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High-performance liquid chromatography of the neuroactive steroids alphaxalone and pregnanolone in plasma using dansyl hydrazine as fluorescent label: application to a pharmacokinetic-pharmacodynamic study in rats

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Abstract

This report describes a rapid and sensitive analytical method for the quantification of the neuroactive steroids alphaxalone and pregnanolone in rat plasma using derivatization with dansyl hydrazine as fluorescent label. The method involves protein precipitation, alkaline derivatization and extraction of the compounds and internal standard pregnenolone with dichloromethane, followed by isocratic reversed-phase high-performance liquid chromatography on a 3-μm Microsphere C_{18} column with fluorescence detection at wavelengths 332 nm and 516 nm for excitation and emission, respectively. The mobile phase consists of a mixture of 25 m*M* acetate buffer (pH 3.7)–acetonitrile (45:55, v/v for alphaxalone and 40:60, v/v for pregnanolone) with a flow-rate of 1 ml/min. The total run time was ~35 min. In the concentration range of 0.010–10 μg ml^{-1}, the intra- and inter-assay coefficients of variation were less than 17% for both methods. In 50 μl plasma samples the corresponding limits of detection were 10 ng ml^{-1} (signal-to-noise ratio=3). The utility of the analytical method was established by analyzing plasma samples from rats, which had received an intravenous administration of 5 mg kg^{-1} alphaxalone or pregnanolone. Values for clearance, volume of distribution at steady state and terminal half life were 71.9 ml min^{-1} kg^{-1}, 814 mg kg^{-1} and 13.5 min for alphaxalone and 69.2 ml min^{-1} kg^{-1}, 1638 ml kg^{-1} and 27.8 min for pregnanolone, respectively. Due to its simplicity and sensitivity this method can be used on a routine basis for pharmacokinetic analysis of neuroactive steroids.

Keywords: Steroids; Alphaxalone; Pregnanolone; Dansyl hydrazine

1. Introduction

Neuroactive steroids are a novel class of steroids that do not interact with any of the classical cytostolic hormonal steroid receptor, but can modulate

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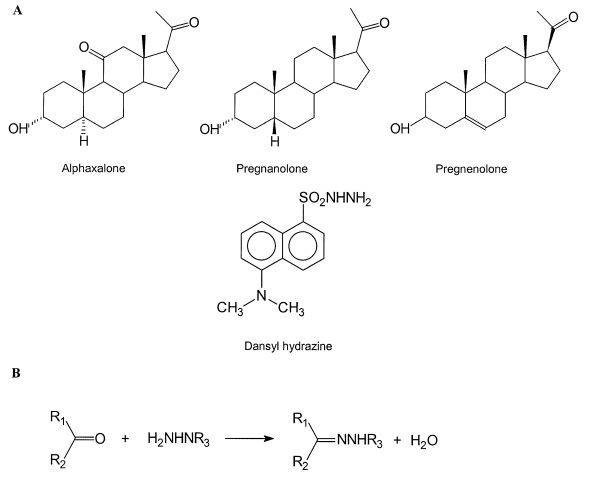

Fig. 1. Chemical structures of alphaxalone (5α-pregnan-3α-ol-11,20-dione), pregnanolone (5β-pregnan-3α-ol-20-one), pregnenolone (5-pregnen-3β-ol-20-one) and dansyl hydrazine (A). Reaction scheme for the derivatization of the carbonyl group with dansyl hydrazine (B).

$GABA_A$ receptor function via a specific, allosteric binding site [1]. Although neuroactive steroids such as alphaxalone and pregnanolone (Fig. 1) have long been known to produce anesthesia, there is a renewed interest for neuroactive steroids as potential drugs for the induction and maintenance of anesthesia and the treatment of epilepsy, anxiety, insomnia, migraine and drug dependence [2]. The synthetic neuroactive steroid alphaxalone and the endogenous neurosteroid pregnanolone (eltanolone) have both been formerly used for general anesthesia [3]. Nowadays these and related compounds are commonly used as reference compounds to study neurosteroid/$GABA_A$ receptor pharmacology in vitro and in vivo [4]. To date, however, the mechanisms that determine the time course of the in vivo effect of neuroactive steroids are still unclear.

An integrated pharmacokinetic-pharmacodynamic approach can provide insight in the factors that determine pharmacodynamic behavior in vivo [5]. Such an approach, however, requires a convenient, rapid and sensitive analytical assay to determine concentrations in small plasma samples. Published assays for alphaxalone and pregnanolone in plasma use expensive equipment such as GC–MS and GLC with complex derivatization with trimethylsilyl ethers [6,7]. Radioimmunoassays have been described for endogenous neurosteroids but show high cross reactivity and are not commercially available [8,9]. For a simple determination an HPLC method would be preferable. A major problem, however, is that neuroactive steroids lack any saturable bonds making direct detection with spectrophotometric techniques impossible. However, fluorimetric derivatization shows promise as a reliable detection method. Dansyl hydrazine (Fig. 1) is a fluorescent reagent frequently used for derivatization of carbonyl compounds [10,11]. Shimada et al. used fluorescence detection for qualitative detection of derivatized ketosteroids in the brain, however mass spectrometry was used for quantitative detection [12,13]. A complex HPLC method has been developed for detection of several ketosteroids using pre- and post-column derivatization and with peroxyoxalate chemiluminescence [14,15]. In an attempt to develop a more simple assay for pregnanolone, Jones et al. have used 2,4 dinitrophenyl hydrazine as UV labelling [16]. However, in that study large sample volumes of 1 ml were used and detection limits were not sufficient for preclinical pharmacokinetic experiments.

Therefore, we have developed a simple method using dansyl hydrazine as fluorescent label that can be used to detect alphaxalone and pregnanolone in 50 μl plasma samples with sufficient high sensitivity and that also can be applied to other neuroactive steroids with only minor modifications. This novel assay was applied to a pharmacokinetic-pharmacodynamic study in rats of alphaxalone and pregnanolone, in which the electroencephalogram (EEG) was used as a measure of $GABA_A$-receptor activity.

2. Experimental

2.1. Chemicals

Alphaxalone (5α-pregnan-3α-ol-11,20-dione), pregnanolone (5β-pregnan-3α-ol-20-one) and pregnenolone (5-pregnen-3β-ol-20-one) were purchased from Sigma Alldrich BV (Zwijndrecht, The Netherlands). Dansyl hydrazine was obtained from Fluka Chemie AG (Buchs, Switzerland). Acetonitrile (DNA synthesis grade) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Dichlorome-

thane Chromasolv© was obtained from Riedel-de Haën GmbH (Seelze, Germany). Methanol was purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). All other chemicals used were of analytical grade (Baker, Deventer, The Netherlands).

2.2. Instrumentation

The HPLC system for alphaxalone (system 1) consisted of Waters 501 solvent delivery pump (Millipore-Waters, Milford, MA, USA), a Waters 717plus autosampler (Millipore-Waters) and a Jasco FP 1520 intelligent fluorescence detector (Jasco Co., Tokyo, Japan). The HPLC system for pregnanolone (system 2) consisted of a Spectroflow 400 solvent delivery system (Applied Biosystems, Ramsey, NJ, USA), a Waters 712 autosampler (Millipore-Waters) and a Perkin-Elmer LC240 fluorescence detector (Perkin-Elmer Ltd., Beaconsfield, UK).

Chromatography was performed on a stainless steel Microsphere C_{18} 3-μm cartridge column (100 mm×4.6 mm I.D.) (Chrompack, Bergen op Zoom, The Netherlands) equipped with a guard column (20 mm×2 mm I.D.) (Upchurch Scientific, Oak Harbor, WA, USA) packed with C_{18} (particle size 20–40 μm) (Chrompack).

The mobile phase consisted of a mixture of 25 m*M* acetate buffer (pH 3.7) and acetonitrile (45:55, v/v for alphaxalone; 40:60, v/v for pregnanolone). Mobile phase solvents were filtered through a 0.45 μm nylon filter (Gelman Scientific, Ann Arbor, MI, USA), mixed and degassed with helium. Flow-rate was 1 ml/min. Fluorescence detection occurred at excitation wavelength of 332 nm and emission wavelength of 516 nm.

Data acquisition and processing was performed using a Chromatopac C-R3A reporting integrator (Shimadzu, Kyoto, Japan).

2.3. Reagent and standard solutions

The dansyl hydrazine solution (20%, w/v) was prepared by dissolving 20 mg dansyl hydrazine in 1 ml methanol, to which 40 μl sulphuric acid was slowly added. The dansyl hydrazine solution was freshly prepared each week and stored at 4°C.

Stock solutions of alphaxalone, pregnanolone and pregnenolone were prepared at concentrations of 1 μg ml^{-1} in acetonitrile. The alphaxalone and pregnanolone solutions were diluted with water to concentrations in the range of 0.01–10 μg ml^{-1}. Pregnenolone was diluted with water to a concentration of 2.5 and 7.5 μg ml^{-1} for use as internal standard in the assay of alphaxalone and pregnanolone, respectively. Stored at 4°C these solutions remained stable for at least two months.

2.4. Extraction procedure

To 50 μl plasma in a glass centrifuge tube, 50 μl of the internal standard was added. After mixing, 200 μl acetonitrile was added to precipitate plasma proteins and vortexed for 30 s. After centrifugation for 10 min at 4500 *g* the supernatant was transferred to a clean tube. Twenty-five μl dansyl hydrazine solution and 50 μl 2 *M* NaOH were added and the mixture was vortexed for 30 s. After centrifugation for 10 s at 4500 *g*, the mixtures were stored at a dark place at room temperature for 20 h or 4 h for alphaxalone and pregnanolone, respectively. Subsequently, 500 μl 1 *M* NaOH and 5 ml dichloromethane were added and the mixture was vortexed for 5 min. The phase system was centrifuged for 15 min at 4500 *g*. The water phase was disposed by means of suction. The remaining water was removed by freezing and the organic phase was transferred to a clean tube and evaporated under reduced pressure on a vortex vacuum evaporator (Buchler Instruments, Fort Lee, NJ, USA) at 37°C. The residue was dissolved in 100 μl mobile phase of which a volume of 50 μl was injected into the HPLC system.

2.5. Calibration and validation

On each day of analysis a twelve point calibration curve was prepared by spiking 50 μl of plasma with 50 μl of an alphaxalone solution and 50 μl of the internal standard pregnenolone at 2.5 μg ml^{-1} or 50 μl of a pregnanolone solution and 50 μl of the internal standard pregnenolone at 7.5 μg ml^{-1}. This resulted in alphaxalone and pregnanolone plasma concentration ranges of 0.01–10 μg ml^{-1}. Samples were processed as described above and peak-height ratios of alphaxalone/pregnenolone and pregnanolone/pregnenolone were calculated. Calibration curves were constructed by weighted linear

regression [weight factor=1/(peak height ratio)^2]. Quality control samples of fixed concentrations were prepared to determine intra- (500 and 5000 ng ml^{-1}) and inter-variability (50, 500 and 5000 ng ml^{-1}).

2.6. Study in rats

Chronically instrumented male Wistar rats, weighing 285±10 g (mean±SEM, n=17) were used in the experiments. Ten days before the experiment, seven cortical electrodes were implanted into the skull. Three days before the experiment the abdominal aorta was canulated by an approach through the right femoral artery for serial blood sampling. The right jugular vein was implanted with a canula for administration of the infusion solution. The surgical procedures were performed under anesthesia of medetomidine HCl (0.1 ml kg^{-1} of a 1 mg ml^{-1} solution, intra muscularly) and ketamine base (1 ml kg^{-1} of a 50 mg ml^{-1} solution, subcutaneously). After surgery rats received a single dose of ampicilline trihydrate (0.6 ml kg^{-1} of a 200 mg ml^{-1} solution, A.U.V., Cuijk, The Netherlands).

At the day of experiment the rats (fully recovered, unrestrained, conscious) received an intravenous infusion of either alphaxalone (5 mg/kg in a 5 min infusion) or pregnanolone (5 mg/kg in a 5 min infusion). Twenty 100 μl blood samples were collected over a period of 2 h at predetermined time intervals and added to 40 μl heparinized saline. After centrifugation for 15 min at 4500 g, 50 μl plasma was transferred to a glass centrifuge tube and stored at −20°C until analysis as described above.

Following the administration of either alphaxalone or pregnanolone the EEG was recorded continuously. After off-line fast Fourier Transformation using the data analysis software Spike2 (version 4.60, CED, Cambridge, UK) the absolute amplitude in the β-frequency range in 5 s epochs were averaged over 1 min intervals.

The pharmacokinetics of alphaxalone and pregnanolone were quantified for each individual rat using the least squares minimization algorithm [weight=1/(y predicted)^2] of the WinNonlin Pro package V.1.5 (Pharsight Corporation, Mountain View, CA, USA). For both alphaxalone and pregnanolone a standard two-compartment model [17] best described the concentration-time profile, as judged by the Akaike Information Criteria [18].

3. Results and discussion

3.1. Chromatography

Derivatization of alphaxalone and pregnanolone with dansyl hydrazine followed by sample pre-treatment with liquid–liquid extraction resulted in absence of interference by endogenous compounds, as demonstrated by the representative chromatograms in Figs. 2 and 3, respectively. All peaks were well separated. Although alphaxalone and pregnanolone

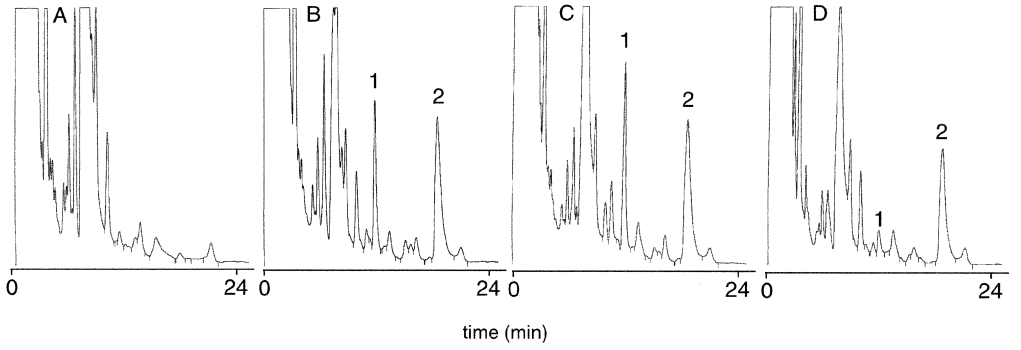

Fig. 2. Chromatogram obtained using system 1 of an extract of blank plasma (A), blank plasma spiked with alphaxalone (2500 ng ml^{-1}, peak nr. 1) and internal standard pregnenolone (2500 ng ml^{-1} peak nr. 2) (B), plasma obtained from a rat 19 min (C) and 99 min (D) after having received an infusion of 5 mg kg^{-1} alphaxalone during 5 min (alphaxalone concentration were 2219 ng.ml^{-1} and 254 ng.ml^{-1}, respectively.

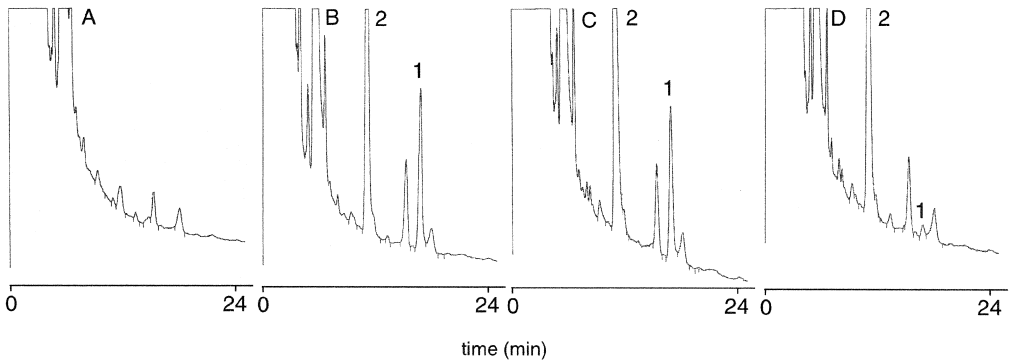

Fig. 3. Chromatogram obtained using system 2 of an extract of blank plasma (A), blank plasma spiked with pregnanolone (2500 ng ml^{-1}, peak nr. 1) and internal standard pregnenolone (7500 ng ml^{-1}, peak nr. 2) (B) and plasma obtained from a rat 7.1 min (C) and 114 min (D) after having received an infusion of 5 mg kg^{-1} pregnanolone during 5 min (pregnanolone concentration were 1557 ng.ml^{-1} and 68 ng.ml^{-1}, respectively).

could be detected simultaneously in a single run, two systems (1 and 2; see Experimental) were used. Retention times for alphaxalone, pregnenolone and pregnanolone were 12.3, 18.9 and 30 min on system 1, respectively. However, to study pregnanolone pharmacokinetics, it was desired to shorten run time, which also improved peak symmetry and sensitivity. On system 2, retention times for pregnenolone and pregnanolone were 11.5 and 17.2 min, respectively; the alphaxalone peak was not separated sufficiently from the injection peak. Total run time for both methods was 35 min. Mobile phase was not circulated during detection since recirculation resulted in immediate loss of sensitivity due to fluorescent waste. Excess of reagent was removed using liquid–liquid extraction and none of the co-extracted substances seemed to interfere significantly with the determination of alphaxalone and pregnanolone in plasma. Table 1 summarizes reproducibility and accuracy of both analysis methods. For alphaxalone and pregnanolone intra- and inter-day coefficients of variability were less than 17% in the concentration range 0.010–10 μg ml^{-1}. The weighted linear regression equations (mean±SEM) for alphaxalone and pregnanolone were $y=(0.0013\pm0.0001)\ x+(0.053\pm0.017)$ ($n=17$), and $y=(0.0001\pm0.0000)\ x+0.0108\pm0.0044)$ ($n=9$), respectively. Corresponding coefficients of correlation were 0.9901 ± 0.0017 ($n=17$) alphaxalone and 0.9886 ± 0.0019 ($n=9$) for pregnanolone, indicating

Table 1
Validation of the determination of alphaxalone and pregnanolone: intra-assay and inter-assay variability, coefficients of variation and accuracy

Compound	Added (ng ml^{-1})	Intra-assay			Inter-assay		
		Found (ng ml^{-1}) (mean±SEM, $n=5$)	C.V. (%)	Accuracy (%)	Found (ng ml^{-1}) (mean±SEM, alphaxalone: $n=25$, pregnanolone: $n=14$)	C.V. (%)	Accuracy (%)
Alphaxalone	50	–	–	–	48.1±1.8	12.7	103.9
	500	487.0±41.7	17.1	102.6	499.9±17.8	17.8	100.0
	5000	5383.1±182.3	7.6	92.3	5377.3±112.2	8.3	92.5
Pregnanolone	50	–	–	–	50.7±3.3	17.2	98.6
	500	546.5±17.4	6.4	90.7	539.7±9.7	7.0	92.1
	5000	5050.8±186.8	7.4	99.0	4628.0±160.1	12.5	107.4

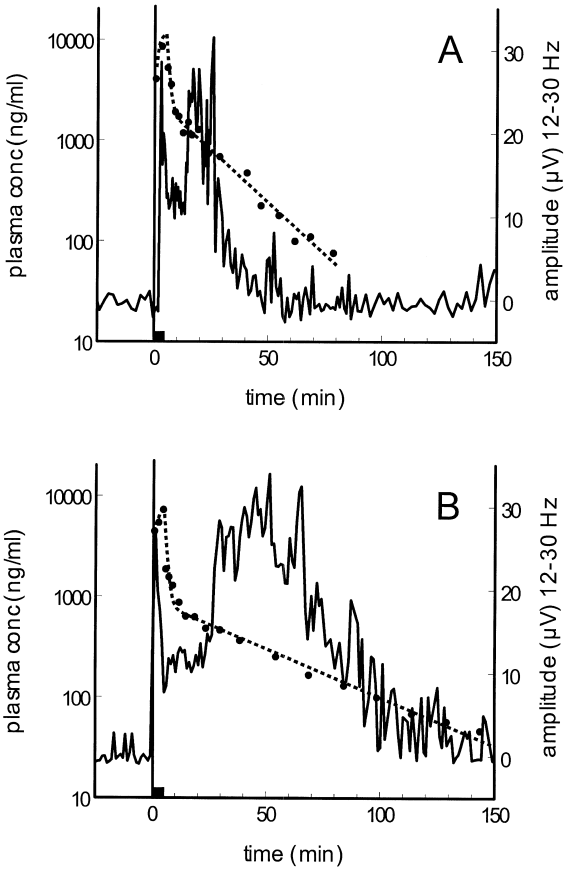

Fig. 4. Typical plasma concentration-time profiles (dashed line, left ordinate) and amplitudes in β-frequency range vs. time (solid line right ordinate) in rats following intravenous infusion of 5 mg kg^{-1} alphaxalone (A) and 5 mg kg^{-1} pregnanolone (B) in 5 min. The dashed line represents the best description of the plasma concentrations according to a two-compartment pharmacokinetic model.

the linearity of the method. Using 50 μl plasma, the limit of detection for alphaxalone and pregnanolone was 10 ng ml^{-1} (signal-to-noise ratio=3).

3.2. Optimization of reaction

It was necessary to remove plasma proteins by precipitation with acetonitrile before dansyl hydrazine was added in order to improve reproducibility and to avoid interfering peaks. Tri-chloro acetic acid (TCA) could not be used for protein precipitation since the optimum pH for the reaction of alphaxalone and pregnanolone with dansyl hydrazine is over pH 12. For alphaxalone the reaction time was prolonged to 20 h, which improved reaction yield and detection limit significantly. Extraction with dichloromethane resulted in the highest absolute recovery compared to other extraction solvents. Recovery of extraction could not be determined since extraction was required for sample clean-up prior to injection onto the HPLC to be able to detect the neuroactive steroids quantitatively.

3.3. Study in rats

Fig. 4 shows representative plasma concentration time profiles for an intravenous infusion of 5 mg kg^{-1} if alphaxalone in 5 min and 5 mg kg^{-1} of pregnanolone in 5 min in rats. The values for clearance, volume of distribution at steady state and terminal half life were estimated for each individual rat (Table 2). Fig. 4 also shows the time-course of the change in amplitude of the β-frequency range of the EEG during administration of alphaxalone and

Table 2
Average pharmacokinetic parameter estimates (mean±SEM) obtained with a 2-compartment pharmacokinetic model for alphaxalone and pregnanolone after 5 mg kg^{-1} infusion in 5 min

Compound	Infusion dose/time (mg kg^{-1}/min)	*n*	Clearance (ml min^{-1} kg^{-1})	Volume of distribution at steady state (ml kg^{-1})	Elimination half life (min)
alphaxalone	5/15	8	71.9±4.0	814.0±68.6	13.5±1.2
pregnanolone	5/15	7	69.2±8.7	1638.2±242.4	27.8±2.1

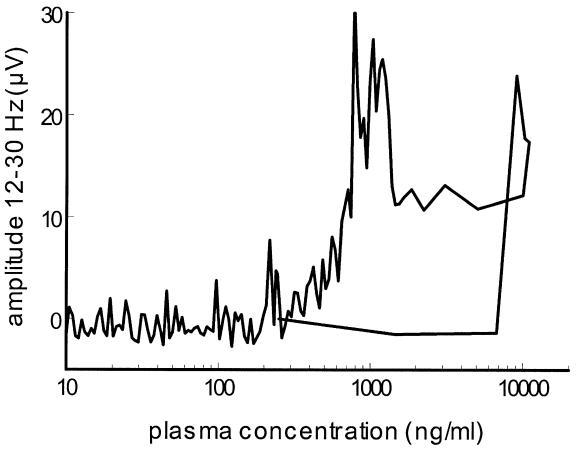

Fig. 5. Complex concentration-effect relationship for alphaxalone in a typical rat, obtained from the data presented in Fig. 4A.

pregnanolone. After the start of the infusion the amplitude increased, followed by a partial decrease and increase again and eventually the amplitude gradually returned to baseline within 50 and 100 min, respectively. Combining pharmacokinetic and pharmacodynamic data revealed a complex, biphasic concentration-effect relationship (Fig. 5).

4. Conclusion

We have developed a simple and sensitive HPLC assay using the fluorescent label dansyl hydrazine for the determination of the neuroactive steroids alphaxalone and pregnanolone in plasma. The short time of analysis, the sensitivity, the reproducibility and the simplicity of the methodology used make this assay particularly useful for pharmacokinetic-pharmacodynamic studies in which large numbers of samples need to be analyzed. After slight modifications in the mobile phase composition and pH of extraction this bio-analysis may also be applied in disposition studies of other neuroactive steroids. In combination with EEG measurements, the assay allows for the generation of concentration-effect relationships of alphaxalone and pregnanolone in individual rats which can be used for quantitative analysis of the $GABA_A$-receptor-mediated effects in vivo.

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