Fluorescence Polarization Immunoassay for the Detection of Drugs of Abuse in Human Whole Blood

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ABSTRACT
Fluorescence polarization immunoassay (FPIA) is a technique which has been known for a number of years. Since the development of the fundamental principles of fluorescence polarization by Perrin in a series of papers beginning in 1926, immunological techniques using labelled reactants have gained an extraordinary importance in the field of medical research and in routine diagnosis. As one of the non-radioactive immunological techniques, FPIA has found broad application in clinical and forensic toxicology. The authors report a new method to quickly screen autopsy, police and hospital blood samples for opiates, benzodiazepines, benzoylecgonine, barbiturates and methadone after Extrelut extraction utilising the FPIA methodology.

INTRODUCTION

Simple and rapid drug screening methods for whole blood samples are of great practical value in forensic toxicology. After the principles of FPIA had been developed by Perrin (1926) this technique was first applied to biological systems by Weber (1953). The application of FPIA to the antigen-antibody reaction was originally described by Dandliker et al. (1961). The principles and theory of fluorescence polarization and fluorescence polarization immunoassay have been intensively described in a number of papers (Dandliker et al., 1970; Dandliker et al., 1973; Gunzer et al., 1980; Jolly, 1981; Smith et al., 1981; Stewart, 1986; DFG, 1988; Liu, 1994) and will not be discussed in detail in this report. When screening large groups of individuals for drugs of abuse, urine samples are usually the material of choice for analysis. Unfortunately, urine samples are often unavailable in forensic toxicology. In cases of driving under the influence of drugs, blood samples are of great importance, since they provide the forensic chemist with information more relevant to the actual driving ability at the time of sampling. Therefore existing urine assays had been adapted for the examination of blood samples. Previously reported methods for the screening of whole blood for common drugs of abuse include EMIT and FPIA immunoassays after precipitation of the blood samples with either methanol (Peel et al., 1981; Asselin et al., 1988; Gjerde et al., 1990), acetone (Lewellen et al., 1988; Bogusz et al., 1990; Maier et al., 1992); N,N-dimethylformamide (Blum et al., 1989; Klinger et al., 1990), trichloroacetic acid (McCord, 1988) or liquid/liquid extraction (Slightom et al., 1978; Slightom et al., 1982). The purpose of this paper was to develop a method based on a homogenous immunoassay for the rapid screening of opiates, benzodiazepines, benzoylecgonine, barbiturates and methadone from autopsy, police and hospital blood samples. Applying a

simple sample pre-treatment (Extrelut extraction) prior to the immunological examination, the authors were able to screen these blood samples within a short period of time.

MATERIALS AND METHODS

Reagents and standards

Morphine hydrochloride and methadone hydrochloride, flunitrazepam, benzoylecgonine and phenobarbitone were obtained from Sigma Chemie (Buchs, Switzerland). Chloroform was of analytical grade and was purchased from E. Merck (Darmstadt, Germany). 3 mL Extrelut glass columns, 25% aqueous NH_4OH solution and acetate buffer (pH 4.6) were also obtained from E. Merck.

Apparatus

FPIA determinations were performed using an ADx Analyzer (Abbott, Zug, Switzerland) and the complete ADx reagent kits.

Toxicological analyses

1.5 mL of an aqueous 5% NH_4OH solution (pH 11.8) was added to 1 mL of the blood specimens in a 10 mL teflone screw-capped glass tube. The sample mixture was then mixed with a hand vortex mixer for about 10 seconds. The mixture was applied on a 3 mL Extrelut glass column and allowed to stand for 10 minutes. The substances under consideration were then eluted with 15 mL $CHCl_3$ (2 × 7.5 mL) into another glass vessel. Under a gentle stream of nitrogen the organic layer was evaporated to dryness at 40°C and the residue reconstituted in 0.5 mL ADx buffer. 50 μL of each sample was used for FPIA analysis. Standards of known amounts of reference substance were prepared by spiking blank blood to obtain final concentrations in the ranges of 40-200 ng morphine/mL, 5-100 ng flunitrazepam/mL, 50-400 ng benzoylecgonine/mL and 100-1200 ng methadone/mL. The extraction procedure for barbiturates was the same as described above except that 1.5 mL acetate buffer (pH 4.6) was used. Blank blood was spiked with phenobarbitone as reference substance in the range of 10-50 μg/mL.

RESULTS

Typical calibration curves of ADx assays applied to blank blood and spiked blood samples subjected to Extrelut extraction prior to the immunoassay are depicted in Figure 1. All immunological measurements were conducted as recommended by the manufacturers' manuals. Net polarization values of the ADx measurements and concentrations of the corresponding reference substances were taken for construction of the calibration curves. For each batch of blood samples to be screened, new calibration curves for the substances mentioned above were constructed. This way the stability of the calibration values of the FPIA determinations could be checked. Cannabinoids were excluded from the ADx assay. Amphetamines were included in the ADx assay. However, the polarization values obtained were too poor to be used for the construction of the calibration curve. Amphetamine immunoassay after Extrelut extraction of whole blood or serum samples is not reliable and was stopped after a series of experiments using both amphetamine or methamphetamine as reference substances. This result is in agreement with the findings of Maier et al. (1992) as well as with Bogusz and co-workers (1990). Both authors had used an acetone precipitation prior to their FPIA measurements. Moreover, Maier et al. (1992) found that even ultrafiltration could not be used for pretreatment of the blood samples for general screening with immunological tests. On the basis of the calibration curves obtained, the cut-off limits shown in Table I were utilised in the screening of autopsy, police and hospital blood samples on the mentioned groups of drugs of abuse.

DISCUSSION

Drugs are often screened by RIA, GC, GC/MS or HPLC. Some of these methods involve laborious, time-consuming pretreatment procedures. The goal of this report was to set up a simple and reliable extraction procedure which would also allow the detection of low-dosed benzodiazepines like flunitrazepam. In contrast to Bogusz and co-workers (1990) who used oxazepam as a reference substance, we were able to extract flunitrazepam from spiked blood samples and draw a reasonable calibra-

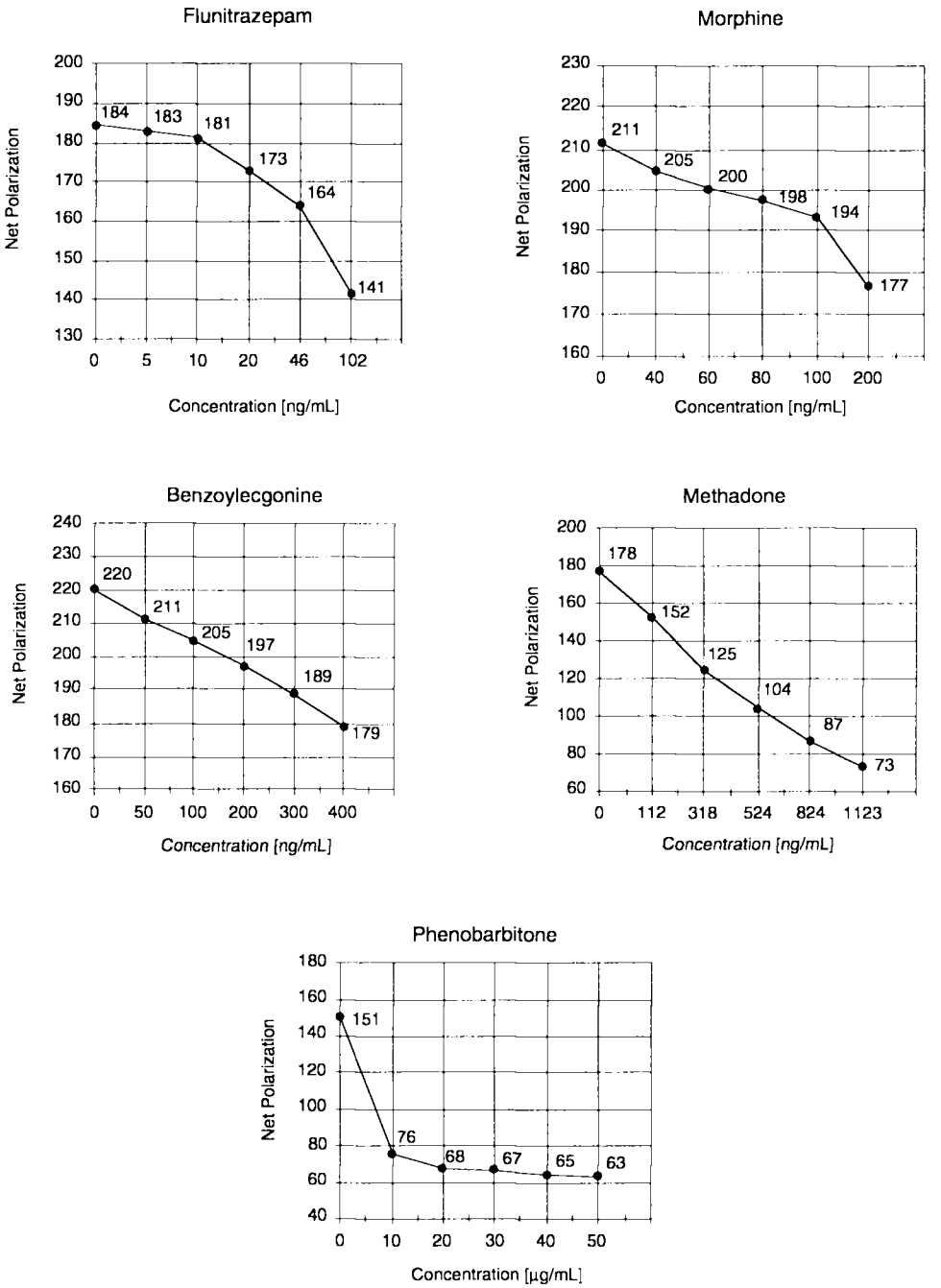

Figure 1. Calibration curves of spiked blood samples using the FPIA methodology.

Table I. Net polarization values, cut-off limits and cut-off concentrations of the tests performed with ADx immunoassay in whole blood samples.

Test	Net pol. (blank blood)	Net pol. (cut-off)	Cut-off concentration
Opiates	211	200	60 ng/mL
Benzodiazepines	184	173	20 ng/mL
Benzoylecgonine	220	211	50 ng/mL
Methadone	178	152	110 ng/mL
Barbiturates	151	75	10 μg/mL

tion curve after FPIA measurements. The cut-off limit for benzodiazepines could therefore be set at 20 ng/mL. This cut-off limit is ten times lower than the one used by Bogusz et al. in their screenings. Bogusz and co-workers state cut-off limits for benzoylecgonine and opiates at a concentration of 100 ng/mL each. According to them the cut-off limit for opiates could be lowered to 20 ng/mL in an individual test procedure. In our experiments we set the cut-off limits for benzoylecgonine at 50 and for opiates at 60 ng/mL, respectively. All samples negative with the FPIA screen also gave negative results with other analytical methods. More sensitive methods (GC/NPD and GC/MS) were applied in cases of suspected cannabinoid and amphetamine misuse. All positive samples with the FPIA screen could be confirmed by either GC/ECD, GC/PND or GC/MS. A difference between autopsy blood samples and blood samples obtained from living subjects could not be observed. In conclusion, our Extrelut extraction method is easy to perform (although the barbiturates have to be extracted separately), reliable and therefore suitable in general unknown cases.

REFERENCES

Asselin W.M., Leslie J.M. and McKinley B. (1988) Direct detection of drugs of abuse in whole haemolyzed blood utilizing the EMIT-dau urine assay. *J. Anal. Toxicol.* **12**, 207–15.

Blum L. M., Klinger R.A. and Rieders F. (1989) Direct automated EMIT-dau analysis of N,N-dimethylformamide-modified serum, plasma and postmortem blood for benzodiazepines, benzoylecgonine, cannabinoids and opiates. *J. Anal. Toxicol.* **13**, 285–8.

Bogusz M., Aderjan R., Schmitt G., Nadler E. and Neureither B. (1990) The determination of drugs of abuse in whole blood by means of FPIA and EMIT-dau immunoassays - a comparative study. *Forensic Sci. Int.* **48**, 27–37.

Dandliker W.B. and Feigen G.A. (1961) Quantification of the antigen-antibody reaction by the polarization of fluorescence. Biochem. *Biophys. Res. Commun.* **5**, 299–304.

Dandliker W.D., Kelly R.J. and Dandliker J. (1973) Fluorescence polarization immunoassay: theory and experimental method. *Immunochemistry* **10**, 219–27.

Dandliker W.D. and Saussure V.A. (1970) Review Article: Fluorescence polarization in immunochemistry. *Immunochemistry* **7**, 799–828.

Deutsche Forschungs Gemeinschaft (DFG) (1988), Empfehlungen zur klinisch-toxiko-logischen Analytik. VCH-Verlag **1**, 3–27.

Gjerde H., Christophersen A.S., Skuterud B., Klemetsen K. and Morland J. (1990) Screening for drugs in forensic blood samples using EMIT® urine assays. *Forensic Sci. Int.* **44**, 179–85.

Gunzer G. and Rieke E. (1980) Homogener Immunoassay. *Kontakte* **3**, 3–11.

Jolly M.E. (1981) Fluorescence polarization immunoassay for the determination of therapeutic drug levels in human plasma. *J. Anal. Toxicol.* **5**, 236–40.

Klinger R.A., Blum L.M. and Rieders F. (1990) Direct automated EMIT-dau analysis of N,N-dimethylformamide-modified serum, plasma and postmortem blood for amphetamines, barbiturates, methadone, methaqualone, phencyclidine and propoxyphene. *J. Anal. Toxicol.* **14**, 288–91.

Lewellen L.J. and McCurdy H.H. (1988) A novel procedure for the analysis of drugs in whole blood by homogenous enzyme immunoassay (EMIT®). *J. Anal. Toxicol.* **12**, 260–7.

Liu R.H. (1994) Comparison of common immunoassay kits for effective application in workplace drug urine analysis. *Forensic Sci. Rev.* **6(1)**, 19–57.

Maier R.D., Erkens M., Hoenen H. and Bogusz M. (1992) The screening for common drugs of abuse in whole blood by means of EMIT-ETS and FPIA-ADx urine immunoassays. *Int. J. Leg. Med.* **105**, 115–19.

McCord C.E. and McCutcheon J.R. (1988) Preliminary evaluation of the Abbott TDx for benzoylecgonine and opiate screening in whole blood. *J. Anal. Toxicol.* **12**, 295–7.

Peel H. W. and Perrigo B. J. (1981) Detection of cannabinoids in blood using EMIT. *J. Anal. Toxicol.* **5**, 165–7.

Perrin F. (1926) Polarization de la lumiere de fluorescence. Vie moyenne de molecules dans l'état exite. *J. Phys. Radium* **7**, 390–401.

Slightom E.L., Cagle J.C., McCurdy H.H. and Castagna F. (1982) Direct and indirect homogenous enzyme immunoassay of benzodiazepines in bio-logical fluids and tissues. *J. Anal. Toxicol.* **6**, 22–5.

Slightom E.L. (1978) The analysis of drugs in blood, bile and tissue with an indirect homo-genous enzyme immunoassay. *J. Forensic Sci.* **23**, 292–303.

Smith D.S., Al-Hakiem M.H.H. and Landon J. (1981) A review of fluorescence polarization immunoassay and immunofluorometric assay. *Ann. Clin. Biochem.* **18**, 253–74.

Stewart M.J. (1986) *Immunoassay. In: Clarke's isolation and identification of drugs*. The Pharmaceutical Press, London, pp 148–59.

Weber G. (1953) Rotational brownian motion and polarization of the fluorescence of solutions. *Adv. Protein Chem.* **8**, 415–59.