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# Development of a Fluoroimmunoassay for the Detection of Buprenorphine in Urine

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**ABSTRACT:** The development of a fluoroimmunoassay for the detection of buprenorphine in urine samples is described. Fluore-scein-norbuprenorphine and pseudobuprenorphine, the dimer of buprenorphine, were synthesized as tracer molecules. The antibodies were prepared by coupling the 2-diazobenzoic acid derivative of buprenorphine with bovine serum albumine, using the carbodiimide method. The assay was mainly used for the routine detection of buprenorphine in urine specimens of persons suspected of Temgesic® abuse. The minimum detectable dose of the immunoassay was calculated to be 20 ng/mL.

**KEYWORDS:** toxicology, buprenorphine, fluoroimmunoassay, abuse, urine

Buprenorphine, (2S)-2-[(−)(5R,6R,7R,14S)-9a-cyclopropylmethyl-4,5-epoxy-3-hydroxy-6-methoxy-6,14-ethanomorphinan-7-yl]-3,3-dimethylbutan-2-ol (Temgesic®) is a highly lipophylic derivative of thebaine with opioid partial agonist properties. Very soon after buprenorphine became available in retail pharmacies all over the world, reports of abuse were published. It was observed that at times of shortage of heroin supply, buprenorphine was frequently used as the drug of choice. The first reports of abuse of buprenorphine were published between 1983 and 1986 [*1–6*]. More recently, other studies illustrated the increasing rate of abuse of buprenorphine, especially as a substitute of heroin [*7–9*]. In our laboratory, we indeed encountered cases of positive buprenorphine samples with doses far in excess of a therapeutic dose. These high doses clearly indicated an abuse of Temgesic® [*10*].

For the prescreening of a large amount of urine samples, the availability of a specific and rapid analysis technique is mandatory. For this reason, a sensitive and specific radioimmunoassay (RIA) has been developed in our laboratory [*11*]. This radioimmunoassay was developed on the basis of antibodies, elicited by the 2-diazobenzoic acid derivative of buprenorphine which was coupled to bovine serum albumin (Fig. 1). The spacer between buprenorphine and BSA was established at carbon 2, and therefore, the substituents on carbon 3, 6 and 7 and the substituent on the nitrogen were free to serve as potential antigenic determinants. Although the radioimmunoassay yielded sensitive results for the determination

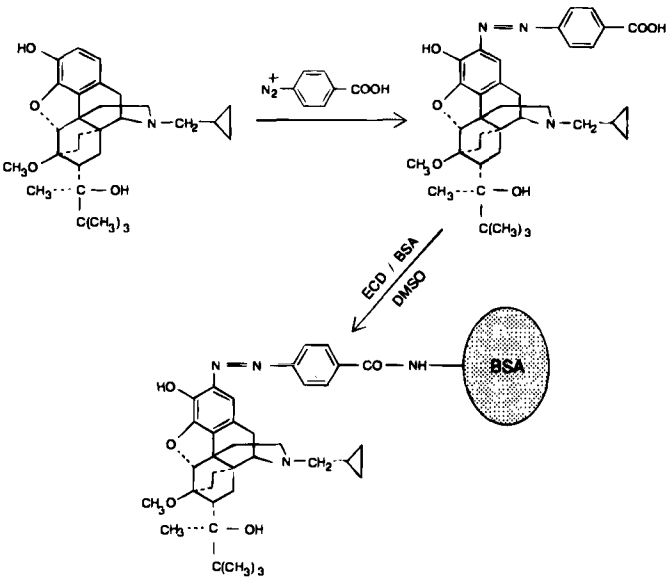


FIG. 1—*Preparation of a specific buprenorphine immunogen.*

of small amounts of buprenorphine in urine samples, this technique has certain disadvantages, inherent to isotopically labelled compounds, particularly the short half life of the $^{125}I$ isotope that necessitates the preparation of the label at frequent intervals.

An alternative to RIA is fluorescence immunoassay (FIA), a technique in which a fluorescent label is substituted for the radioactive label. This paper reports on the synthesis of two tracer molecules and their possible use for the development of a non-isotopic and sensitive fluoroimmunoassay.

## Materials and Methods

### *General Reagents*

Fluorescein isothiocyanate (FITC; Isomer I) was obtained from Pierce (Rockford, Ill. and 5-(6)-carboxyfluorescein-N-hydroxy succinimide ester (FLUOS) from Boehringer (Boehringer Mannheim Biochemica, Mannheim, Germany). Silica gel (0.063 to 0.200 mm), potassium hydroxide, potassium dihydrogen phosphate, disodium hydrogen phosphate dihydrate, anhydrous sodium sulphate, potassium hexacyanoferrate(III), methanol, chloroform, dichlormethane, methylsulfoxide, triethylamine (TEA), and β-glucuronidase/arylsulphatase (Helix Pomatia) were obtained from E. Merck (Darmstadt, Germany), silica gel thin-layer chromatographic (TLC) plates from Machery-Nagel (Duren, Germany), goat

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anti-rabbit gamma globulin (GARGG) and normal rabbit serum (NRS) from Calbiochem® (Calbiochem® Biochemicals and Immunochemicals, San Diego, CA), goat anti-rabbit immunoglobulin immuno-beads® from Bio-Rad (Bio-Rad Laboratories, Richmond, CA) and Triton X-405, 70% from Janssen Chimica (Beerse, Belgium). Norbuprenorphine and buprenorphine were synthesized according to the method of Kleemann and Engel [*12*]. [$^{125}$I] iodobuprenorphine (920 mCi/mmol) was synthesized in the laboratory [*13*].

*Mass Spectrometry*

Liquid surface-assisted ionization mass-spectrometry (L-SIMS) was performed with a Kratos Concept 1H instrument using a 7-keV Cs beam. Pseudobuprenorphine was dissolved in glycerol on the probe tip.

*Synthesis of the Fluorescent Tracers*

*Synthesis of Fluorescein-Labeled Norbuprenorphine*—Fluorescein labeled norbuprenorphine was prepared using activated fluorescein label (FITC). The best results were obtained using the following reaction procedure: to a solution of norbuprenorphine (100 mg) in MeOH-TEA (9-1), 100 mg FITC, dissolved in MeOH, were added and the reaction mixture stirred for 2 h. The solvent was evaporated and the mixture purified with preparative TLC ($CH_2Cl_2$:MeOH-TEA (99-1), 9:1). The major fluorescent zone was scratched off from the plate, dissolved in the $CH_2Cl_2$: MeOH-TEA (99-1), 9:1 solvent and further purified by HPLC with fluorescence detection. A Merck-Hitachi L-6200 pump was coupled to a LiChrosorb® C18 column (25 cm × 0.4 cm i.d.) (E. Merck). The mobile phase consisted of a mixture of acetonitrile: $H_2O$ (8:2) and was pumped at a rate of 1 mL/min. The fluorescence detector (F-1050 Fluorescence Spectrophoto meter, Merck-Hitachi) was set at a sensitivity of 20; $\lambda_{ex}$ at 490 nm and $\lambda_{em}$ at 525 nm. After each injection of the mixture, the most important peak ($R_t$ = 7.2 min) was collected. The collected fluorescent fractions were dried under a stream of nitrogen at 40°C and the residue was examined with infrared spectrophotometry. A stock solution of 1 mg/mL MeOH was stored in the freezer at −18°C. See Fig. 2.

*Preparation of Pseudobuprenorphine*—Buprenorphine can be oxidized to the bimolecular base pseudobuprenorphine by reaction with potassium hexacyanoferrate(III) in aqueous solution. The fluorescence properties of this molecule are greatly enhanced due to an extended conjugated system. Buprenorphine (100 mg) was added to a hot solution of potassium hydroxide (200 mL of a 2 g/L solution), the mixture sonicated and cooled to room temperature. Under continuous stirring, the solution of potassium hexacyanoferrate(III) (120 mg) in water (2 mL) was added over a time period of 60 min. After another 30 min, the mixture was made alkaline (pH 8.5) with borate buffer (0.1 mol/L), and extracted with $CHCl_3$. The organic layer was dried over anhydrous sodium sulphate and evaporated. The obtained residue was further purified by column chromatography (silica gel 0.063–0.200 mm; $CHCl_3$: MeOH/TEA (99–1); 95:5). The column was coupled to an UV detector (SP8-400 UV/VIS spectrophotometer, Pye Unicam, Cambridge, England) through a 1 mL flow cell. 11.9 mg of the fluorescent dimer was isolated. A stock solution of 1 mg/mL MeOH was stored in the freezer. The fluorescence characteristics of the product were measured at pH 7.0 (phosphate buffer 0.1 mol/L). See Fig. 3.

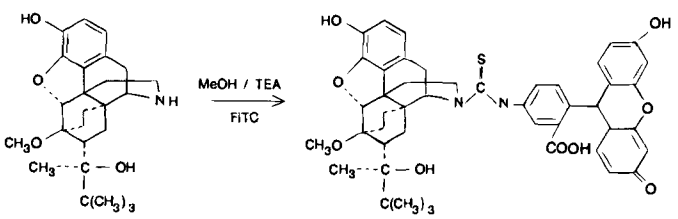


FIG. 2—*Preparation of fluorescein labeled norbuprenorphine.*

*Affinity of the Fluorescent Tracers for the Antibodies*

The fluorescent buprenorphine derivatives were tested for their ability to bind to the buprenorphine antibodies. Dilutions of the fluorescent tracers (fluorescein-norbuprenorphine and pseudobuprenorphine) were prepared in phosphate buffer pH 7.4, 0.1 mol/L to final concentrations of 1, 10, 100 and 1000 ng/100 μL. The cross-reactivity of the synthesized fluorescent tracers to [$^{125}$I] iodobuprenorphine was examined. To 300 μL reaction matrix (2% BSA in phosphate buffer pH 7.4, 0.1 mol/L) 100 μL [$^{125}$I] iodobuprenorphine (±60.000 dpm) and 100 μL of the fluorescence labels, at the different concentrations, were pipetted into duplicate polypropylene tubes. The mixture was vortexed and 100 μL antiserum (1/400) added. The mixture was incubated for 1 h 30 min. 50 μL goat anti-rabbit gamma globulin (GARGG) (1/125 in phosphate buffer) and 50 μL normal rabbit serum (NRS) (1/1000 in phosphate buffer) were added. The mixture was further incubated overnight, centrifuged, decanted and the pellet counted with the gamma counter. Pseudobuprenorphine showed the highest affinity for the antibodies and was selected for the elaboration of a fluoroimmunoassay.

*Assay Procedure*

For the fluoroimmunoassay, all the experiments were conducted in phosphate buffer (0.1 mol/L, pH 7.4 containing 1% BSA and 0.05% Triton X-405). 100 μL urine, first hydrolyzed overnight at 37°C with β-glucuronidase/arylsulphatase, or standard solution (100 μL) was pipetted into the reaction matrix (300 μL phosphate buffer), followed by 100 μL of the tracer (pseudobuprenorphine, 10 ng/100 μL). After vortexing, 100 μL of the antiserum was added. After 1 h 30 min bound tracer was separated from free tracer. For the separation of bound and free tracer, two methods were studied (GARGG method and the Immunobead® method). The latter technique gave a lower non-specific binding and was selected for further experiments. 100 μL of the Immunobead® reagent was added. The reactants were mixed, equilibrated for 2 h and centrifuged at 1880 × g for 10 min; the supernatant was decanted. The particles were washed three times with phosphate buffer (2 mL). Then 2.5 mL of eluting agent (tracer-dissociating agent) was added (MeOH-bicarbonate buffer 0.02 mol/L; pH 9.0:80-20). The mixture was sonicated for 60 s. The particles were separated by centrifugation and the supernatant was filtered through an Acrodisc® filter 0.2 μm (Gelman Sciences, Ann Arbor,

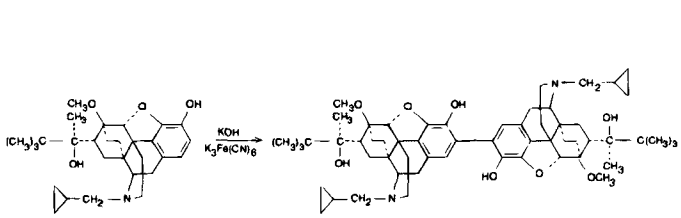


FIG. 3—*Synthesis of pseudobuprenorphine.*

MI), the fluorescence of the filtrate was measured using a 4 mL cuvette that was placed in the "open-close shutter" of the fluorescence spectrophotometer. The detector was coupled to a Merck-Hitachi D-2500 integrator. The sensitivity of the fluorescence detector was set at 100 (maximum sensitivity).

*Calibration Curve*

The standard curve values (20, 50, 100, 200, and 500 ng/mL) were plotted, after logit-log transformation, as % $B/B_0$ (counts bound for arbitrary dose relative to that for zero dose) to the concentrations of buprenorphine standard.

*Study of Field Samples*

A selected number of field samples from persons suspected of Temgesic® abuse was analyzed with the fluoroimmunoassay.

## Results

*Preparation of Fluorescein-Labeled Norbuprenorphine and Pseudobuprenorphine*

Two activated fluorophores (FLUOS and FITC) were tried out for the synthesis of the fluorescent tracer. The applicability of FLUOS for the labeling of norbuprenorphine was examined using the already described reaction procedures for the coupling of the primary amines of proteins with FLUOS [*14*]. Both norbuprenorphine and FLUOS were dissolved in different mixtures of DMSO and phosphate buffer 0.1 mol/L, pH 7.0. The reaction was stopped at different time intervals and checked by TLC. No coupling of FLUOS with the secondary amine group of norbuprenorphine was observed. No better results were obtained using buffers of a higher pH value (pH 8.0 and pH 9.0). In 1972, Cuatrecasas et al. [*14*] succeeded in coupling the N-hydroxysuccinimide ester of fluorescein with the unprotonated form of primary aliphatic or aromatic amino groups.

Using FITC in a mixture of MeOH-TEA, it was possible to bind fluorescein to norbuprenorphine. Isothiocyanates are acylating agents which react with primary and secundary amines, and they generally yield stable thiourea derivatives [*15–17*]. The formation of the thioureum bond in fluorescein-norbuprenorphine was proven by infrared spectrometry (1430 $cm^{-1}$). Alternatively, it was also possible to identify norbuprenorphine after hydrolysis of the synthesized fluorescent compound. The product was therefore dissolved in 2 N NaOH and the solution boiled for 30 min. The pH was adjusted to 8.5 and the mixture extracted with $CHCl_3$. The organic layer was concentrated and spotted on a TLC plate, using norbuprenorphine as a reference. Norbuprenorphine could be identified with an acidified iodoplatinate spray. Due to the lack of affinity of the synthesized compound for the antibodies, no further confirmation, for example, mass spectrometry (L-SIMS) was performed.

The purified pseudobuprenorphine fraction showed an intense blue fluorescence with maxima of respectively: $\lambda_{ex}$ = 326 nm and $\lambda_{em}$ = 435 nm. Similar fluorescence characteristics are described for pseudomorphine: $\lambda_{ex}$ = 324 nm and $\lambda_{em}$ = 434 nm [*18*]. Confirmation of the structure of the molecule has been obtained by high resolution mass spectrometry. The mass spectrum (Fig. 4) illustrates the molecular ion of pseudobuprenorphine at m/z 933.

*Affinity of the Fluorescence Tracers for the Antibodies*

The affinity of the antibodies for the fluorescein norbuprenorphine label was extremely low; a cross-reactivity to [$^{125}$I] iodobuprenorphine of only 4.5% was observed. Pseudobuprenorphine had a much higher affinity for the antibodies (cross-reactivity of 39%). For this reason, the latter product was selected for the elaboration of the fluoroimmunoassay, though the fluorescence characteristics of the fluorescein derivative of norbuprenorphine were superior to those of pseudobuprenorphine.

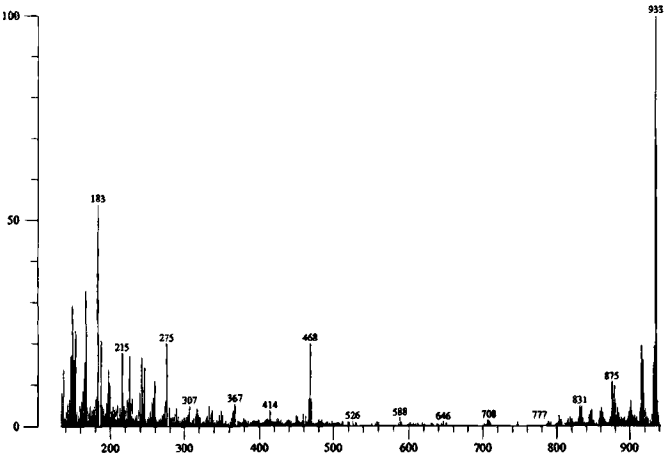

FIG. 4—*Mass spectrum of pseudobuprenorphine.*

*Assay Validation*

Two methods were tried out for the separation of bound and free tracer. The goat anti-rabbit gamma globulin technique showed high non-specific binding values. Another separation method, using highly purified goat anti-rabbit gamma globulins, covalently bound to spherical polyacrylamide particles, yielded better results (lower non-specific binding and higher reproducibility).

A concentration of 20 ng/mL is for 99% of the cases significantly different from the blank value (Student t-distribution; $P$ = 0.01; $d_f$ = 8). The intra-assay precision was assessed by performing 5 assays on each of three urine samples. Mean values were 105, 125, and 178 ng/mL with CV values of 11%, 13%, and 15%, respectively.

The same urine samples were assayed on three separate days, giving CV values of 13%, 15%, and 17%, respectively, for interassay precision.

*Calibration Curve*

After logit-log transformation, a linear calibration graph ($Y = \beta_0 + \beta_1 X$) was obtained with the following regression line $Y = 7.06 - 1.28\ X$ ($Y = \log_e y/1-y$ with $y$ = %$B/B_0$ and $X = \log_e$ conc. buprenorphine), $r$ = 0.991 and the 95% confidence limits ($t_{0.025}$ for $d_f$ = 3) are $\pm 0.14$ for $\beta_1$ and $\pm 1.67$ for $\beta_0$.

*Field Samples*

A number of urine samples of persons suspected of Temgesic® abuse were analyzed with fluorescence-immunoassay. The same urine samples were also analyzed by HPLC with electrochemical detection [*19*] for confirmation. False-negative results were not noticed in any of the samples. The results of the fluorescence-immunoassay clearly showed that the method is applicable for the control of these samples in a screening program. Several of them showed apparent buprenorphine concentrations >100 ng/mL.

## Discussion

Pseudobuprenorphine has shown to be a valuable tracer molecule in the fluoroimmunoassay for the detection of buprenorphine in urine samples. The affinity of the fluorescent derivative for buprenorphine antibodies is acceptable and superior to that of a fluorescein norbuprenorphine label. A fluoroimmunoassay has been developed that permits specific detection of buprenorphine down to 20 ng/mL in urine specimens following enzymatic hydrolysis. The absence of radioactivity and a much longer useful lifetime of the assay kit are the major advantages of this immunoassay. The sensitivity of the method is however low in comparison to the radioimmunoassay due to several reasons. First, pseudobuprenorphine has some structural features (substituents on the nitrogen and on C-7 and C-6) which cause a large decrease in fluorescence intensity. Indeed, the fluorescence intensity of pseudomorphine is some 15 times higher than for pseudobuprenorphine [*18*]. Furthermore, relative high non-specific binding values were obtained due to the interference of endogenous compounds of the urine matrix. Finally, the affinity of the antibodies for pseudobuprenorphine is about three times lower than for [$^{125}$I] iodobuprenorphine. This lower sensitivity necessitates enzymatic deconjugation of the urine samples prior to analysis. After hydrolysis, the FIA can be used for the routine detection of buprenorphine in urine specimens of persons suspected of Temgesic® abuse. In field samples high concentrations of apparent buprenorphine have been detected. The non-isotopic assay lacks the sensitivity, necessary for pharmacokinetic studies, but is a valuable method in a screening program.

## References


[*1*] Harper, I., "Temgesic Abuse," *New Zealand Medical Journal*, Vol. 96, 1983, p. 777.

[*2*] Quigley, A. J., Bredemeyer, D. E., and Seow, S. S., "A Case of Buprenorphine Abuse," *Medical Journal of Australia*, Vol. 140, 1984, pp. 425–426.

[*3*] Rainey, H. B., "Abuse of Buprenorphine," *New Zealand Medical Journal*, Vol. 134, 1986, p. 72.

[*4*] Richert, S., Strauss, A., Von Arnim, T., Vogel, P., and Zech, A., "Medikamentenabhangigkeit von Buprenorphin," *Munchener Medizinische Wochenschrift*, Vol. 125, 1983, pp. 1195–1198.

[*5*] Robertson, J. R. and Bucknall, A. B. V., "Buprenorphine: Dangerous Drug or Overlooked Therapy," *British Medical Journal*, Vol. 292, 1986, p. 1465.

[*6*] Strang, J., "Abuse of Buprenorphine," *The Lancet*, Vol. 25, 1985, p. 725.

[*7*] Chowdhury, A. N. and Chowdhury, S., "Buprenorphine Abuse: Report from India," *British Journal of Addiction*, Vol. 85, 1990, pp. 1349–1350.

[*8*] O'Connor, J. J., Moloney, E., Travers, R., and Campbell, A., "Buprenorphine Abuse Among Opiate Addicts," *British Journal of Addiction*, Vol. 83, 1988, pp. 1085–1089.

[*9*] Robertson, J. R. and Roberts, J. J. K., "Management of Drug Abuse," *The Lancet*, Vol. 2, 1987, pp. 284–285.

[*10*] Debrabandere, L., Van Boven, M., and Daenens, P., "Analysis of Buprenorphine in Urine Specimens," *Journal of Forensic Sciences*, Vol. 37, No. 1, January 1992, pp. 82–89.

[*11*] Debrabandere, L., Van Boven, M., and Daenens, P., "Development of a Radioimmunoassay for the Determination of Buprenorphine in Biological Samples," *The Analyst*, Vol. 118, 1993, pp. 137–143.

[*12*] Kleemann, A. and Engel, J., *Pharmazeutische Wirkstoffe, Ergängzungsband 1982–1987*, Georg Thieme, Stuttgart, New York, 1987.

[*13*] Debrabandere, L., Van Boven, M., and Daenens, P., "Synthesis of Iodobuprenorphine for Use in Radioimmunoassay," *Journal of Labelled Compounds and Radiopharmaceuticals*, Vol. 31, 1992, pp. 575–588.

[*14*] Cuatrecasas, P. and Parikh, I., "Adsorbents for Affinity Chromatography. Use of N-hydroxysuccinimide Esters of Agarose," *Biochemistry*, Vol. 11, 1972, pp. 2291–2299.

[*15*] Brandtzaeg, P., "Conjugates of Immunoglobulin G with Different Fluorochromes. Specific and Non-Specific Binding Properties," *Scandinavian Journal of Immunology*, Vol. 2, 1973, pp. 333–348.

[*16*] Brandtzaeg, P., "Conjugates of Immunoglobulin G with Different Fluorochromes. Characterization by Anionic-Exchange Chromatography," *Scandinavian Journal of Immunology*, Vol. 2, 1973, pp. 273–290.

[*17*] Steinbach, G. and Von Mayersbach, H., "Characterization of Fluorescein Isothiocyanate," *Acta Histochemica*, Vol. 55, 1976, pp. S 110–123.

[*18*] Darwin, W. D. and Cone, E. J., "Fluorescence Properties of Pseudomorphine and Congeners: Structure-Activity Relationships," *Journal of Pharmaceutical Sciences*, Vol. 69, 1980, pp. 253–257.

[*19*] Debrabandere, L., Van Boven, M., and Daenens, P., "High-Performance Liquid Chromatography with Electrochemical Detection of Buprenorphine and Its Major Metabolite in Urine," *Journal of Chromatography*, Vol. 564, 1991, pp. 557–566.



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