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Determination of [^{3}H]brifentanil, a potent narcotic analgesic, from rat serum by high-performance liquid chromatography with on-line radioactive detection

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ABSTRACT

A method for rapid analysis of [^{3}H]brifentanil extracted from rat serum is described that has the advantages of sensitivity, speed and specificity. The method is based on extraction from serum via solid-phase extraction followed by chromatographic separation on a reversed-phase high-performance liquid chromatographic column. Detection of [^{3}H]brifentanil is accomplished with an on-line radioactive detector, thus the laborious step of peak collection and subsequent liquid scintillation counting is eliminated. The developed method is sensitive to 0.1 ng/ml and has been successfully applied to pharmacokinetic studies in rats. *In vivo* metabolites retaining the radiolabel have been detected with the method and were more polar than the parent compound as based upon the elution order on the reversed-phase system.

INTRODUCTION

Brifentanil, a potent narcotic analgesic structurally similar to alfentanil (Fig. 1), is currently undergoing clinical trials. Based upon preliminary studies [1], anticipated blood level projections for adequate pharmacokinetic evaluation in rats and dogs were between 200 and <1 ng/ml. Therefore, a highly reproducible and sensitive method of analysis from serum was required. Other methods developed for alfentanil and fentanyl include high-performance liquid chromatography (HPLC) with UV detection [2], gas chromatography (GC) [3–7] as well as radioimmunoassay [8,9]. All of these methods are either labor- and time-intensive or lack the selectivity which is required for adequate pharmacokinetic analysis.

We report here that a reproducible, sensitive and selective method for the analysis of [^{3}H]brifentanil in serum has been developed using solid-phase extraction followed by reversed-phase HPLC with on-line radioactive detection. From 1 ml of serum, the detection limit (defined as a 3:1 signal-to-noise ratio) was 0.1 ng/ml based on a [^{3}H]brifentanil specific activity of 25.7 Ci/mmol. The internal standard selected for the method is a structurally similar compound, ocfentanil (Fig. 1).

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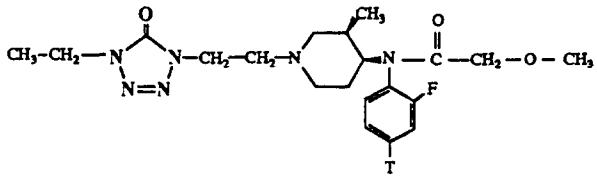
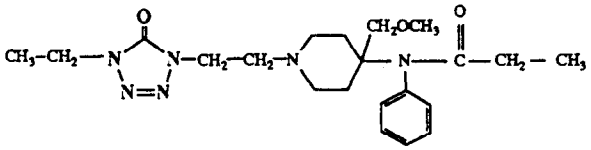


(I)



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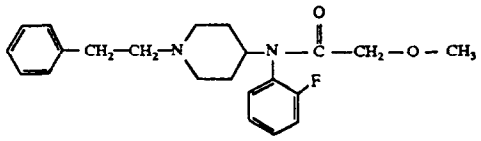

(III)

Fig. 1. Chemical structures for [^{3}H]brifentanil (I), showing position of the radiolabel, alfentanil (II) and ocfentanil, the internal standard (III).

EXPERIMENTAL

Materials and reagents

[^{3}H]Brifentanil (4′-tritio-2′-fluorophenyl, 25.7 Ci/mmol, $\geq$97% radiochemical purity as determined by radio-HPLC) was custom-synthesized by DuPont, NEN Research Products (Boston, MA, U.S.A.). The internal standard, ocfentanil, was synthesized in-house. All solvents were of HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Monobasic potassium phosphate and 85% phosphoric acid were of reagent grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The liquid scintillation fluid, Flo-Scint III (Radiomatic, Tampa, FL, U.S.A.), was selected for its compatability with reversed-phase systems.

Instrumentation and chromatographic conditions

The HPLC system (Fig. 2) consisted of a Milton Roy CM4000 multiple solvent delivery system (Riviera Beach, FL, U.S.A.) for reversed-phase HPLC solvent, a Perkin Elmer ISS 100 automatic sampler (Norwalk, CT, U.S.A.) attached to a Rheodyne injection valve (200-μl sample loop) which was coupled to a Hypersil (Keystone Scientific, Bellefonte, PA, U.S.A.) octadecylsilane (25 cm $\times$ 4.6

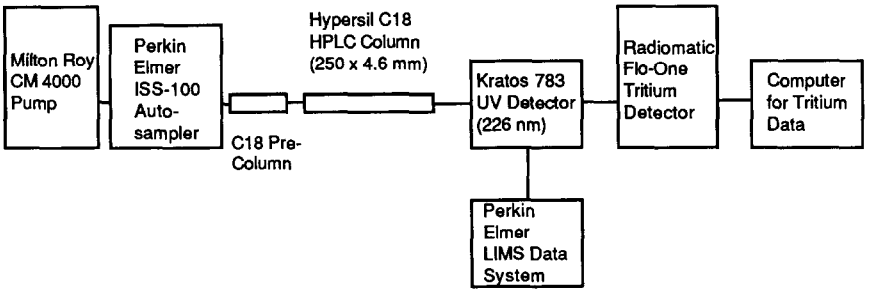

Fig. 2. Schematic representation of HPLC system.

mm, 5 μm particle size) reversed-phase column. The column was preceded by a Waters μBondapak C_{18} Guard-Pak precolumn cartridge (Milford, MA, U.S.A.). Radioactivity in the column effluent was detected by a Radiomatic Flo-One IC Beta (Radiomatic) radioactive flow detector consisting of a 0.3-ml sample chamber. Prior to reaching the flow cell, the effluent was mixed with Flo-Scint III liquid scintillation fluid in a 1:1 (v/v) ratio yielding a total flow-rate of 2 ml/min through the flow cell. This flow ratio was experimentally determined as the optimum flow ratio providing a maximum radioactive detection efficiency of 52%. Calculation of the radioactive efficiency was determined by dividing the cpm response of the analyte by the on-column dpm injected. The injected dpm was simultaneously confirmed via scintillation counting.

The reversed-phase mobile phase consisted of acetonitrile–buffer (40:60, v/v) with a flow-rate of 1.0 ml/min. The buffer, 0.05 *M* monobasic potassium phosphate, was first titrated to pH 4.0 with 85% phosphoric acid. Prior to use, the mobile phase was filtered through a 0.45-μm Nylon 66 membrane (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and degassed via helium sparging.

Detection of the internal standard was accomplished using a Kratos Spectroflow 783 variable-wavelength UV detector that preceded the radioactive detector and was set at a fixed wavelength of 226 nm. Data for the internal standard responses were recorded on a Perkin Elmer 3212 LAS/LIMS (Laboratory Information Management System) data information system (Norwalk, CT, U.S.A.). Data generated for radioactive chromatograms were stored on 2S-HC double-sided soft-sector diskettes.

Serum extraction

All serum samples (1 ml) containing [^{3}H]brifentanil were spiked with 50 μl of internal standard stock solution to yield a final concentration of 10 μg/ml prior to solid-phase extraction. A Speed-Wiz automated solid-phase extraction system (Applied Separations, Bethlehem, PA, U.S.A.) was used for all sample extractions. C_{18} Bond-Elut solid phase extraction columns (1 ml) (Analytichem, Harbor City, CA, U.S.A.) were initially conditioned with two column volumes of

methanol followed by two column volumes of HPLC water, keeping the sorbent wet following the last rinse. Serum samples were added to and then aspirated through the columns. [^{3}H]Brifentanil was retained on the columns and further removal of endogenous material was accomplished via three column rinses of HPLC water. After the final water rinse the columns were allowed to dry. Elution of [^{3}H]brifentanil was accomplished with two 0.6-ml rinses of methanol and collection into 100 × 16 mm borosilicate glass culture tubes. The eluent was evaporated under nitrogen at 50°C and then resuspended in 1 ml of HPLC mobile phase prior to injection (100 μl) onto the HPLC system.

Precision

Variability of the method was determined by within-day and between-day percent coefficients of variation (C.V.). System variability was determined from six replicate injections of a mobile phase standard containing 10 ng/ml [^{3}H]brifentanil with 10 μg/ml internal standard. The serum extraction method variability was determined from six separate serum samples spiked with identical concentrations of [^{3}H]brifentanil and internal standard and then carried through the extraction procedure described above.

Linearity and recovery

Linearity and recovery from serum were evaluated by adding known amounts of [^{3}H]brifentanil to serum samples (0.4–200 ng/ml) and extracting as described above. Results of regression analysis and serum recovery values were compared for standard curves generated on three different days. Absolute recovery of extracted standards was based upon comparison to detector responses of unextracted standards.

Accuracy

The accuracy of the method was determined from replicate standard curves and a blind analysis of eight samples containing unknown amounts of [^{3}H]brifentanil previously spiked by another analyst. Calculated values using the method were compared to theoretical values and the percentage error was determined.

Stability

Stability of [^{3}H]brifentanil in serum stored at −20°C was evaluated throughout a 30 day period (intervals of 0, 1, 3, 7, 14 and 30 days). Triplicate stored samples containing 5.0 ng/ml [^{3}H]brifentanil were analyzed and compared to freshly prepared serum standards on each day of analysis. A drop in sample response $>10\%$ was considered significant.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms of control and spiked serum containing [^{3}H]brifentanil are shown in Fig. 3. Elution of [^{3}H]brifentanil from the HPLC column occurred at approximately 6.2 min while the internal standard eluted at approximately 7.8 min. The enhanced selectivity provided by the radioactive detector allows for a detection limit of 0.1 ng/ml [^{3}H]brifentanil from 1 ml of serum based upon a signal-to-noise ratio of 3:1.

An additional feature of radioactive detection is on-line isolation of *in vivo* metabolites that retain the radiolabel. This is exemplified by Fig. 4 which shows radiochromatograms of extracted rat serum obtained 45 and 90 min following a 230 μg/kg intravenous dose of [^{3}H]brifentanil. All radioactive metabolites observed were more polar than [^{3}H]brifentanil as indicated by their earlier elution from the reversed-phase column.

Validation

Results for precision of the assay are represented in Table I. The C.V. was based on the response ratio of six replicates for each test. Response ratios were calculated as the ratio of cpm response to the internal standard peak-height response. The C.V. for the system ranged from 3.4 to 6.9% for samples analyzed

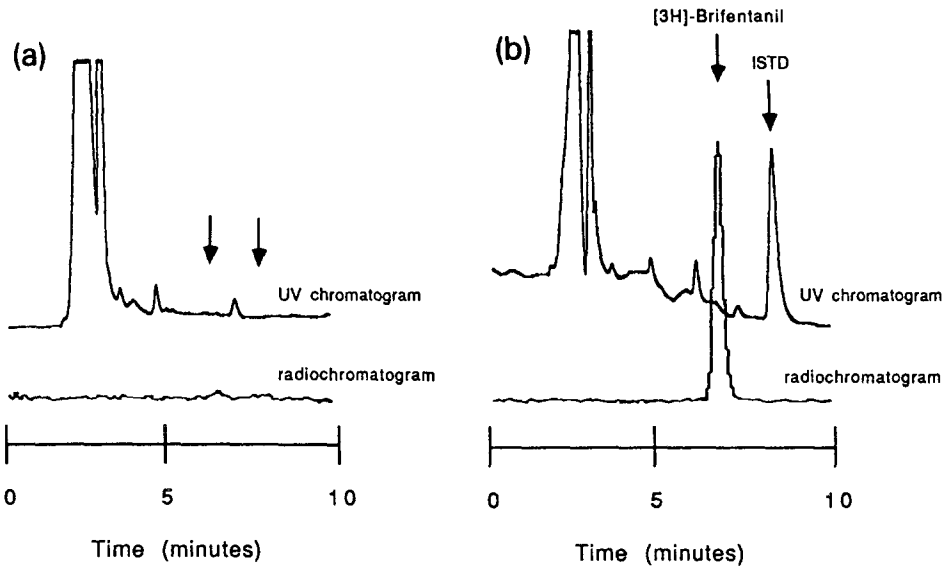

Fig. 3. Representative chromatograms of [^{3}H]brifentanil and ocfentanil (ISTD) in serum. (a) Control serum with arrows showing lack of interfering peaks at [^{3}H]brifentanil and ISTD retention times; (b) spiked serum containing 1 ng/ml [^{3}H]brifentanil and 10 μg/ml ocfentanil. Upper traces are UV chromatograms, lower traces are radiochromatograms.

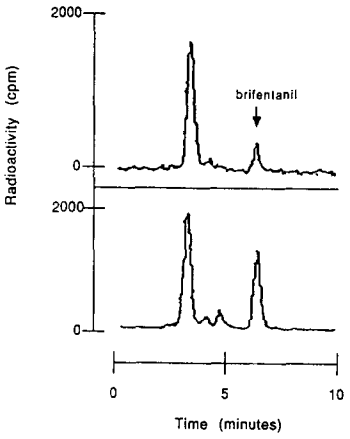

Fig. 4. Rat serum radiochromatograms following an intravenous bolus dose of 230 μg/kg brifentanil (30 μg/kg labelled, 200 μg/kg unlabelled) showing more polar metabolites. Bottom panel = 45 min post-dose; top panel = 90 min post-dose.

on two different days. Similarly, the C.V. range for the extraction method of samples analyzed on two different days was between 2.2 and 10.8%.

Due to the large concentration range required for this assay (0.4–200 ng/ml), two standard curves within this range were constructed and weighted ($1/C$) in order to adequately quantitate unknown samples. Accuracy of the method using this type of calculation is represented in Table II. All calculated error values were less than 10%. Additionally, results from a blind analysis of unknown samples (Table III) were calculated with a percent error range of 2–12%. Mean serum recoveries of [^{3}H]brifentanil (0.4–200 ng/ml, n = 18) and the internal standard (10 μg/ml ocfentanil, n = 18) were 111.2 ± 5 and 101.1 ± 9%, respectively. Reproducibility of the method was indicated by consistent slopes and intercepts obtained from curves generated on three different days (Table IV).

[^{3}H]Brifentanil was stable in serum upon storage at −20°C for up to 30 days

TABLE I

SUMMARY OF WITHIN-DAY AND INTER-DAY PRECISION

Day		n	Response ratio (mean ± S.D.)	C.V. (%)
1	System	6	1.5426 ± 0.0517	3.4
	Method	6	1.6001 ± 0.0354	2.2
2	System	6	1.3041 ± 0.0909	6.9
	Method	6	1.2266 ± 0.1328	10.8

TABLE II

ACCURACY OF THE DETERMINATION OF [^{3}H]BRIFENTANIL FROM SERUM

Numbers in parentheses represent percentage error values.

Concentration added (ng/ml)	Concentration found (ng/ml)		
	Day 1	Day 2	Day 3
0.4	0.44 (10)	N.A.[a]	0.41 (2.5)
1.0	0.97 (−3)	1.01 (1)	0.95 (−5)
5.0	4.97 (−0.6)	5.04 (0.8)	N.A.[a]
10.0	10.05 (0.5)	9.95 (−0.5)	10.01 (1)
100.0	98.91 (−1)	93.82 (−0.6)	99.90 (−0.1)
200.0	201.63 (0.8)	205.81 (2.9)	199.30 (−0.4)

[a] N.A. = not available

TABLE III

ACCURACY OF THE BLIND ANALYSIS OF [^{3}H]BRIFENTANIL EXTRACTED FROM SERUM

Unknown sample No.	Added (ng/ml)	Found (ng/ml)	Error (%)
1	50	48.8	−2.4
2	25	23.1	−7.6
3	10	9.3	−7.0
4	5	4.6	−8.0
5	10	9.1	−9.0
6	2.5	2.6	4.0
7	1.0	0.9	−10.0
8	1.5	1.5	0.0

TABLE IV

REPRODUCIBILITY OF [^{3}H]BRIFENTANIL STANDARD CURVES

Day	Slope	Intercept	r^2
Low curves (0–10 ng/ml)			
1	0.1762	0.0010	0.9998
2	0.1651	0.0008	0.9990
3	0.1634	0.0009	0.9970
High curves (10–200 ng/ml)			
1	0.1268	0.4960	0.9998
2	0.1274	0.3282	0.9980
3	0.1321	0.4021	0.9973

TABLE V

STABILITY OF [^{3}H]BRIFENTANIL IN FROZEN SERUM

Day analyzed	*n*	Mean recovery (%)	C.V. (%)
0	3	103.2	6.2
1	3	90.7	5.3
3	3	103.4	6.3
7	2	102.5	1.6
14	3	93.2	0.3
30	5	92.3	3.8

(Table V). Examination of [^{3}H]brifentanil stability in mobile phase following solid-phase extraction indicated stability for up to 7 days upon storage at −20°C (data not shown).

Application to biological samples

The method has successfully been applied to a pharmacokinetic study in rats. Fig. 5 represents the mean serum concentration *versus* time profile of brifentanil following an intravenous bolus dose of 230 μg/kg (30 μg/kg labelled, 200 μg/kg unlabelled). Serum levels were detected up to 270 min post-dosing and displayed a terminal half-life of 78.9 min.

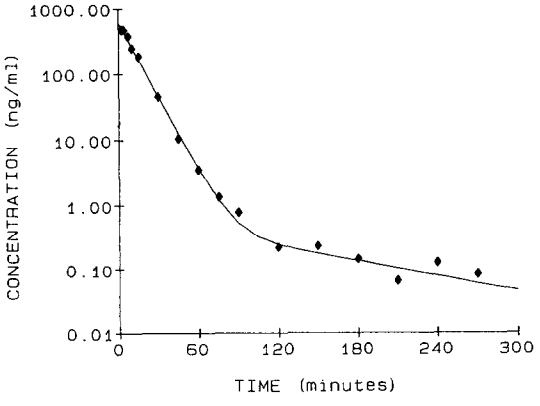

Fig. 5. Mean serum concentration *versus* time profile (four rats per time point) following a 230 μg/kg intravenous bolus dose of brifentanil (30 μg/kg labelled, 200 μg/kg unlabelled) showing the best fitted line through the data points.

CONCLUSIONS

Development of potent opioid analgesics has resulted in the need for very sensitive and selective assays from biological fluids. A sensitive and reproducible assay for [^{3}H]brifentanil determination in rat serum has been developed. Solid-phase extraction combined with reversed-phase HPLC and on-line radioactive detection provided a 0.1 ng/ml limit of detection from 1 ml of serum. Advantages of this detection system include rapid analysis via immediate sample detection without the need for peak collection and subsequent liquid scintillation counting of radioactivity. Peak resolution with an on-line detector is greatly enhanced as compared to manual peak collection due to the rapid sampling times provided by the detector (2 s per scan). Additionally, accumulation of excess scintillation solvent and vials is avoided. Unlike UV detection methods, endogenous serum constituents are not crucial to quantitation since the detector is only specific for radiolabelled species. As a result, an increase in sample sensitivity is observed. In addition, this method may be applied to other biological fluids, *i.e.* urine and bile, with similar sensitivity and selectivity. Since many *in vivo* and *in vitro* metabolism studies utilize radiolabelled drug, this method can also be incorporated early in the drug development process to provide a rapid metabolite screen as well as to serve as a complimentary tool with liquid scintillation counting. The method has been successfully applied to biological samples in a rat pharmacokinetic study and can be implemented in future pharmacokinetic studies, animals or human, that use a radiolabelled drug.

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