafedrine	1000	n.d.	oral	50 mg	0-8 h	770	Ephedrine	100	100
	10000	n.d.			8-24 h	1183	Etafedrine	1000	n.d.
					post 24 h	2139	N-Ethylnorephedrine		?/+
					0-24 h	1093	Norephedrine	1000	17.0
Morazone	1000	n.d.	oral	70 mg		615	Phenmetrazine	1000	15
	10000	n.d.					Norpseudoephedrine	10000	2.9

n.d. = none detected. Concentration less than the sensitivity of the assay (100 ng/mL).
a) Substances were tested by adding a known quantity of the test compound to a drug-free urine. Crossreactivities refered to IMIM and Abbott Laboratories studies.
b) Real urinary excretion studies.

TABLE 3.- Differences between results obtained after analyzing spiked urines and the corresponding excretion study.

AMPHETAMINE/METHAMPHETAMINE
FPIA

SUBSTANCE	STANDARD URINE (a)		GENERATED URINE (b)					STANDARD URINE (c)	
	CONCENTRATION ADDED (ng/mL)	% CROSS REACTIVITY	ROUTE OF ADMINISTRATION	DOSE GIVEN	URINE COLLECTION PERIOD	RESULTS (FPIA) (ng/mL)	OBSERVED SUBSTANCES (GC/NPD)	CONCENTRATION ADDED (ng/mL)	% CROSS REACTIVITY
Clobenzorex	1000	n.d.	oral	30 mg	0-8 h	3348	Amphetamine	300	110
	10000	n.d.			8-24 h	1010	Clobenzorex	10000	n.d.
					post 24 h	1890	Clobenzorex-M		?
					0-24 h	1271			
Fenproporex	1000	n.d.	oral	10 mg	0-8 h	1506	Amphetamine	300	110
	10000	n.d.			8-24 h	2874	Fenproporex	10000	n.d.
					0-24 h	1986			
Mefenorex	1000	n.d.	oral	40 mg	0-8 h	>8000	Amphetamine	300	110
	10000	n.d.			8-24 h	>8000	Mefenorex	10000	n.d.
					post 24 h	3591			
					0-24 h	>8000			

n.d. = none detected. Concentration less than the sensitivity of the assay (100 ng/mL).
a) Substances were tested by adding a known quantity of the test compound to a drug-free urine (Ref. Intramural studies, Abbott Laboratories).
b) Real urinary excretion studies.
c) Crosss-reactivities refered to IMIM and Abbott Laboratories studies.

TABLE 4.- Differences between results obtained after analyzing spiked urines and the corresponding excretion study.

INTERNATIONAL OLYMPIC COMMITTEE LIST OF DOPING CLASSES (revision May 1992)

Bold face indicates urines included in the present study.

CLASSE A: STIMULANTS

Amfepramone
Amfetaminil
Amineptine
Amiphenazole
Amphetamine
Benzphetamine
Caffeine
Cathine
Chlorphentermine
Clobenzorex
Clorprenaline
Cocaine
Cropropamide *
Crotetamide *
Dimetamfetamine
Ephedrine
Etafedrine
Ethamivan
Etilamfetamine
Fencamfamin
Fenetylline
Fenfluramine **
Fenproporex
Furfenorex
Mefenorex
Mephentermine **
Mesocarb
Methamphetamine
Methoxyphenamine
Methylephedrine
Methylphenidate
Morazone
Nikethamide
Pemoline
Pentetrazole
Phendimetrazine
Phenmetrazine
Phentermine
Phenylethylamine **
Phenylpropanolamine
Pholedrine **
Pipradrol
Prethcamide **
Prolintane
Propylhexedrine
Pseudoephedrine **
Pyrovalerone
Strychnine

and related compounds

* component of "MICOREN"
** not explicitly included in I.O.C. lists.

TABLE 5.- List of excretion studies evaluated by Amphethamine Class and Amphethamine/Methamphetamine tests.

AMPHETAMINE TESTS

- **Detected Substances**

Amfepramone
Amfetaminil
Amineptine
Amphetamine
Cathine
Clobenzorex
Dimethamphetamine
Ephedrine
Etafedrine
Fenetylline
Fenfluramine
Fenproporex
Mefenorex
Mephentermine
Mesocarb
Methamphetamine
Methoxyphenamine
Morazone
Phendimetrazine
Phenmetrazine
Phentermine
Phenylpropanolamine
Pholedrine
Pseudoephedrine

- **Non Detected Substances**

Amiphenazole
Etamivan
Fencamfamin
Methylphenidate
Nikethamide
Pipradrol
Prethcamide
Prolintane
Strychnine

TABLE 6.- List os stimulants detected or non detected by combining Amphethamine Class and Amphethamine/Methamphetamine tests.

AMPHETAMINE TESTS

- Amphetamine Class Positive &
  Amphetamine/Methamphetamine Negative

  Amfepramone
  Cathine
  Ephedrine
  Etafedrine
  Phendimetrazine
  Phenmetrazine
  Phenylpropanolamine
  Pseudoephedrine

- Amphetamine/Methamphetamine Positive &
  Amphetamine Class Negative

  Amineptine
  Pholedrine

TABLE 7.- List of stimulants that can only be detected by one of the evaluated tests.

INTERNATIONAL OLYMPIC COMMITTEE LIST OF DOPING CLASSES

Bold face indicates urines included in the present study.

CLASSE B: NARCOTIC ANALGESICS

Alphaprodine
Anileridine
Buprenorphine
Codeine
Dextromoramide
Dextropropoxyphene
Diamorphine
Dihydrocodeine
Dipipanone
Ethoheptazine
Ethylmorphine
Levorphanol
Methadone
Morphine
Nalbuphine
Pentazocine
Pethidine
Phenazocine
Trimeperidine

and related compounds

TABLE 8.- List of excretion studies evaluated by Opiates test.

AMPHETAMINE CLASS

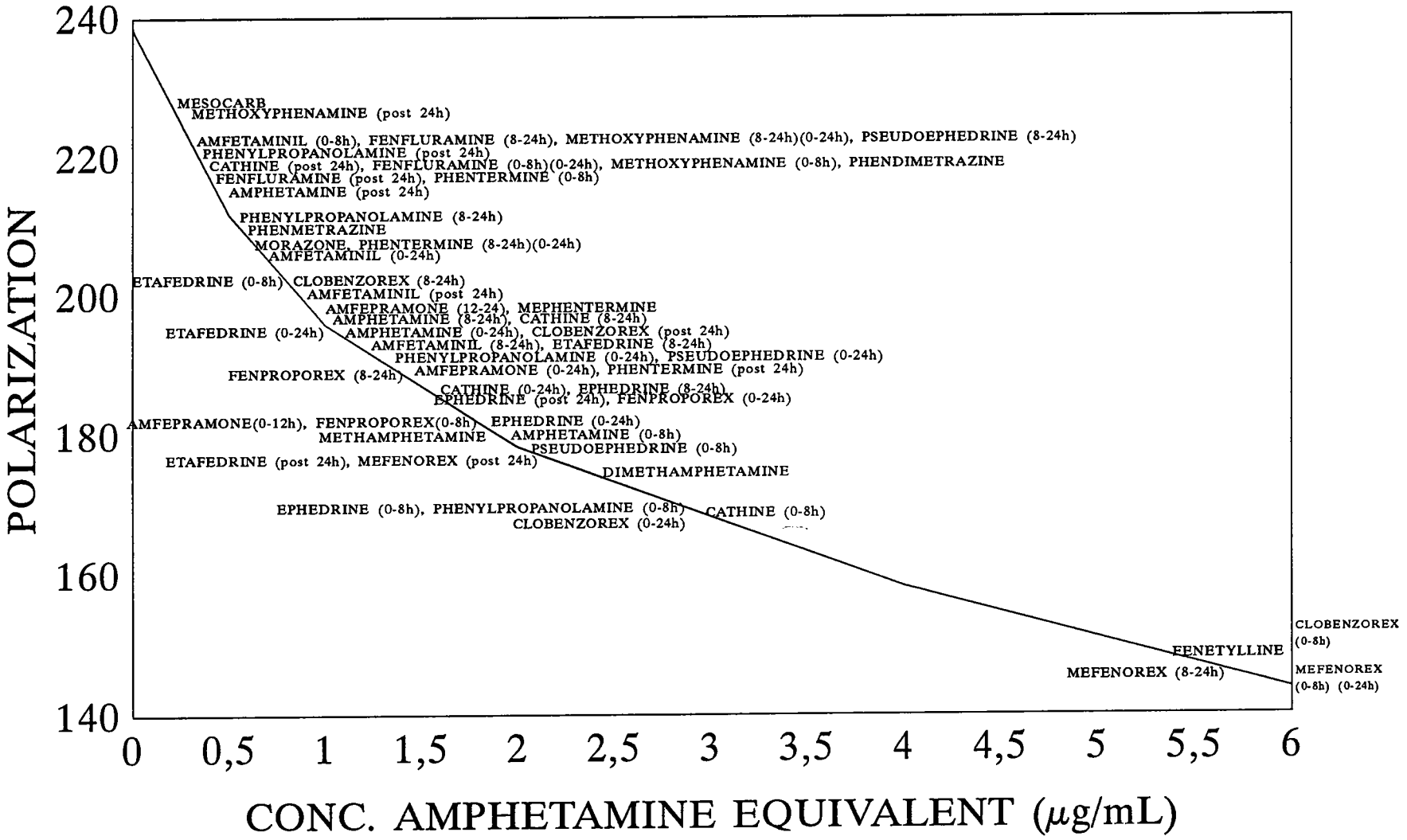

FIGURE 1

AMPHETAMINE/METHAMPHETAMINE

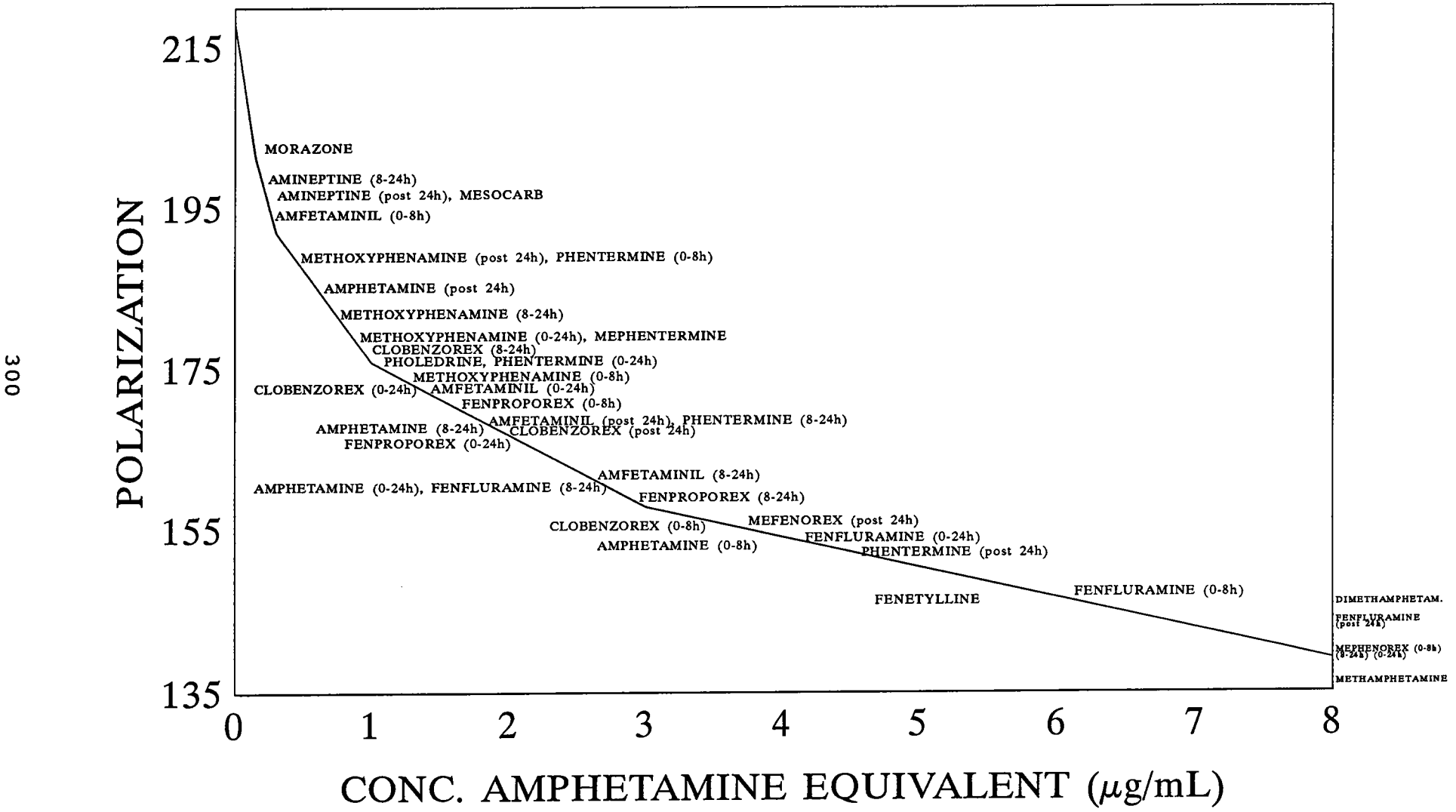

FIGURE 2

OPIATES

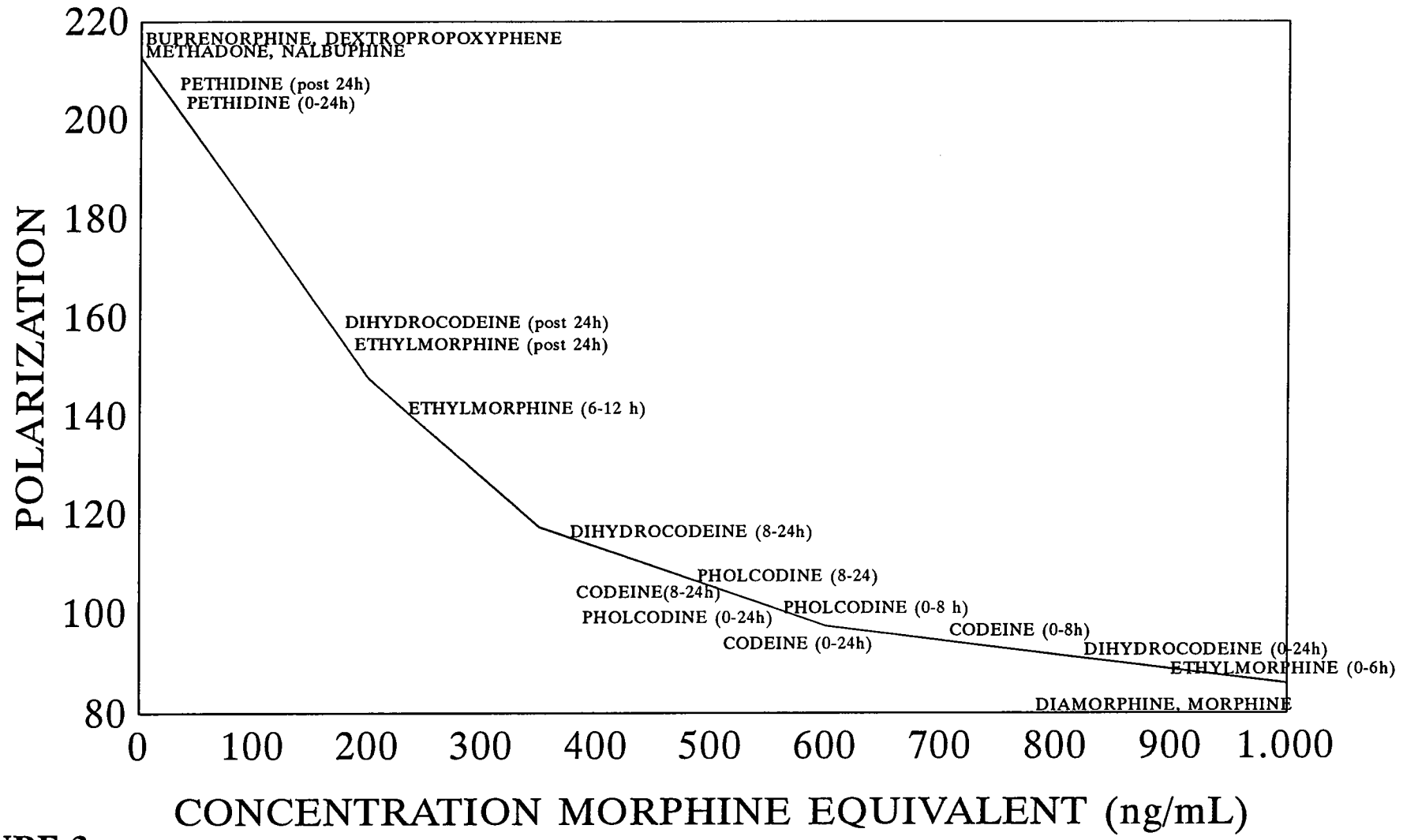

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FIGURE 3